UNIVERSITÀ DEGLI STUDI DI TRIESTE
Sede Amministrativa del Dottorato di Ricerca

XIX Ciclo del

Dottorato di Ricerca in

SCIENZE BIOMOLECOLARI

Biopolymers for Cartilage Tissue-Engineering

(Settore scientifico-disciplinare BIO/10)

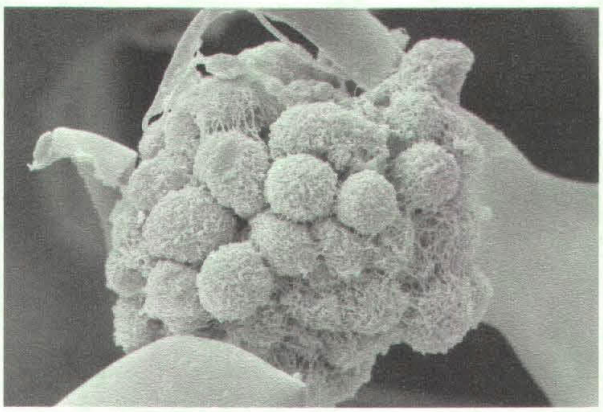

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Table of Contents

CHAPTER 1
Introduction

1.1 Cartilage

Cartilage is a connective tissue of mesenchymal origin displaying three key functions within the body. Firstly, it acts as a template for the growth and development of long bones, forming a large part of the foetal skeleton and playing an important role in endochondral ossification. In addition, cartilage is present at the articulating surfaces of bones, where it provides a low-friction surface. It also acts as a supporting framework in some organs within the body, for example in the trachea where it prevents airway collapse. Cartilage differs with respect to biochemical composition, structure and location within the body (Serafini-Fracassini and Smith, 1974).
Cartilage is a tissue with unique properties consisting of a small number of highly specialized cells (chondrocytes) surrounded by a large amount of extracellular matrix (ECM).

Cartilage tissue in the human body can be divided into three subtypes. Hyaline cartilage is the predominant type, is present on the articulated surfaces of all joints and in large parts of the respiratory tract; elastic cartilage is the main component of the outer ear and is found in some parts of the respiratory tract; fibrocartilage is located in the menisci and in the intravertebral discs.

Hyaline cartilage, found in all the diarthrodal joints in the human body, is rich in type-II collagen fibres. It can be divided into four zones with different cell shape and size, collagen fibril diameter and orientation, proteoglycan concentration and water content.

- **Superficial zone**: it's the outer layer of the articular cartilage; the collagen fibrils are oriented parallel to the surface and are very closely packed. The dense arrangement of collagen fibres in this region of the tissue provides it with its low friction surface and high tensile strength (Guilak and Mow, 2000). Chondrocytes in the superficial zone are flat in shape, with the main axis of the cells oriented along the articular surfaces: for this reason it is also called "tangential zone"; the proteoglycan content of the extracellular matrix is low, and the water content is high.
- **Middle or transitional zone**: at this level the collagen fibrils are thicker and randomly oriented; the chondrocytes have a more rounded profile.
- **Deep zone**: collagen fibrils are thick and oriented perpendicular to the articular surface. The chondrocytes in this zone are rounded and arranged in vertical columns. The proteoglycan content is high, while the water content decreases.
- **Calcified Zone**: the tidemark separates the deep zone from the calcified cartilage zone, which represents the boundary between the articular cartilage and the subchondral bone. In this region the chondrocytes are small and the extracellular matrix is calcified.

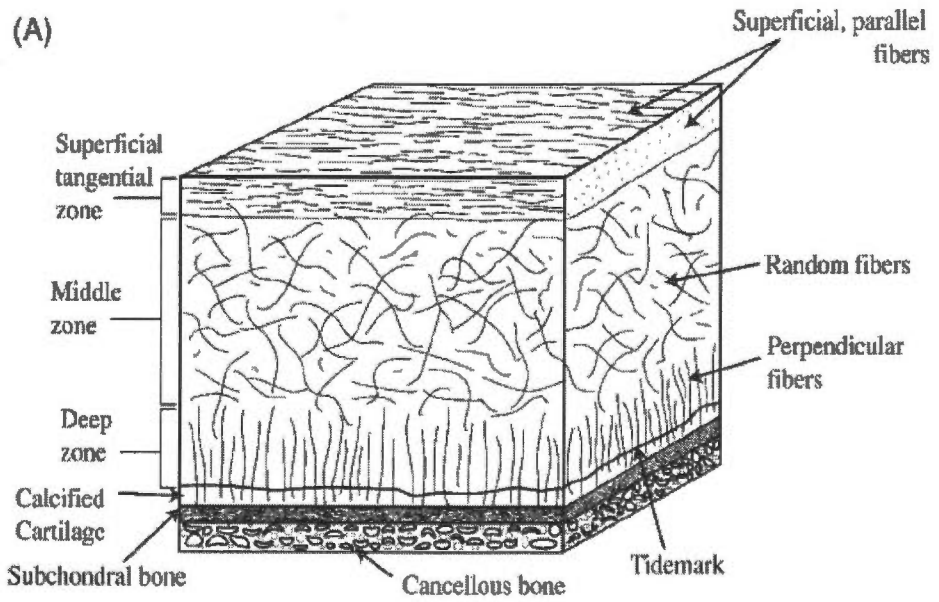


Fig. 1.1: Different zones of articular cartilage (Aufderheide and Athanasiou, 2004).

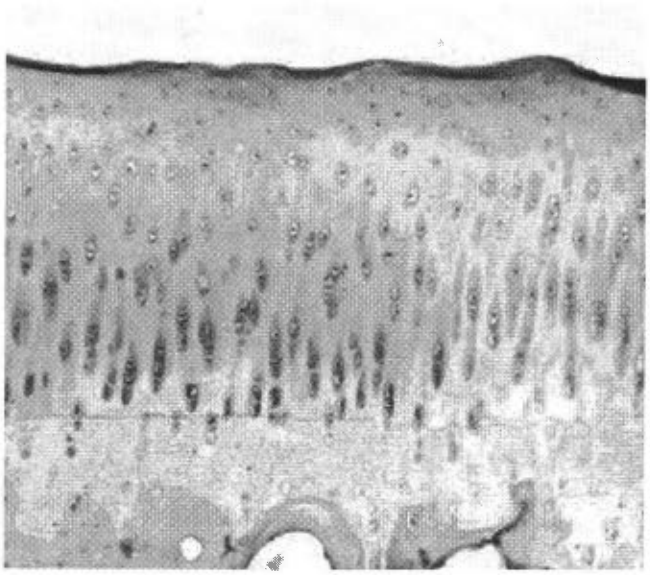


Fig. 1.2: The organization of chondrocytes and ECM within articular cartilage.

Elastic cartilage, in the ear and in the respiratory tract, is lined by perichondrium. The extracellular matrix of this tissue is a network of elastic fibres. Elastin comprises approximately 20% of the dry tissue weight. While the structure of articular cartilage changes from top to bottom, elastic cartilage has a more homogeneous, symmetrical structure. In this type of cartilage chondrocyte morphology is different, with larger cells, often characterized by two nucleoli and lipid vesicles.

Fibrous cartilage is found in the meniscus and in intravertebral disc. Fibrocartilage contains lower glycosaminoglycans (GAGs) levels than other types of cartilage, possesses highly organized collagen fibres and is found at the ends of ligaments and tendons. This tissue has intermediate features, between connective tissue and hyaline cartilage. There is a significant amount of type-I collagen; chondrocytes have a morphology similar to fibroblasts. This is the predominant repair tissue found in articular cartilage defects.

1.2 Articular Cartilage

Articular cartilage forms a durable layer 0.5 to 7.0 mm thick at the surface, reducing friction between the bones and distributing loads across the entire joint surface (Carver and Heath, 1999). The exact biochemical composition of articular cartilage varies with species, age and location within the tissue (McDevitt and Webber, 1990). The tissue is composed of cells within an extracellular matrix (ECM) composed of fibrillar components, for example collagen, proteoglycans, non-collagenous proteins and water (Alberts et al., 2002). Articular cartilage is considered to be one of the simplest tissues within the body since it possesses a single cell type, the chondrocyte; it is

aneural and has no vascular or lymphatic supply (Buckwalter and Mankin, 1998b).

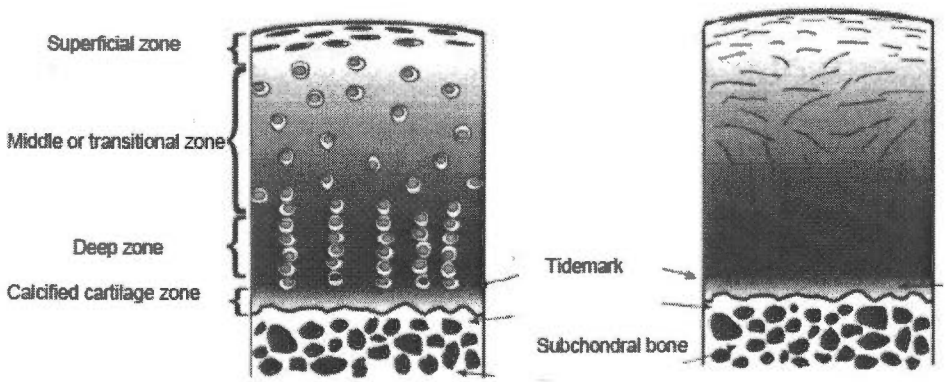


Fig. 1.3: Chondrocyte morphology and collagen orientation in hyaline cartilage.

1.3 Articular Cartilage Components

1.3.1 Cells

Chondrocytes form a very small proportion of articular cartilage, typically around 1% of the tissue dry weight (Buckwalter and Mankin, 1998b). Chondrocytes are responsible for synthesis of the cartilage ECM macromolecules, the assembly and organization of these macromolecules into an ordered framework and the continual replacement of degraded matrix components (Buckwalter and Mankin, 1998b). Within the tissue, the cells vary in number, shape, size and metabolic activity (Aydelotte, 1988). One of the key differences between chondrocytes and fibrochondrocytes is that the predominant collagen secreted by fibrochondrocytes is type I, whereas that of chondrocytes is type II (Benjamin and Ralphs, 2004).

1.3.2 Extracellular Matrix of Articular Cartilage

Water makes up to 65 to 80% of the wet weight of the tissue.

Collagen

Collagen is the main component of the extracellular matrix in articular cartilage. The fibrous network of collagen accounts for 50-60% of the tissue dry weight (LeBaron and Athanasiou, 2000). The predominant collagen of articular cartilage is type II, which represents up to 90-95% of the total collagen (Heath and Magari, 1996). Type II collagen, having a glycine rich amino acid sequence of glycine-X-Y, where X and Y are hydroxyproline and proline arbitrarily, forms rope-like fibrils which aggregate into larger cable-like bundles or fibres (Alberts et al., 2002).

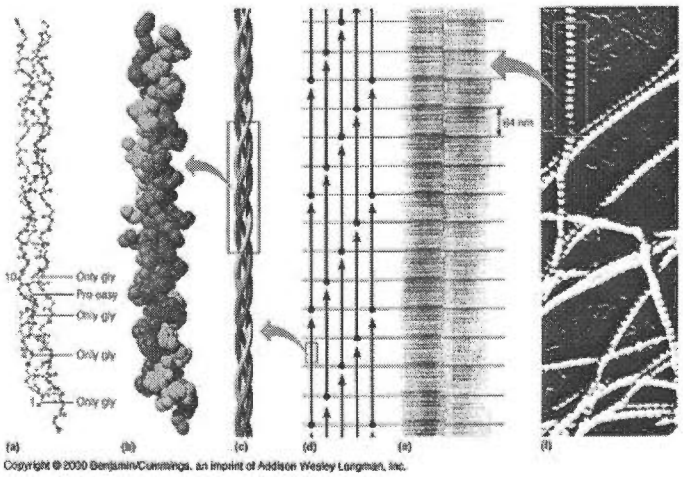


Fig. 1.4: Collagen fibres molecular organization.

Other collagen types present in the cartilage are collagen V, VI, IX, X and XI, all having a triple-helical structure and contributing to the ECM network organization. Type-XI is a fibrillar collagen involved in the establishment of a fibre network; type-IX is a fibril-associated collagen thought to aid linkage of

the collagen fibrils to the rest of the ECM; type-VI is found in the matrix immediately surrounding chondrocytes and is believed to help attachment of the cells to the ECM; type-X collagen is involved in chondrocyte hypertrophy (Loeser, 1993).

The collagen network covalently binds the proteoglycans, providing articular cartilage tensile and shear properties.

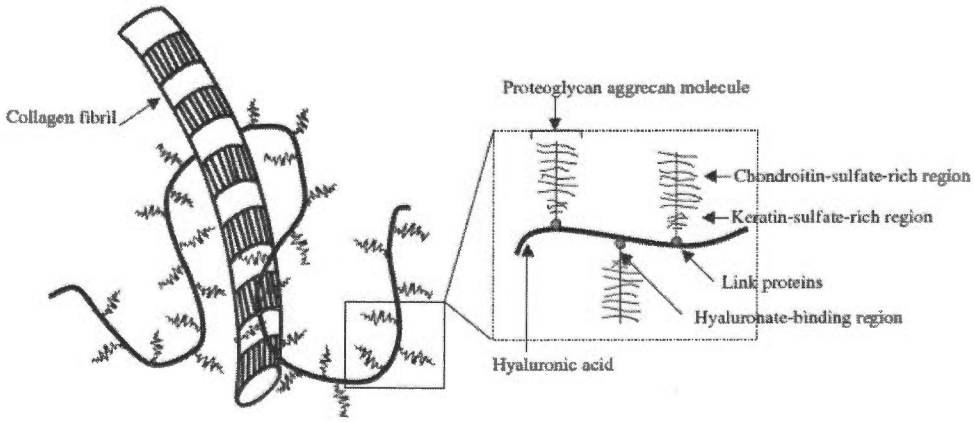


Fig. 1.5: Composition of the ECM of cartilage; interactions between collagen, proteoglycan and hyaluronic acid.

Proteoglycans

Proteoglycans are the third important component of the extracellular matrix, constituting between 15 and 30% of the dry weight of articular cartilage (Freed et al., 1998). Proteoglycans are complex macromolecular aggregates, consisting of a core protein to which one or more glycosaminoglycan chains (GAGs) are attached (Buckwalter and Mankin, 1998b). GAGs are unbranched polysaccharide chains which contain repeating disaccharide units where one of the sugars within the repeating unit is an amino sugar, for example N-acetylglucosamine, and the second is usually a uronic acid, for example glucuronic acid (Alberts et al., 2002). Since each of the disaccharides contains at least one ionizable carboxylic or sulphate group, GAGs contain

long chains of negative charge which attract cations and repel anions (Buckwalter and Mankin, 1998b). In solution these groups become negatively charged. Water molecules are bound to the negatively charged glycosaminoglycans leading to an osmotic swelling pressure. Constraining forces of the collagen network counteract this swelling pressure.

There are four groups of GAGs: i) hyaluronan, ii) chondroitin sulphate and dermatan sulphate, iii) keratan sulphate and iv) heparan sulphate, the first three groups of which are present in articular cartilage (Buckwalter and Mankin, 1998b).

Two classes of proteoglycan are present: large aggregating proteoglycan monomers, for example aggrecan, and small proteoglycans, such as decorin, biglycan and fibromodulin (Buckwalter and Mankin, 1998b; Nakano et al., 1997).

About the 80-90% of the total amount of proteoglycans can be found as large aggregates, as aggrecan. Aggrecan consists of chains of chondroitin and keratan sulphate bound to core proteins. Individual aggrecan monomers interact with hyaluronan, to form high molecular weight aggregates. These interactions are stabilized by link protein, which binds to both the hyaluronan and a specific binding site at the N-terminus of the aggrecan (Hardingham, 1979). The GAG/proteoglycan aggregates form gels which occupy a large volume relative to their mass. These hydrophilic gels draw in considerable quantities of water that confer high compressive strength properties to the tissue (Bryant and Anseth, 2001).

The smaller non-aggregating proteoglycans are involved in binding macromolecules, for example decorin and fibromodulin bind with type II collagen and therefore it is postulated that they may play a role in organizing and stabilizing the collagen meshwork (Hasler et al., 1999). The smaller proteoglycans are also able to bind transforming growth factor-β (TGF-β), a cytokine known to stimulate cartilage matrix synthesis (Buckwalter and Mankin, 1998b).

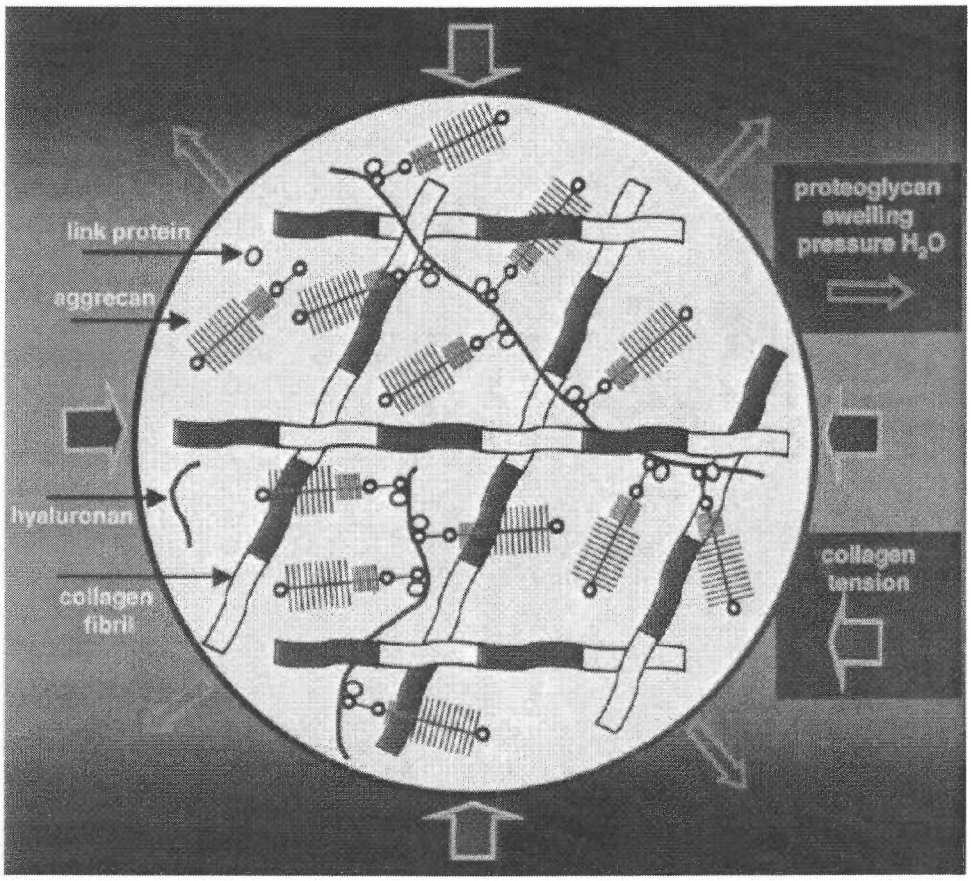


Fig. 1.6: The combined properties of collagen and aggrecan in articular cartilage (Kiani et al., 2002).

Non-collagenous Proteins and Glycoproteins

In addition to proteoglycans and collagens, articular cartilage contains non-collagenous proteins and glycoproteins. Some of these molecules are thought to be involved in the organization and maintenance of the ECM structure (Buckwalter and Mankin, 1998b). Anchorin CII, for example, is a collagen binding protein found at the surface of chondrocytes that is believed to help anchor chondrocytes to collagen fibrils (von der Mark and Kuhl, 1985). Another example is fibronectin, a protein that has been identified in many other tissues. It has been shown that chondrocytes attach to fibronectin and

that the binding is mediated by integrins (Loeser, 1993). Whilst the exact role of fibronectin in cartilage is not fully understood, it is postulated that it may be involved in matrix organization or cell-matrix interactions (Hayashi et al., 1996). Three adhesion glycoproteins have been identified within meniscal fibrocartilage all of which have been found to contain the arginine-glycine-aspartic acid (RGD) peptide sequence: type VI collagen, fibronectin and thrombospondin (McDevitt and Webber, 1990).

Tissue Fluid Components

Besides water, tissue fluid contains gases, small proteins, metabolites and a large number of cations. Interactions between the negative charge of the large aggregating proteoglycans and the cations within the tissue fluid help retain water within the tissue and contribute to the mechanical properties of cartilage (Buckwalter and Mankin, 1998b).

Articular cartilage can be divided into regions according to the distance of matrix from the cells: the pericellular, territorial and interterritorial compartments (Newman, 1998). In general, the pericellular and territorial regions are thought to facilitate attachment of chondrocytes to the ECM and to protect them during loading of the tissue (Buckwalter and Mankin, 1998b). The pericellular region occurs where the membranes of cells appear to be attached to the ECM (Temenoff and Mikos, 2000b). The matrix in this region contains a high concentration of proteoglycans. Anchorin CII and type VI collagen are present in this region of articular cartilage, supporting the hypothesis that this matrix region is involved in attachment of chondrocytes to the ECM (Buckwalter and Mankin, 1998b). The pericellular matrix of each chondrocyte is contained within envelopes of territorial matrix known as lacunae (Temenoff and Mikos, 2000b). It is believed that these collagenous

envelopes protect the cells from mechanical stresses experienced within the tissue (Buckwalter and Mankin, 1998b). The interterritorial region comprises the majority of the ECM and is considered to be responsible for the mechanical properties of articular cartilage (Temenoff and Mikos, 2000b).
Although articular cartilage of human adults has no blood supply, articular chondrocytes show a high level of metabolism. Chondrocytes derive their nutrition mainly from the synovial fluid and to a lesser extent from the underlying bone. They synthesize and assemble extra-cellular matrix components and direct their distribution within the tissue. All this is done in order to maintain the structure and function of the extracellular matrix. The high level of metabolism is mainly due to proteoglycan turnover. Although collagen turnover does take place, its level is much lower.

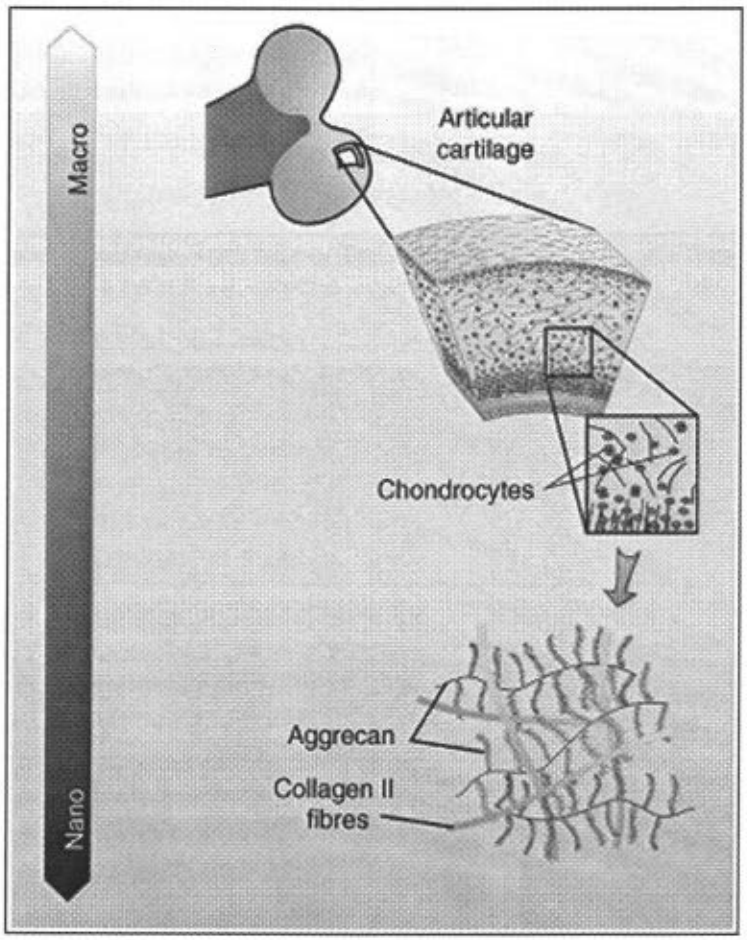


Fig. 1.7: Hierarchical structure of articular cartilage (Bonzani et al., 2006).

1.4 Cartilage Lesions

Since articular cartilage has a poor intrinsic capacity for repair, two major problems need to be addressed in the regeneration process. The first is to fill the defect void with a tissue that has the same mechanical properties as articular cartilage. The second is to trigger successful integration between the repair tissue and surrounding articular cartilage (Redman et al., 2005).
Although the presence of undifferentiated chondrocyte precursor cells in cartilage has been demonstrated (Alsalameh et al., 2004), chondrocytes show a rather poor proliferation capacity, being virtually locked in the extracellular matrix that strongly impedes cell migration toward the defect.
The most important factor limiting the response of articular cartilage to injury is, however, the lack of blood supply. When a vascularized tissue is damaged a fibrin clot is formed. Inflammatory cells and undifferentiated stem cells migrate with the blood to the tissue defect. Removal of necrotic tissue by inflammatory cells, accompanied by proliferation and differentiation of undifferentiated cells lead to the tissue repair (Jackson et al., 2001; Shapiro et al., 1993). When studying articular cartilage defects it is therefore essential to discriminate between superficial **chondral defects** (limited to the cartilage layer), and **osteochondral defects** extending into the subchondral bone. In superficial defects no blood clot is formed and as a consequence no repair tissue occurs. Cells surrounding the defect will die in a process called apoptosis (Shapiro et al., 1993). Only when a cartilage injury perforates the subchondral bone can a fibrin clot be formed. Inflammatory and mesenchymal stem cells from the bone marrow can enter the defect and initiate a repair response, influenced by growth factors released from the platelets. But even then the repair is insufficient. The defect is filled with fibrocartilage, the tidemark is not restored and there is no integration with the intact native cartilage. Diseased cartilage shows a distinctly different ultrastructure compared to healthy tissue. The long-term

performance of fibrocartilage is inferior to that of normal hyaline cartilage. It lacks mechanical strength and the collagen fibril network is disorganized (Shapiro et al., 1993; Heath and Magari, 1996; Buckwalter and Mankin, 1998b). The articulating surface is less smooth compared to healthy adult tissue. The water content of the diseased tissue is higher, increasing the permeability and reducing the compressive modulus of cartilage. Failure of the intrinsic repair capacity has led to several surgical techniques to treat isolated articular cartilage defects.

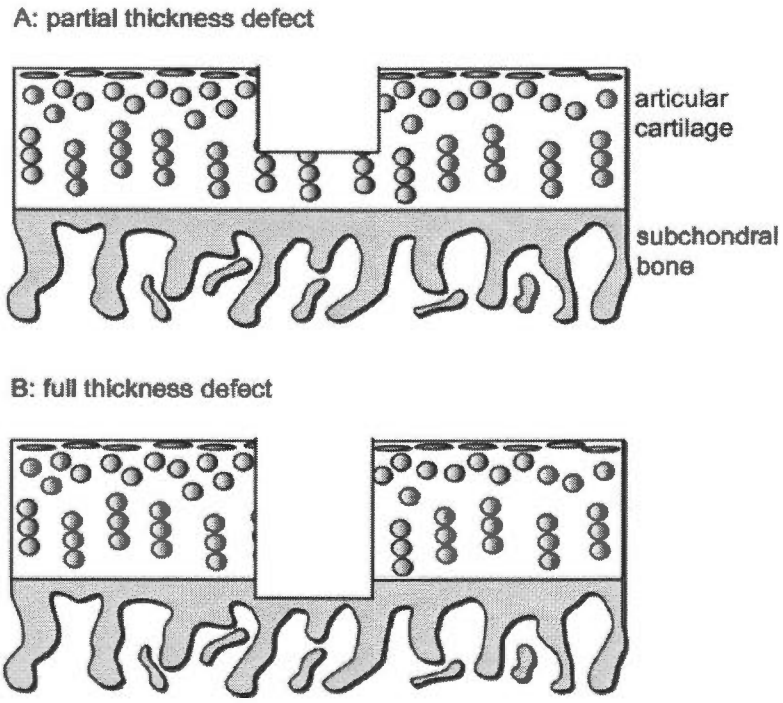


Fig. 1.8: Diagram illustrating a partial thickness defect in articular cartilage (A) and full thickness defects that penetrates to the subchondral bone (B) (Redman et al., 2005).

1.5 Repair Strategies

In general, repair strategies involve one or more of the following: (i) surgical intervention; (ii) a space-filling device e.g. a tissue graft; or (iii) a treatment to stimulate a healing response and chondrogenesis (e.g. penetration of the subchondral bone to allow infiltration of inflammatory and progenitor cells into the defect site (O'Driscoll, 1999)). Something that all current treatment options have in common is the variability in their success – functional repair that can be achieved in some joints in some patients, but no one treatment allows complete healing of all defects in all patients (Lohmander, 2003).
A variety of surgical procedures have been used in the treatment of articular cartilage defects. Arthroscopic lavage and debridement are often used to reduce joint pain. Lavage implying irrigation of the joint during arthroscopy appears to alleviate pain through an unclear mechanism (Livesley et al., 1991). Debridement is the arthroscopic removal of damaged tissue from the joint, which has shown to reduce pain as well. When used in conjunction, these two procedures have shown to produce a longer effect in pain reduction. However both lavage and debridement do not stimulate chondrogenesis or repair of the injury site since there is no penetration of the subchondral bone (Chen et al., 1999).

Many arthroscopic techniques used to induce repair of articular cartilage exploit the intrinsic healing properties of the tissue when the subchondral bone is exposed. These procedures include abrasion arthroplasty, Pridie drilling and microfracture. Abrasion arthroplasty uses an automated burr to access the vasculature and is used combined with debridement (Friedman et al., 1984). Pridie drilling stimulates bleeding by drilling the subchondral bone. Microfracture is based on the debridement of damaged tissue down to the subchondral bone, and the following perforation with small awls. The aim of these techniques is to induce the formation of blood clots that, in turn, will

induce fibrous tissue synthesis that promote cartilage regeneration. The clinical outcome of these methods varies according to the unpredictable composition of the tissue that fills the defect, the age and activity level of the patient (Hunt et al., 2002).

Soft tissue grafts involve the transplantation of periosteum and perichondrium. These methods have been used in animal models and human clinical trials. It has emerged that there is no significant difference between the repair tissue produced from periosteum and perichondrium (Carranza-Bencano et al., 1999). Anyway, periosteum is more easily obtained and more frequently used for transplantation. Periosteum has a chondrogenic potential during development, due to the presence of chondrocyte precursor cells in the cambial layer (O'Driscoll, 1999). However, clinical experience with periosteal grafts has shown poor results.

To treat predominantly large defects, osteochondral transplantation (mosaicplasty) of autogenic and allogenic tissue has been widely used (Hangody et al., 2001). Allogenic material derived from cadaveric donors have been applied (Czitrom et al., 1986). Experiments have demonstrated that fresh tissue is more successful than frozen tissue (Tomford et al., 1992). An immune response is still a potential problem with this approach. Autologous osteochondral transplantation consists of cylindrical osteochondral pieces removed from non-weight bearing areas of the articular cartilage and their transfer into debrided full thickness defects. Results have shown decreased pain and improved joint function, mainly in small and medium sized full-thickness defects (Hangody et al., 2004).

Autologous chondrocyte implantation (ACI) was first applied in clinical practice in 1994 (Brittberg et al., 1994). This surgical procedure involves two stages. The first one involves the excision of healthy biopsy by arthroscopy

from a non-load bearing region of the articular cartilage. Following enzymatic digestion, the cells are expanded in culture. In the second stage, the lesion is cleared to healthy cartilage and with a separate incision a periosteal graft is taken from the medial tibia and sutured over the defect. Cultured autologous chondrocytes are then injected under the periosteal flap. Results showed reduced pain and improved joint function in many patients.

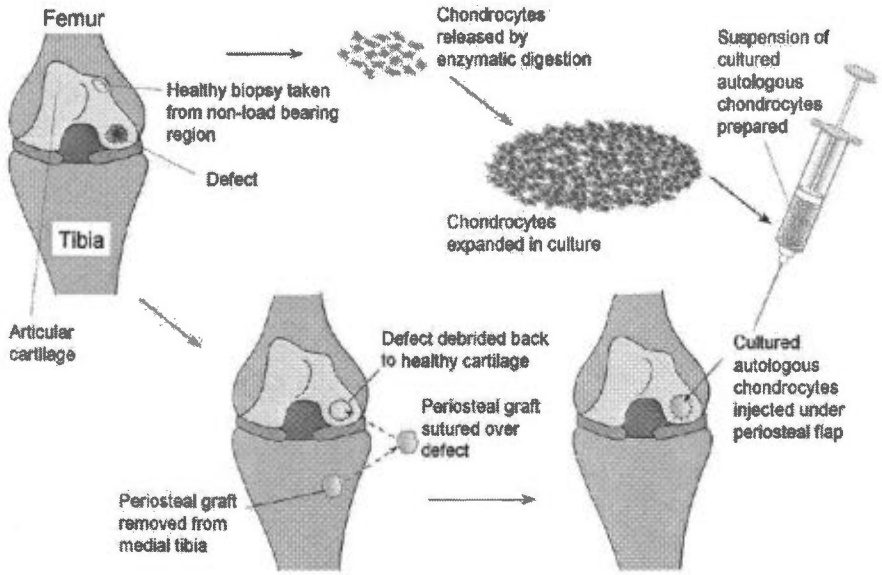


Fig. 1.9: Schematic diagram showing the different phases of autologous chondrocyte implantation.

1.6 Osteoarthritis

Osteoarthritis is one of the most common forms of arthritis characterized by the degeneration of cartilage. This debilitating and progressive syndrome of joint pain and dysfunction caused by joint degeneration affects more people than any other joint disease (Brooks, 2002). Joint degeneration is a process that includes progressive loss of articular cartilage accompanied by attempted repair of articular cartilage, remodelling and sclerosis of subchondral bone, and osteophyte formation (Buckwalter and Mankin, 1998a).

Current therapies do not prevent or cure osteoarthritis, and symptomatic treatments often fail to provide satisfactory pain relief. Once patients develop osteoarthritis, this disease remains for their entire lives and the severity of pain and disability generally increases.

The frequency and chronicity of osteoarthritis and the lack of effective preventive measures or cures make this disease a substantial economic burden for patients, health care systems, businesses, and nations (Reginster, 2002).

The World Health Organization (WHO) estimates that 10% of the world people over the age of 60 years suffers from osteoarthritis, and the 80% of people with osteoarthritis have limitation of movement and 25% cannot perform major daily activities (WHO, 2001).

Osteoarthritis represents a combination of several disorders in which biomechanical properties of cartilage are altered, leading to tissue softening and ultimately to degradation (Pool, 2001). The main feature of osteoarthritis is an imbalance between chondrocyte anabolic (synthesis) and catabolic (resorptive) activities. Degradation of cartilage is characterized by two phases: phase one, where the chondrocytes attempt to repair the damaged ECM with little success, and phase two, where the activity of the enzymes

produced by chondrocytes digests the ECM and matrix synthesis is inhibited. In osteoarthritic cartilage, chondrocytes continue to produce the matrix components; however they are unable to face the rate of degradative catabolic activity and tissue degradation results (Nesic et al., 2006). Enzymes such as collagenases and aggrecanases cause the degradation of the cartilage matrix. Collagenases (also known as matrix metalloproteinases) cause the degradation of the triple helix of collagen allowing for further degradation by aggrecanases (Mort and Billington, 2001). Collagenases make an initial cleavage in a weak point in the collagen fibril and then the aggrecanases cleave the core protein. Aggrecanases, in tandem with other enzymes, cause the degradation of aggrecan. These changes in the matrix components of cartilage cause it to weaken and to lose its biomechanical function.

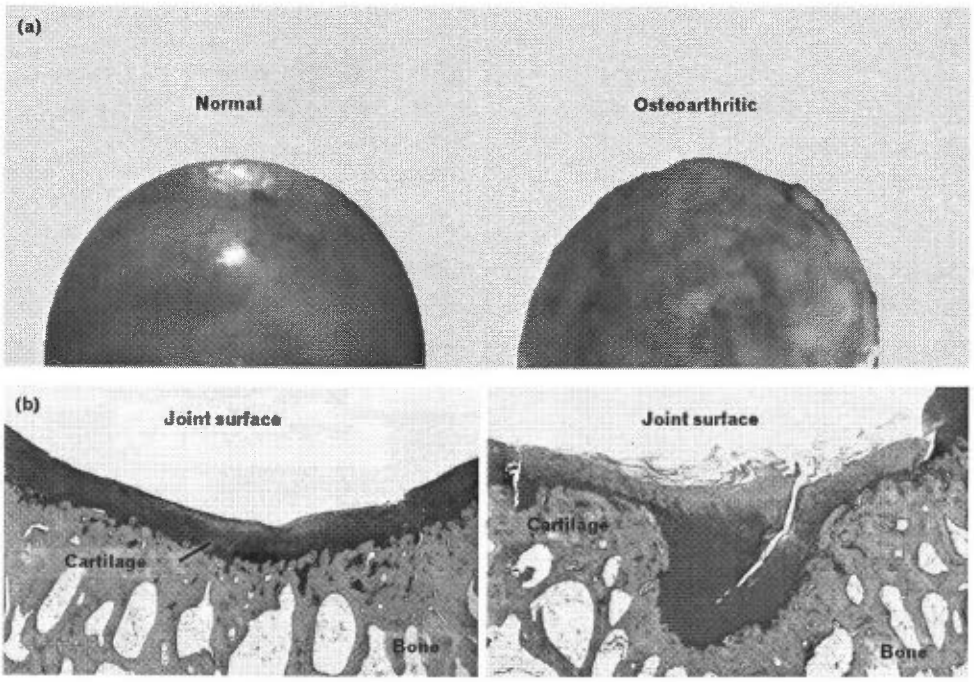


Fig. 1.10: Macroscopic view (a) and histology (b) of normal versus osteoarthritic cartilage (Kuo et al., 2006).

The etiology of osteoarthritis, although not fully understood, is comprised of several interconnected factors: age, programmed cell death (apoptosis), local inflammatory processes and mechanical stress (Nesic et al., 2006).

Risk Factors for osteoarthritis (Neustadt, 2006; Felson, 1988):

- aging
- genetic factors
- overweight and obesity
- overuse of the joints
- trauma
- misalignment of the knee
- muscle weakness

The prevalence of osteoarthritis in all joints increases with age. More than a third of people over 45 years report joint symptoms that vary from a sensation of occasional joint stiffness to permanent loss of motion and constant deep pain (Buckwalter and Mankin, 1998a). In some populations more than 75% of the people over 65 have osteoarthritis that involves one or more joint (Felson, 1990).

After the age of 40 the incidence of osteoarthritis increases rapidly with each passing decade in all joints, and in most joints the incidence is greater in women than in men (Felson et al., 1995).

The total economic burden of arthritis is 1% to 2.5% of the gross national product of western nations, and osteoarthritis accounts for the major share of this burden (Reginster, 2002). Osteoarthritis is second to ischemic heart disease as a cause of work disability in men over the age of 50 years (Lawrence et al., 1998).

In addition to the disorders associated with secondary osteoarthritis (e.g. joint injuries, joint dysplasias, and joint infections) genetic predisposition, obesity, female gender, greater bone density, and joint laxity have been identified as risk factors.

The most important risk factor in all populations is age. The percent of people with evidence of osteoarthritis in one or more joints increases from less than 5% of people between 15 and 44 years, to 25% to 30% percent of the people 45 to 64 years of age, and to more than 60% and as high as 90% in some populations, of the people over 65 years of age (Elders, 2000). Mechanical loading that exceeds the ability of a joint to repair or maintain itself is another universal risk factor.

The radiographic changes associated with osteoarthritis include narrowing of the cartilage space, increased density of subchondral bone and the presence of osteophyte (Buckwalter and Martin, 2006).

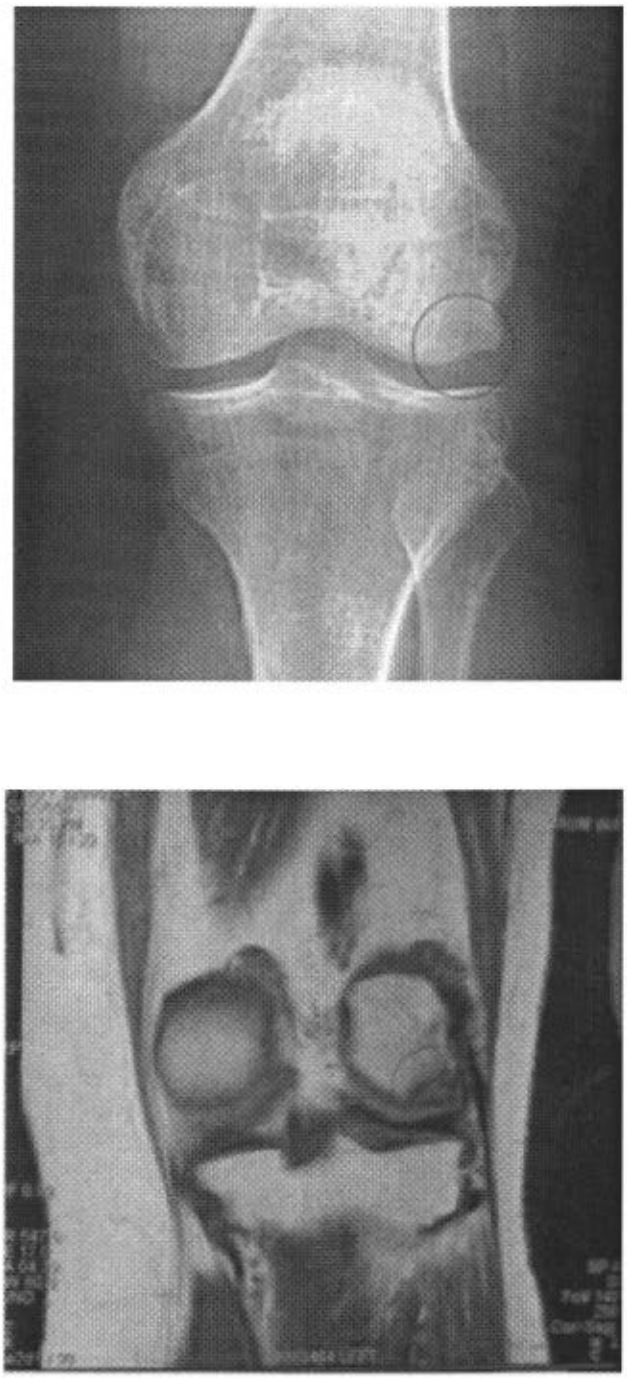
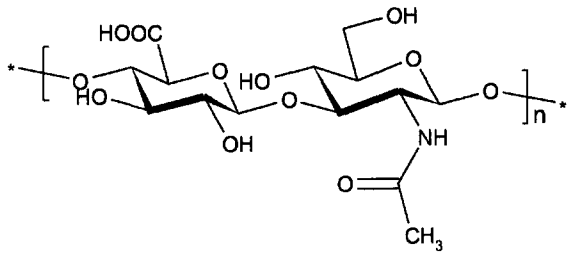


Fig. 1.11: X-ray (top) and MRI (bottom) images of lesions of the articular cartilage.

1.7 Viscosupplementation

The main function of synovial fluid (a highly viscoelastic solution) is to surround and protect the synovial tissue and surface structure of the articular cartilage from mechanical damage. Hyaluronic acid (HA) is the molecule responsible for the synovial fluid's rheological properties, enabling it to act as a lubricant or shock-absorber in dependence of the forces exerted upon it (Lo et al., 2003).



Hyaluronic Acid

Fig. 1.12: Hyaluronic acid chemical structure.

Hyaluronan is a glycosaminoglycan polysaccharide composed of repeating disaccharide units of a β(1-4) glucuronic acid α(1-3) linked to an N-acetylglucosamine. It differs from other glycosaminoglycans in that it is unsulfated, and it does not bind covalently with proteins to form proteoglycans monomers, serving instead as the backbone of proteoglycan aggregates. In joints it is produced by chondrocytes and synovial cells. At high molecular weights hyaluronic acid is viscoelastic, aiding weight-bearing joints in lubrication, shock-absorption, and fluid retention during movement (Carney and Muir, 1988; Lo et al., 2003).

In the synovial fluid of patients with osteoarthritis there is an increase in the relative number of hyaluronic acid molecules of molecular weight lower than

normal and a reduction in the concentration of hyaluronic acid due to exudation. These changes reduce the viscoelasticity of the synovial fluid and its ability to protect the joint, resulting in the destruction of cartilage and bone (Conrozier and Vignon, 2005).

Viscosupplementation is the symptomatic treatment of osteoarthritis by intra-articular injection of exogenous hyaluronic acid or its derivatives. This therapy aims to restore the physiological homeostasis of the pathologically altered joint and induce a restoration of normal hyaluronic acid metabolism (Balazs and Denlinger, 1993). Hyaluronic acid possesses chondroprotective effects and is reported to inhibit the loss of proteoglycans from the extracellular matrix of joint cartilage. Hyaluronic acid is also reported to protect the cartilage against proteoglycan loss, against chondrocyte cell death caused by free oxygen radicals, interleukin-1 (IL-1), and against other alterations (Goldberg and Buckwalter, 2005).
Injections of exogenous hyaluronic acid induce a decrease of inflammatory and proliferative processes within the synovium. The mechanisms of action include control of synovial permeability, blockade of inflammatory scavenging oxygen free radicals, and inhibition of matrix metalloproteinases. Hyaluronan protects also chondrocytes and promote cartilage matrix synthesis. *In vitro* studies of human synoviocytes from osteoarthritic joints reveal that exogenous hyaluronan stimulates *de novo* synthesis of hyaluronan (Neustadt, 2006).

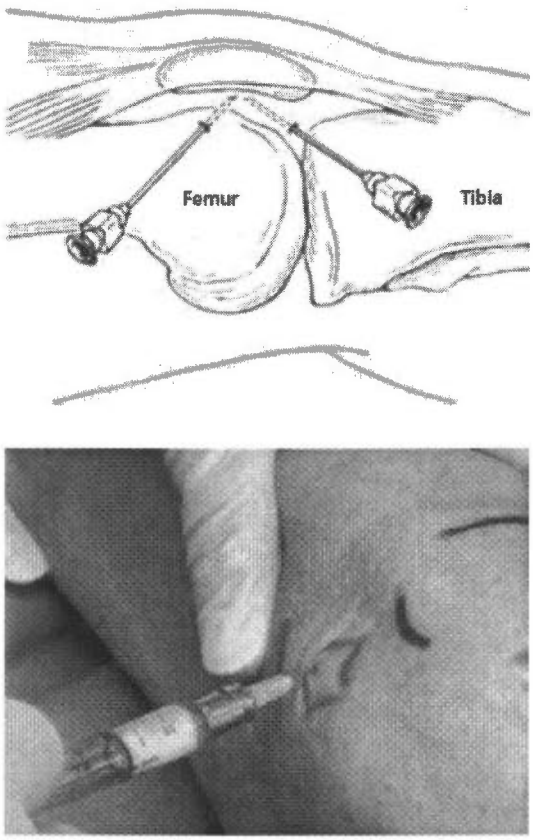


Fig. 1.13: Arthrocentesis of the joint (top). Injection into the joint via medial approach (bottom) (Neustadt, 2006).

More than 20 commercial viscosupplement formulations are available worldwide from different manufacturers. These products vary in their molecular weight and residence times in the joint, and recommended dosing regimens range from one to five injections at weekly intervals. An intra-articular preparation was approved by the US Food and Drug Administration (FDA) in 1997 for use in osteoarthritis of the knee. In 2006 four FDA-approved hyaluronan preparations, extracted from rooster combs were available in United States (Neustadt et al., 2005):

- sodium hyaluronate (Hyalgan)
- sodium hyaluronate (Supartz)
- Hylan G-F 20 (Synvisc)
- high molecular weight hyaluronan (Ortho-visc).

Approved hyaluronan preparations

	SODIUM HYALURONATE (HYALGAN)	SODIUM HYALURONATE (SUPARTZ)	HIGH-MOLECULAR-WEIGHT HYALURONAN (ORTHOVISC)	HYLAN G-F 20 (SYNVISC)
Type of product	Natural	Natural	Natural	Chemically cross-linked
Molecular weight (X 10^6 daltons)	0.5–0.73	0.6–1.2	1.0–2.9	6.0
Concentration (mg/mL)	10	10	15	8
Dose volume (mL)	2	2.5	2	2
Dose interval	1 week	1 week	1 week	1 week
Number of doses	3 to 5	5	3 or 4	3

Table 1.1: Approved hyaluronan preparations (Neustadt, 2006).

All are highly purified native hyaluronan except for Hylan G-F 20, which is chemically crosslinked hyaluronan with added formaldehyde and vinylsulfone to increase its stability and retention in the joint cavity. Non-animal-derived hyaluronan preparations, obtained by a bacterial fermentation process, are available in Europe. One of this products, Euflexxa, recently approved in the United States is a highly purified hyaluronan sample of high molecular weight (2.3-3.6x10^6 Da) (Kirchner and Marshall, 2006).

Four meta-analyses of randomized clinical trials with viscosupplementation in symptomatic knee osteoarthritic patients have been published to date and provide the most robust evaluation of efficacy.

Lo et al. 22 analyzed placebo-controlled randomized clinical trials in knee osteoarthritis patients. The authors concluded that viscosupplementation has a small effect when compared with intra-articular placebo. They further noted that the highest MW products may be more efficacious than those with lower MW, but significant study heterogeneity limited definitive conclusions (Lo et al., 2003).

The analysis of Wang et al. of 20 blinded viscosupplementation randomized clinical trials in knee osteoarthritis found significant improvements in pain and functional outcomes with few adverse events. Although between-study differences in efficacy estimates were observed, the trials of high MW

products demonstrated greater pooled estimates of efficacy than those involving low MW products (Wang et al., 2004).

Modawal et al. evaluated the efficacy of viscosupplementation by conducting a meta-analysis of 11 trials and concluded that moderate symptomatic relief was achieved up to 10 weeks after the final injection (Modawal et al., 2005).

Most recently, and most extensively, a Cochrane review by Bellamy et al. has analysed 63 randomized clinical trials of 17 viscosupplement products in osteoarthritis of the knee. This systematic review concludes that viscosupplementation is superior to placebo, demonstrates comparable efficacy to non-steroidal anti-inflammatory drugs (NSAIDs) and has a more prolonged effect than intra-articular corticosteroids. In general, few adverse events were reported in the hyaluronan/hylan trials included in the analyses. Again, the review noted considerable between-product, between-variable and time dependent variability in clinical response, and the majority of studies using high MW products had more robust effect sizes than those using low MW viscosupplements (Bellamy et al., 2006).

These four meta-analysis vary in methodology, selection criteria, data extraction and data analysis, resulting in significant heterogeneity. However, there is general agreement in considering that viscosupplementation is effective and safe, and that higher MW preparations may be more effective than lower MW preparations.

As a local therapy administered by intra-articular injection, viscosupplementation has a much lower risk of systemic effects than other osteoarthritis treatments, such as NSAIDs with their risk of gastric, hepatic and renal side-effects. Due to the method of delivery, some local reactions usually involving mild transient discomfort, swelling and/or effusion are to be expected (Pagnano and Westrich, 2005).

In conclusion, viscosupplementation is an effective treatment for osteoarthritis of the knee and appears to be a promising option for osteoarthritis of the hip. Anyway, as with all therapies, appropriate diagnosis,

effective patient selection and correct technique can determine the best results in term of clinical outcome with this treatment.

1.8 Cartilage Tissue Engineering

In 1993 Langer and Vacanti defined tissue engineering as a multidisciplinary research area that exploits both biological and engineering principles for the purpose of generating new, living tissues to replace the diseased or damaged tissue and restore tissue function (Langer and Vacanti, 1993). Ideally, the biological substitute should structurally and morphological resemble native tissue and be able to perform similar biological functions. In comparison to artificial implants, biologically engineered tissue may offer a better long-term performance due to the enhanced biocompatibility, integration into surrounding tissues and the ability to remodel according to the body requirements.

The ultimate aim of cartilage tissue engineering is the *in vitro* generation of cartilaginous constructs for implantation. These constructs should be able to remodel upon implantation into the patient so that functional cartilage with the required biochemical composition and mechanical properties is able to fully integrate with the host tissue (Vunjak-Novakovic, 2003).

It has been proposed that the tissue may either be grown entirely *in vitro* and implanted into the defect as hyaline cartilage or that the developing tissue within the scaffold structure may be implanted and allowed to form cartilage *in vivo* (Hutmacher, 2000).

Cartilage tissue engineering is critically dependent on selection of appropriate cells (differentiated or progenitor cells), fabrication and utilization of biocompatible and mechanically adequate scaffolds, stimulation with chondrogenically bioactive molecules and application of dynamic, mechanical loading regimens for conditioning of the engineered tissue constructs, including the design of specialized biomechanically active bioreactors.

1.8.1 Cells

Cells used in tissue engineering must be biosynthetically active and have nutrients, metabolites and other regulatory molecules readily available (Jackson and Simon, 1999). The donor age and differentiation state have all varied in the cells used in cartilage tissue engineering studies to date (Buckwalter and Mankin, 1998b; Vunjak-Novakovic, 2003). Mature, differentiated chondrocytes are advantageous for cartilage regeneration as they are the native cell population within cartilage and synthesize the appropriate ECM components (Freed et al., 1999). Articular chondrocytes are therefore the most obvious choice of cell for articular cartilage tissue engineering. Whilst articular chondrocytes can easily be isolated, obtaining an appropriate number of cells with the capacity to regenerate cartilage is one of the challenges facing tissue engineers (Huckle et al., 2003). It is possible to expand cell populations using *in vitro* cell culture techniques; although it has been observed that in monolayer culture articular chondrocytes dedifferentiate, become fibroblastic in appearance and secrete a fibrous matrix. It has been documented that culturing the cells within a 3-dimensional environment such as a porous scaffold can help them retain their chondrocytic phenotype (Freed et al., 1999). A population of progenitor cells have recently been isolated from the superficial zone of articular cartilage. In addition it has been shown that these cells retain their ability to produce articular cartilage following several population doublings (Dowthwaite et al., 2004). The use of chondrocytes from other cartilage types for engineering articular cartilage has also been studied (Huckle et al., 2003). Kafienah and co-workers have published data showing that chondrocytes from nasal cartilage can be used to engineer articular cartilage following *in vitro* expansion (Kafienah et al., 2002). Other cell types that have been used in cartilage tissue engineering studies include stem cells isolated from a variety of tissues, such as muscle (Deasy et al., 2002) and

adipose (Huang et al., 2005); mesenchymal stem cells, MSCs (Caplan and Bruder, 2001). Despite these cells having greater proliferative capacities than adult articular chondrocytes they do not have the intrinsic ability to differentiate into chondrocytes unless given specific stimuli (Huckle et al., 2003).

Adult MSCs (Song et al., 2004) are easily obtained from bone marrow and possess a multi-lineage potential, allowing them to be induced to differentiate into bone, cartilage, and adipose-like cell types, even after many doublings in culture. Chondrogenic differentiation of MSCs has been effected by the application of transforming growth factor (TGF)-β family members (Johnstone et al., 1998) in a variety of three dimensional scaffolds (Worster et al., 2001; Awad et al., 2004).

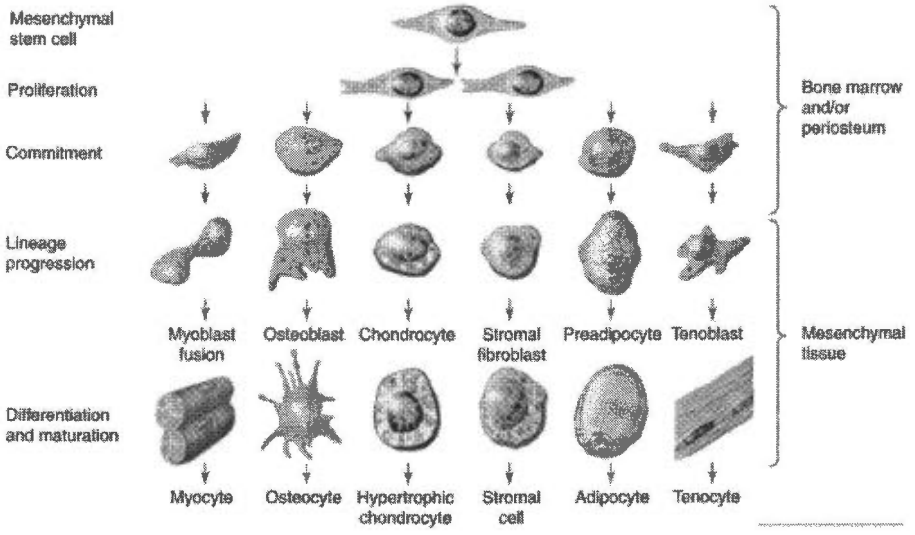


Fig. 1.14. The schematic drawing depicting mesengenic process. Mesenchymal stem cells (MSCs) differentiate into a variety of tissues including muscle, bone, cartilage, marrow, fat and ligaments etc.

1.8.2 Scaffolds

The basic function of a tissue engineering scaffold is to provide a temporary structure while cells seeded within the biodegradable matrix synthesize new, natural tissue. New tissue regeneration takes place during scaffold degradation, with the new tissue gradually growing off the mould of the original scaffold (Kuo et al., 2006). Therefore, design criteria include controlled biodegradability, suitable mechanical strength and surface chemistry, ability to be processed in different shapes and sizes, and the ability to regulate cellular activities, such as proliferation, differentiation, and more (Kuo and Tuan, 2003).

A wide range of scaffolds have been used in cartilage tissue engineering studies. These scaffolds may be classified with respect to the types of material used (natural or synthetic, degradable or non-degradable), the geometry of the scaffold (gels, fibrous meshes or porous sponges) and their structure (total porosity, pore size, connectivity and distribution) (Vunjak-Novakovic, 2003). It is crucial that a tissue engineering scaffold is fabricated from a material that is biocompatible, allowing attachment of cells, ECM secretion and tissue formation without the induction of an inflammatory or toxic response (Freed et al., 1994b; Temenoff and Mikos, 2000b; Agrawal and Ray, 2001). In order for cells to be able to infiltrate the structure uniformly, it should contain a large number of interconnected pores (Freed et al., 1994b). The size of the pores is important to the infiltration and attachment of the cells, for chondrocytes an optimum pore size of between 100 and 200 μm has been suggested (Agrawal and Ray, 2001). The scaffold must also be permeable, to allow diffusion of nutrients into the matrix and the removal of metabolic and degradation by-products from it (LeBaron and Athanasiou, 2000). Finally, it is important that the scaffold has mechanical properties that allow it to withstand implantation and the loads experienced *in vivo* (Agrawal and Ray, 2001). The material used should be easily

processed into the required structure and shape and be able to withstand sterilization processes (Freed et al., 1999).

Natural Materials

Many naturally derived polymers had been used to support chondrocytes growth, because of their similarity with cartilage ECM components. There are two kinds of naturally derived polymers. The first is carbohydrate-based polymers, such as alginate (Fragonas et al., 2000), agarose (Saris et al., 2000), hyaluronan (Brun et al., 1999) and chitosan (Suh and Matthew, 2000). The other is protein-based matrices, such as collagen (Fujisato et al., 1996) and fibrin. Natural polymers are advantageous in tissue engineering applications as they can undergo cell-specific interactions (Grande et al., 1997). The use of natural materials, however, is limited by the large variation between batches, the lack of large supplies for commercial use and as they are often derived from non-human tissue they carry the risk of transferring pathogens (Temenoff and Mikos, 2000a).

Protein-based Matrices

Collagen: Type I collagen-based structures have generally been used as carriers for chondrocytes or mesenchymal stem cells (Kawamura et al., 1998; Frenkel et al., 1997). Many preparations of collagen type I are soluble under acidic conditions. The neutralization of collagen solutions results in the formation of a hydrated collagen gel. When cells are included in the neutralizing solution, they are effectively encapsulated in the collagen gel. Collagen, as a natural bodily constituent, is made up of fibrils with cell adhesive surface and carries the required biological information for their activity. Furthermore, the degradation products of collagen are physiological and non-toxic. One of the main shortcomings in using these systems in

clinical practice is the poor mechanical strength. An additional concern associated with the use of type I collagen as a scaffold material is that most cartilages do not contain type I collagen. Some authors suggested type II collagen from native cartilage matrix may be better than type I collagen (Nehrer et al., 1997). Finally, it should be recalled that all collagens from mammalian sources are under severe scrutiny by the regulatory Authorities in relation to threatens connected to bovine spongiform encephalopathy (BSE).

Fibrin: Fibrin clots or fibrin glues are the resulting products from the reaction of fibrinogen and thrombin. Fibrin is proinflammatory and induces its own degradation and substitution by cellular components of the extravascular tissue spaces. Its degradation products are non-toxic physiological substances. Fibrin has been employed extensively as a scaffold material for incorporating chondrocytes into the exogenous fibrin clot, both *in vitro* (Fortier et al., 1997) and *in vivo* (Hendrickson et al., 1994). However, some immunological reactions to exogenous fibrin have been observed in several animal studies (Haisch et al., 2000).

Carbohydrate Polymers

Alginate: Alginate is an anionic polysaccharide extracted from seaweeds and from bacterial culture broths. In the presence of calcium cations, alginate chains are crosslinked by ionic bonding through guluronic acid blocks (Wong et al., 2001). Typical methods for *in vitro* chondrocyte culture in alginate involve encapsulation of cells in beads made by dropping cells suspended in 1-20% alginate solution into a bath of $CaCl_2$. If $CaSO_4$ were used as a crosslinking agent, the time required for crosslinking will be lengthened. This allows for injectable delivery of chondrocytes in alginate and results in *in situ* gelation (Paige et al., 1995). Within three dimensional alginate cultures, dedifferentiated chondrocytes can readily redifferentiate (Bonaventure et al., 1994), although chondrocytes do not proliferate in

alginate. Likewise, bone marrow derived mesenchymal stem cells can differentiate into chondrocytes when seeded within this matrix and under the appropriate nutritional and stimulatory conditions, both *in vitro* and *in vivo* (Diduch et al., 2000).

Agarose: Agarose is the compositionally purest polysaccharide component of the agar material used for bacterial cell culture which is isolated and purified from red seaweeds. The best agarose samples contain very little amount of ionic groups (mainly sulfate, but also carboxylate). Typically, agarose is heated in aqueous solution to maximize solubility, and then mixed with cold media containing cells (Buschmann et al., 1992). The mixture forms a hydrogel when cooled, effectively encapsulating cells. *In vitro* cultures of chondrocytes in agarose have demonstrated the production of significant amounts of cartilage ECM and increases in the mechanical properties of the constructs. But it has poor biodegradability because there is no enzyme degradation system for agarose in mammalian tissues.

Hyaluronan: Hyaluronan is a physiological component of the articular cartilage matrix and can induce embryonic mesenchymal stem cells to differentiate into chondrocytes (Kujawa and Caplan, 1986). Theoretically, hyaluronan would be an ideal matrix to support articular cartilage repair. However, crosslinking by esterification or other chemical methods are usually necessary to fabricate hyaluronan-based scaffold, which will compromise its biocompatibility. Matrices composed of hyaluronan have been frequently used as carriers for chondrocytes or bone marrow derived mesenchymal stem cells (Brun et al., 1999). Hyaluronan matrices loaded with such cells have been shown to form a cartilage-like tissue both *in vitro* and *in vivo* (Solchaga et al., 1999). Hyalograft C is a hyaluronan-based scaffold which has been used clinically in the treatment of articular cartilage lesions, and a recent clinical study has showed that 96.7% of the repaired tissue was hyaline cartilage (Pavesio et al., 2003). Such constructs have likewise been shown to support cell differentiation processes.

Chitosan: Chitosan is a cationic polymer whose biological properties have been extensively demonstrated. Its activity towards chondrocytes and its processability have rapidly increased the interest of the researchers on the application of structures based on this polymer as scaffolds in cartilage tissue engineering. Sechriest et al. have utilized a hydrogel scaffold comprised of ionically crosslinked chondroitin sulfate A and chitosan to support chondrogenesis (Sechriest et al., 2000). Several *in vitro* studies yield evidence of its potential value as a matrix to facilitate articular cartilage repair. It efficiently supports not only chondrogenic activities (Suh and Matthew, 2000), but also the *in vitro* expression of cartilage extracellular matrix proteins by human chondrocytes (Lahiji et al., 2000). It can also serve as a carrier for growth factors. Chitosan has excellent biodegradability.
Since the work described in this thesis involves the characterization and the application of a chitosan-derived polymer in protocols of cartilage regeneration, chitosan features and application as biomaterial in cartilage tissue engineering will be analyzed in detail further on, in dedicated paragraphs.

Synthetic Materials

The main feature of synthetic polymers is the chance of mass-producing polymers with custom-designed properties. Poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and co-polymers of PLA and PGA (PLGA) are commonly used in tissue engineering studies (Vacanti et al., 1991; Freed et al., 1993), as they have Food and Drug Administration (FDA) approval for use within the human body. These materials degrade through hydrolysis of the ester bond in the polymer backbone. Controls of the physical characteristics of the scaffolds, such as fibre diameter, pore size, and polymer crystallinity can regulate the scaffold degradation rates, which can range from 6-8 weeks in the case of highly porous PGA fibrous mesh to 6-18

months in the case of a highly crystalline PLA (Hooper et al., 1998). The degradation profiles of synthetic polymers can therefore be controlled to match the rate at which the tissue develops, hence ensuring the structural integrity of the construct is maintained throughout tissue regeneration (Woodfield et al., 2002).

Similarly, the mechanical properties of these scaffolds can be regulated and the elastic modulus has been shown to range from 5 kPa to 1 GPa. The most common material used for cartilage tissue engineering has been non-woven PGA mesh (Freed et al., 1994a). PLA and PLGA sponges and foams are stiffer than PGA scaffolds and are in general easier to process than PGA owing to their solubility in common organic solvents. However, there are some shortcomings of these materials. These polymers have relatively poor cell adhesion and tissue-integration properties and are potentially poorly biocompatible. Further, there is a significant foreign body giant cell reactions associated with these systems. This is particularly true in applications for reconstructive surgery, where acute reactions to subcutaneous implants involve a significant inflammatory response (Cao et al., 1998). Inflammatory agents such as interleukin-1 (IL-1) have been shown to inhibit cartilage formation and degrade cartilage ECM. Even in a non-inflammatory environment, the natural breakdown products of these materials are acidic and will lower local pH, rendering the environment cytotoxic. Chondrocytes are known to be acutely sensitive to changes in environmental pH (Gray et al., 1988).

Other synthetic polymers that have been used in tissue engineering applications include poly(ethyleneterephalate) (PET), poly(caprolactone) (PCL) and poly(tetrafluoroethylene) (PTFE). Ideally a scaffold that is to be implanted into the human body should be bioderodible and biodegradable (Freed et al., 1999) and the degradation products should be non-toxic (Freed et al., 1994b; Agrawal and Ray, 2001).

Bioactive Factors

Signalling molecules, including growth factors, cytokines, and non-proteinaceous chemical compounds, are used to trigger tissue growth in cartilage tissue engineering. Indeed, signalling molecules, which bind surface receptors to activate intracellular signal pathways, are the main source of different stimuli towards the cells, such as proliferation, differentiation, extracellular matrix synthesis during the overall process of tissue regeneration (Kuo et al., 2006).

Growth factors responsible of regulatory effects on chondrocytes or stem cells for cartilage tissue engineering include members of the TGF-β superfamily, insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), and epidermal growth factor (EGF) family. Among these growth factors, TGF-βs (Li et al., 2005; Lee et al., 2004a; Barbero et al., 2004) are the most potent stimulators of chondrogenesis in MSCs, and enhance the synthesis of cartilage ECM in chondrocytes. Recently, bone morphogenetic protein (BMP)-2 (Park et al., 2005), a member of the TGF-β superfamily, and FGF-18 (Davidson et al., 2005) have also been shown to promote chondrogenesis of MSCs and limb bud mesenchymal cells, respectively. Growth factors that mediate chondrocytic physiology rather than promote chondrogenesis of MSCs include IGF, FGF, and PDGF. To enhance cartilage growth, these factors commonly work in tandem with TGF-βs (Stevens et al., 2004). A recent trend in cartilage tissue engineering is to exploit a "cocktail" of growth factors to improve their effect and to simulate the physiological growth factors' environment. In a recent study, the combinations of TGF-β3/BMP-6, and TGF-β3/IGF-1 effectively promoted chondrogenesis of MSCs (Indrawattana et al., 2004). IGF-1 together with bFGF and TGF-β2 increased cartilage-specific ECM expression and improved the histological features of engineered cartilage (Chua et al., 2004).

1.9 Chitosan

Chitosan is a partially deacetylated derivative of chitin, the second most abundant natural biopolymer on earth, which is the main component of the exoskeleton of marine crustaceans and cell walls of fungi. Chitosan is a linear polysaccharide consisting of β(1-4) linked D-glucosamine residues ($GlcNH_2$) with a variable number of randomly located N-acetylglucosamine groups (GlcNAc). The main parameters influencing the characteristics of this copolymer are its molecular weight (MW) and the degree of deacetylation (DD), representing the proportion of deacetylated units. These parameters are determined by the conditions of preparation but can be further modified: the degree of deacetylation can be lowered by reacetylation (Sorlier et al., 2001) and the molecular weight can be lowered by acidic depolymerization (Dong et al., 2001).

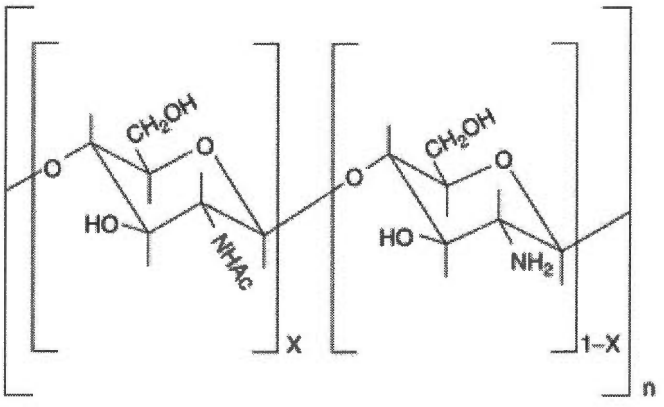


Fig. 1.15: Chitosan chemical structure.

Depending on the source and preparation procedure, chitosan's average molecular weight may range from 300 to over 1000 kDa, with a degree of deacetylation from 30 to 90%. Chitosan is a semi-crystalline polymer and the degree of crystallinity is a function of the degree of deacetylation. While crystallinity is maximum for chitin (0% deacetylated) and fully deacetylated

(100%) chitosan, intermediate degrees of deacetylation are characterized by minimum crystallinity.

In its crystalline form, chitosan is normally insoluble in aqueous solutions above the pH 7; however, in dilute acids (pH<6), the protonated free amino groups on glucosamine facilitate solubility of the molecule (Athanasiou et al., 2001; Madihally and Matthew, 1999).

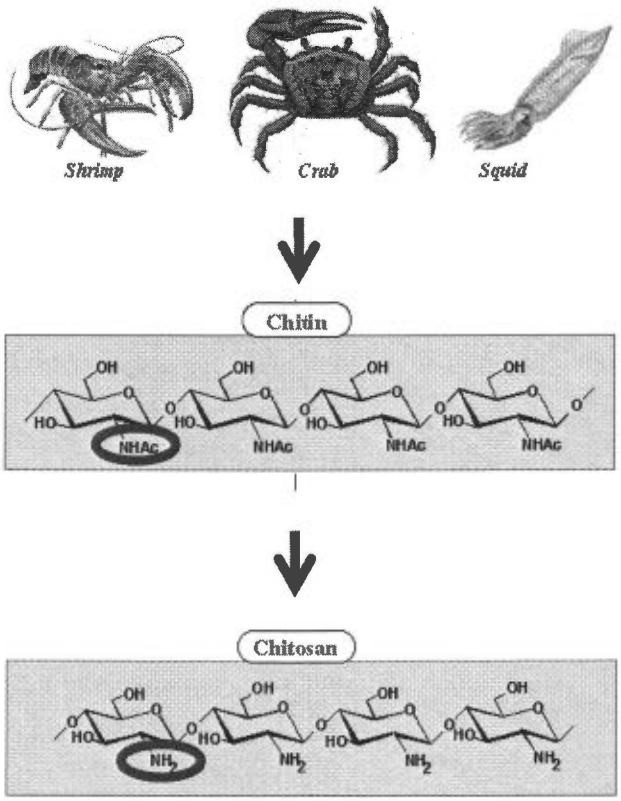


Fig. 1.16: Schematic diagram of the chemical structure of chitin and chitosan.

1.9.1 Chitosan in Biomedical Sciences

Nowadays chitosan is receiving a great deal of interest for medical and pharmaceutical applications. The main reasons for this increasing attention are the interesting intrinsic properties of this polymer (Berger et al., 2004a). Indeed, chitosan is known for being biocompatible allowing its use in different medical applications such as topical ocular application (Felt et al., 1999), implantation (Patashnik et al., 1997) or injection (Song et al., 2001). Moreover, chitosan is considered as biodegradable because it is metabolized by certain human enzymes, especially lysozyme (Muzzarelli, 1997). In addition, it has been demonstrated that chitosan acts as a penetration enhancer by opening epithelial tight-junctions (Kotze et al., 1999). Due to its positive charges at physiological pH, chitosan is also bioadhesive, a property that determines an increasing in retention at the site of application [11,12]. Chitosan also promotes wound-healing [13,14] and has bacteriostatic effects [15,16]. Finally, chitosan is very abundant, and its production is of low cost and ecologically interesting [17]. In medical and pharmaceutical applications, chitosan is used as a component in hydrogels.

Chitosan is a cationic polymer, with chemical groups responsible for electrostatic interactions with anionic glycosaminoglycans (GAG), proteoglycans and other negatively charged molecules. This property is of paramount interest because a large number of bioactive molecules, such as cytokines/growth factors are linked to GAG (mostly with heparin and heparan sulphate), and therefore a chitosan–GAG complex may retain and concentrate growth factors secreted by colonizing cells (Madihally and Matthew, 1999). Moreover, the presence of the N-acetylglucosamine moiety on chitosan also suggests related bioactivities. In fact, chitosan oligosaccharides have a stimulatory effect on macrophages, and both chitosan and chitin are chemo-attractants for neutrophils both *in vitro* and *in*

vivo. Lysozyme is the primary enzyme responsible for *in vivo* degradation of chitosan through hydrolysis of acetylated residues, other proteolytic enzymes have shown low level of degradation activity on the molecule. The degradation rate of chitosan is inversely related to the degree of crystallinity, and thus on deacetylation. Highly deacetylated forms may thus last several months *in vivo*; eventual degradation of the polymeric chain produces chitosan oligosaccharides of variable length. A direct correlation between degree of deacetylation of the chitosan and cell adhesion has been reported (Mao et al., 2004). Therefore, careful selection of chitosan grade is crucial while developing a scaffold for tissue engineering. Host tissue response to chitosan-based implants has been characterised widely: in general, these materials stimulate a minimal foreign body reaction, with little or no fibrous encapsulation (Vandevord et al., 2002). Formation of normal granulation tissue associated with accelerated angiogenesis, appears to be the typical course of the healing response. This immunomodulatory effect has been suggested to stimulate the integration of the implanted material by the host (Suh and Matthew, 2000).

One of the properties of chitosan is that it can be moulded in various forms (Hu et al., 2004). Porous chitosan-based scaffolds are generated by freezing and lyophilizing chitosan solutions (Risbud and Bhonde, 2000) or by processes such as an "internal bubbling process" (IBP) where $CaCO_3$ is added to chitosan solutions to generate chitosan–$CaCO_3$ gels (Chow and Khor, 2000). Ice removal by lyophilization generates a porous material whose pore size and orientation can be controlled by variation of the freezing rate, the ice crystal size and the geometry of thermal gradients during freezing. It is then possible to mould the obtained material as porous membranes, blocks, tubes and beads. Mechanical properties of chitosan scaffolds are closely related to pore size and orientation. Tensile testing of hydrated samples showed that porous membranes have greatly reduced elastic moduli (0.1–0.5 MPa) compared to non-porous membranes (5–7 MPa). The

extensibility (maximum strain) of porous membranes varied from values similar to non-porous chitosan (approximately 30%) to greater than 100% as a function of both pore size and orientation. Tensile strengths of the porous structures were reported to be in the range of 30–60 kPa (Madihally and Matthew, 1999; Suh and Matthew, 2000). Rapid prototyping technique has been applied to fabricate porous chitosan scaffolds (Geng et al., 2005). This procedure sequentially dispenses sodium hydroxide solution and chitosan dissolved in an acetic acid resulting in a gel-like chitosan strand.

Another interesting property of chitosan is its intrinsic antibacterial activity. Studies have shown that chitosan can reduce the infection rate of experimentally induced osteomyelitis by *Staphylococcus aureus* in rabbits (Aimin et al., 1999). Its cationic amino group associates with anions on the bacterial cell wall, suppressing biosynthesis; moreover, chitosan disrupts the mass transport across the cell wall accelerating the death of bacteria.

Chitosan is also a preferred carrier for drug delivery (Aimin et al., 1999), thus combining its intrinsic antibacterial activity with that of the bound antibiotic. When added to HA and plaster of Paris to obtain a composite for sustained vancomycin or fosfomycin release, the composite material was able to inhibit methicillin-resistant *S. aureus in vitro* for as long as 3 months, a period compatible with the treatment of most orthopedic infections (Buranapanitkit et al., 2004).

Chitosan has been combined with a variety of delivery materials such as alginate, hydroxyapatite, hyaluronic acid, calcium phosphate, PMMA, poly-L-lactic acid (PLLA), and growth factors for potential application in orthopaedics. In conclusion, chitosan offers a broad possibilities for cell-based tissue engineering (Hu et al., 2004). Possible matrix preparations for cell cultures include gels (Chenite et al., 2000), sponges, fibres (Tuzlakoglu et al., 2004), or porous compositions of chitosan with ceramic (Zhang and Zhang, 2004) or other polymeric materials such as collagen or gelatin

(Risbud et al., 2001) to adjust cell seeding properties and mechanical behaviour of cell transplants for the intended clinical application.

Chitosan has been used as a scaffolding material in articular cartilage engineering (Suh and Matthew, 2000; Lahiji et al., 2000), due to its structural similarity with various GAGs found in articular cartilage. This is of paramount importance given GAGs fundamental role in modulating chondrocyte morphology, differentiation, and function. Chondrocytes cultured *in vitro* on chitosan substrates maintained round morphology and preserved synthesis of cell–specific ECM molecules (Lahiji et al., 2000). Chitosan was used to improve chondrocyte attachment to PLLA films with a positive effect either on cell adhesion, than in proliferation and biosynthetic activity (Cui et al., 2003). Similarly, to increase the cellular adhesiveness of chitosan, Hsu et al. have developed chitosan–alginate–hyaluronan complexes with or without covalent attachment with RGD containing protein. Cell-seeded scaffolds showed neocartilage formation *in vitro*. When chondrocyte seeded scaffolds were implanted into rabbit knee cartilage defects, partial repair was observed after 1 month both in the presence or absence of RGD indicating potential of this composite material for cartilage regeneration (Hsu et al., 2004). Chitosan-based scaffolds can deliver growth factors to promote the ingrowth and biosynthetic potential of chondrocytes. Lee et al. reported porous collagen/CS/GAG scaffolds loaded with TGF-β1 (Lee et al., 2004b). This scaffold exhibited controlled release of TGF-β1 and promoted cartilage regeneration.

1.9.2 Covalently-crosslinked Chitosan Hydrogels

Hydrogels based on covalently crosslinked chitosan can be divided into three types with respect to their structure:

- chitosan crosslinked with itself;
- hybrid polymer networks (HPN);
- semi- or fully-interpenetrating polymer networks (IPN).

In the simplest structure, chitosan crosslinked with itself, crosslinking involves two structural units that may or may not belong to the same chitosan polymeric chain (Monteiro, Jr. and Airoldi, 1999). The resulting structure could be considered as a crosslinked gel network dissolved in a second entangled network formed by chitosan chains of restricted mobility.

In hydrogels formed by a HPN, the crosslinking reaction takes place between a structural unit of a chitosan chain and a structural unit of a polymeric chain of another type, even if crosslinking of two structural units of the same type and/or belonging to the same polymeric chain cannot be excluded.

Finally, semi- or full-IPNs contain a non-reacting polymer added to the chitosan solution before crosslinking. The result is the formation of a crosslinked chitosan network in which the non-reacting polymer is entrapped (semi-IPN). At this point it is possible to further crosslink this additional polymer in order to have two entangled crosslinked networks forming a full-IPN, whose microstructure and properties can be quite different from its corresponding semi-IPN.

In each of the three different structures, covalent bonds are the main interactions that form the networks, but other interactions cannot be excluded. In fact, secondary interactions, such as hydrogen bridges and hydrophobic interactions, occur between acetylated units of chitosan and lead to a more solid-like gel if the degree of deacetylation is low enough, but as crosslinking density increases, the hydrogel rigidity is predominantly determined by covalent bonds.

When a different polymer is included, additional secondary interactions between this polymer and chitosan arise and participate in the formation of the hydrogel, as it happens in the case of HPN formed with gelatine (Zhao et al., 2002) or in semi-IPN with polyether (Beena et al., 1995), silk fibroin (Gobin et al., 2005) or PEO (Khalid et al., 2002).
Preparation of a hydrogel containing a covalently crosslinked chitosan requires chitosan and a crosslinker in an appropriate solvent, usually water. If the purpose is the formation of a HPN or a semi- or full-IPN other polymeric components need to be added.

Crosslinking agents are molecules with at least two reactive functional groups that allow the formation of bridges between polymeric chains (Berger et al., 2004a). Direct crosslinking in aqueous media is of course desirable, but the addition of potentially toxic auxiliary molecules is often required to initiate or catalyse polymerization or crosslinking.
To date, the most common crosslinkers used with chitosan are dialdehydes such as glyoxal (Khalid et al., 2002; Patel and Amiji, 1996) and in particular glutaraldehyde (Yamada et al., 2000; Denkbas et al., 1999). Their reaction with chitosan is well-documented; the aldehyde groups form covalent imine bonds with the amino groups of chitosan, due to the resonance established with adjacent double ethylenic bonds (Monteiro, Jr. and Airoldi, 1999) via a Schiff reaction. However, the reaction with hydroxyl groups of chitosan cannot be excluded. Dialdehydes allow direct reaction in aqueous media, under mild conditions and, in principle, without the addition of auxiliary molecules such as reducers (Khalid et al., 2002).
The main drawback of dialdehydes (such as glutaraldehyde) as crosslinkers is that they are generally considered to be toxic (Ballantyne and Jordan, 2001; Leung, 2001). It's been demonstrated that glutaraldehyde is neurotoxic, and its fate in the human body is not fully understood

(Beauchamp, Jr. et al., 1992) and glyoxal is known to be mutagenic (Murata-Kamiya et al., 1997).

It is therefore of paramount importance an extensive purification of the hydrogels before administration, to ensure the complete removal of unreacted dialdehydes which could otherwise disclose toxic effects. The unavoidable extensive purification can then suggest the use of reducing agents, a slight excess of which can simultaneously produce more stable crosslinks and safer reduction products of aldehydes, i.e. alcohols. Other covalent crosslinkers for chitosan have been investigated as alternatives. Besides dialdehydes, crosslinkers such as diethyl squarate (DES) (De Angelis et al., 1998), dimethylsuberimidate (DMS) (Charulatha and Rajaram, 2003; Charulatha and Rajaram, 1997), 1,4 butanediol diglycidyl ether (BDGE) (Subramanian et al., 2004) or genipin (Jin et al., 2004) can exhibit direct crosslinking mechanisms. However, there is a lack of data regarding the biocompatibility of diethyl squarate, while oxalic acid has shown *in vitro* toxicity in rats (Klug et al., 2001). The use of genipin is an interesting alternative to dialdehydes. It is a naturally occurring material, which is commonly used in herbal medicine and as a food dye (Mi et al., 2002). The biocompatibility of genipin in humans has not been assessed yet, but it is not cytotoxic *in vitro* (Sung et al., 2001) and has been shown to be biocompatible after injection in rats (Mi et al., 2002).

Another approach is the formation of covalently linked networks, close to HPN, by use of water soluble, biocompatible polymers *ad hoc* functionalized to act as crosslinker. Such functionalized biopolymers can be poly(ethylene glycol) (PEG) diacrylate, oxidised β-cyclodextrin, telechelic-PVA or dialdehydes derived from PEG or scleroglucan. However, even if these products are known to be biocompatible before functionalization, data concerning the biocompatibility of their functionalized derivatives are lacking. In any case, the preparation of a HPN requires the use of additional polymers

that bear reactive groups, able to undergo crosslinking with chitosan. When gelatine, collagen or a sialylating agent, all bearing amine groups are used, they are crosslinked via glutaraldehyde with the amine groups of chitosan (Monteiro, Jr. and Airoldi, 1999). For the preparation of a semi-IPN, the additional polymers do not need functional groups able to react with the crosslinker used. Examples are polyether (Beena et al., 1995), poly(vinyl pyrrolidone) (PVP) (Risbud et al., 2000), silk fibroin (Gobin et al., 2005), PEO (Khalid et al., 2002), poly(N-isopropylacrylamide) (varez-Lorenzo et al., 2005) and PEG (Bhattarai et al., 2005). The latter two polymers can also serve for the preparation of a full-IPN.

The formation of a full-IPN requires crosslinking also of the additional polymer. This can be performed by UV irradiation to polymerize and crosslink for instance PEG (Bhattarai et al., 2005) or by the addition of a second crosslinker, such as methylene bis-acrylamide to crosslink poly(N-isopropylacrylamide) networks. However, the addition of a second crosslinker certainly decreases the biocompatibility of the resulting scaffold. Covalent crosslinking can also be performed after chitosan (polyelectrolytic) complexation with chondroitin sulfate (Shahabeddin et al., 1990), collagen (Zhang et al., 2006) or poly(acrylic acid) (PAA) (Rossi et al., 2003). In addition, chitosan/PVA complexes (Koyano et al., 1998) and grafted chitosan networks of poly(N-isopropylacrylamide)- (Lee et al., 2004c), fructose- (Yagi et al., 1997) or N,O-carboxymethyl–chitosan (Costain et al., 1997) can be crosslinked. In these cases, crosslinking is added in order to reinforce the complexed network and to avoid dissolution during swelling (Berger et al., 2004a).

It is important to characterize the conditions of the crosslinking reaction, since they determine and allow the modulation of the crosslinking density, which is the main parameter influencing interesting properties of hydrogels

such as drug release and mechanical strength. Covalent crosslinking, and therefore the crosslinking density, is influenced by various parameters, but mainly dominated by the concentration of the used crosslinker (Peppas et al., 2000; Mi et al., 2000). It is generally favoured when chitosan has high MW and when the temperature is sufficiently high (Mi et al., 2000). Moreover, since crosslinking requires mainly deacetylated reactive units, a chitosan with high degree of deacetylation is preferable (Draget, 1996). Obviously, crosslinking reactions are also influenced by their duration (Knaul et al., 1999). As the main parameters influencing crosslinking density have been identified, the possibilities of monitoring reaction during hydrogel formation should now be investigated to facilitate the development of tailor-made hydrogels.

Covalent crosslinking leads to the formation of a permanent network allowing the free diffusion of water and enhancing the mechanical properties of the gel. As a result of these characteristics, covalently crosslinked chitosan hydrogels have two main applications, as permanent networks used as scaffolds in cell culture and as drug delivery systems allowing release of bioactive materials by diffusion.

1.9.3 Chitosan Derivatization and Chitlac Synthesis

One of the most interesting feature of chitosan as biomaterial is connected with the presence of amino groups located on the glucosamine units. Chemical derivatization based on the reactivity of the glucosamine residues leads to strong modification of the physico-chemical and biological properties of the polycation. Derivatization examples include acylation (Kubota et al., 2000) (Sorlier et al., 2001), alkylation (Yang et al., 2005) and carboxymethylation (Muzzarelli et al., 1984).

Starting from these assumptions, our group has modified highly deacetylated chitosan by grafting lactose moieties on the free amino groups of the polymer to obtain, by reductive amination, the corresponding lactitol derivative. A low charged, highly hydrophilic chitosan derivative was obtained, namely Chitlac. This synthetic glycopolymer exhibited the ability to induce chondrocyte aggregation leading to the formation of nodules of high dimensions (up to 0.5–1 mm) within 12–24 hours. It stimulated as well the biosynthesis of markers typical of articular cartilage, such as type II collagen and glycosaminoglycan (Donati et al., 2005).

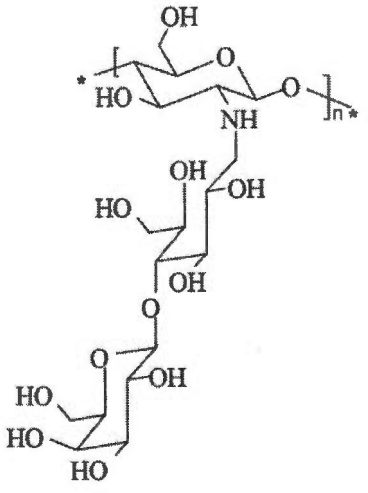


Fig. 1.17: Chitlac chemical structure.

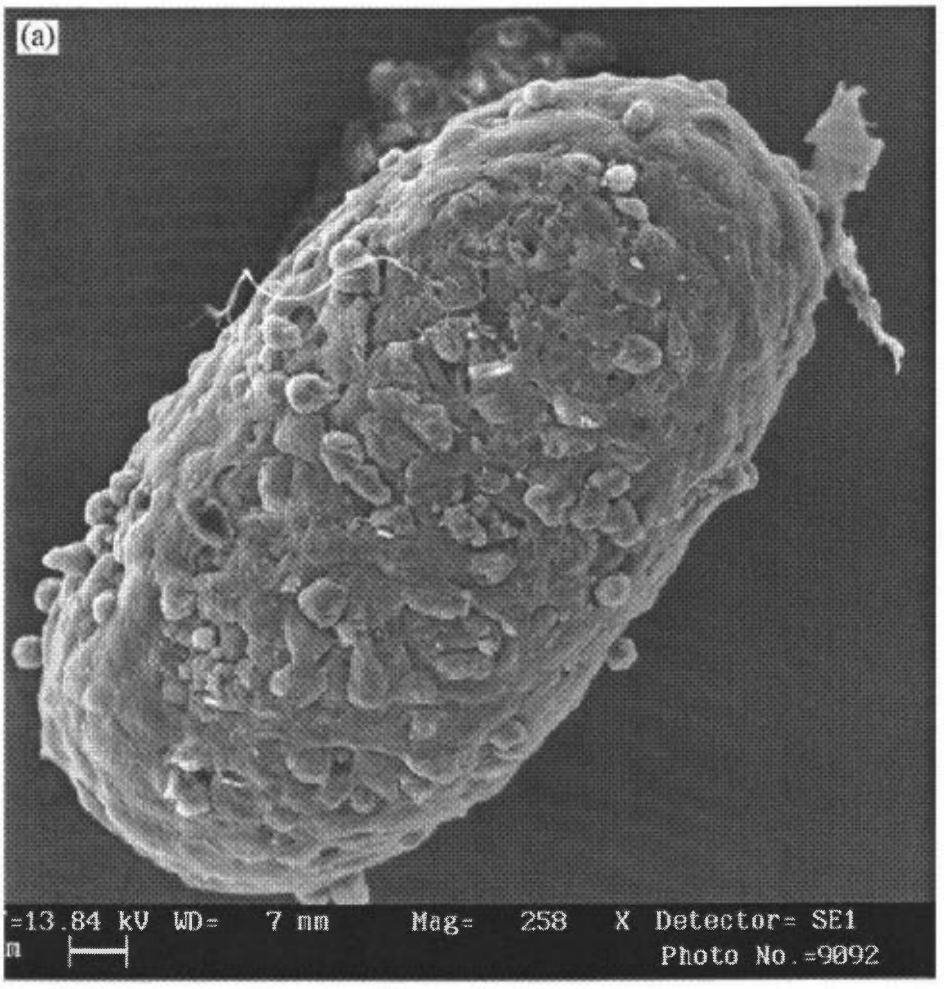


Fig. 1.18: SEM image of a nodule (with a major axis of about 325 nm) formed by aggregated chondrocytes cultured in a dish coated with chitlac; the bar indicates 20 nm (Donati et al., 2005).

These findings seem promising in connection to a possible application of Chitlac in protocols of tissue engineering applied to the regeneration of articular cartilage.

The localization of Chitlac at the plasma membrane of isolated chondrocytes and its permanence at the same site also after nodule formation indicate that the process is mediated by a specific binding of Chitlac to cells, most likely through its β-galactose residues (Donati et al., 2005).

Further experiments demonstrated the involvement of Galectin-1 as a molecular bridge between Chitlac and chondrocyte cell surfaces (Marcon et al., 2005). Galectin-1 is a member of the S-type galactoside-binding animal lectins (Barondes et al., 1994; Cooper and Barondes, 1999). The discovery that Galectin-1 binds with high affinity to polylactosamine-containing ligands (such as laminin) and the co-localization of Galectin-1 with laminin in extracellular matrix suggested that its major function could be to promote cell adhesion to glycoconjugates (Ramkumar and Podder, 2000; Van den Brule et al., 1995; Wasano and Hirakawa, 1997).

CHAPTER 2
General Aim

In 1743, Hunter stated: "From Hippocrates to the present age it is universally allowed that ulcerated cartilage is a troublesome thing and that when destroyed, it is not recovered" (Hunter, 1743). Historically, many scientists and clinicians attempted to develop clinically useful procedures to repair damaged articular cartilage, but these have not yet proved entirely successful. Treatment options are limited and the long-term outcome is still uncertain.

Self-repair of cartilage is limited and the repair tissue that is formed does not perform as well as hyaline cartilage, degrades over time and usually lacks the mechanical properties and ultrastructure to ensure long-term stability (Lonner, 2004).

Tissue engineering, considered as a potential therapeutic option for the regeneration of damaged tissue, has been defined as "an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function" (Langer and Vacanti, 1993). Tissue engineering can perhaps be best described as the use of a combination of interactive factors: cells, engineering materials to either carry or encapsulate the cells, and suitable bioactive factors.

The overall goal of this thesis was the utilization of a lactose-modified chitosan (chitlac) in cartilage tissue engineering. Chitosan-based structures has been extensively used in biomedical sciences and several studies were focused on cartilage regeneration (Di Martino et al., 2005). Bioactive properties of chitlac towards chondrocytes had already been pinpointed: this synthetic glycopolymer exhibited the ability to induce chondrocyte aggregation and stimulated as well the biosynthesis of markers typical of

articular cartilage, such as type II collagen and glycosaminoglycan (Donati et al., 2005).

The first objective of this thesis (CHAPTER 3) was the physico-chemical characterization of this chitosan-derived polymer: the rheological behaviour of chitlac and chitosan aqueous solutions were compared in order to assess the influence of the residues grafted onto the polymer backbone on the hydrodynamic properties of the biopolymer. An important aspect to be investigated was the kinetic of degradation by lysozyme of the lactose-modified polymer, in order to evaluate the overall stability of chitlac.

The second objective (CHAPTER 4) involved the rheological characterization of blends of chitlac and hyaluronic acid (HA). HA is the molecule that being responsible for the synovial fluid's rheological properties (Mazzucco et al., 2002) is widely used in viscosupplementation therapy for the treatment of osteoarthritis through joint injections (Kelly et al., 2004). The viscoelastic properties of the mixture of the two polymers were evaluated in order to define the effect of the chitlac component on the HA network features.

The third objective (CHAPTER 5) was the development of a three dimensional scaffold based on crosslinked chitlac to be used as a carrier of chondrocytes in cartilage tissue engineering protocols. Cell proliferation, morphology and extracellular matrix synthesis were evaluated in order to assess the biocompatibility and the bioactivity of the produced structures.

CHAPTER 3
Synthesis and Physico-chemical Characterization of Chitlac

3.1 Introduction

Following previously reported findings on the biocompatibility and bioactivity properties of the chitlac polymer towards chondrocytes (Donati et al., 2005), a further physico-chemical characterization of the lactose-modified chitosan has been carried out, in order to elucidate the differences existing between the native polymer and its derivative. Rheological and viscometry characterization of native chitosan and lactose-modified chitosan was performed in order to assess the influence of the presence of the lactose moieties (grafted on the polymeric backbone) on the flow properties of the biopolymer. Viscosity measurements were performed to determine the molecular weight of the used chitosan and to assess the hydrodynamic behaviour of chitlac in dilute solution in comparison to the parent polymer.

To get a direct comparison between the two polymeric species, experiments were carried out in acidic aqueous solutions, due to the poor solubility of chitosan at physiological pH. Further investigations on chitlac solution properties were performed in physiological conditions (pH 7.4 and ionic strength 0.15 M), in order to mimic the environment in which the polymer should be used *in vivo*.

Lysozyme is the primary enzyme responsible for the *in vivo* degradation of chitosan, so its action towards chitlac needed to be determined. The stability of chitlac towards enzyme degradation was tested by following the time dependence of viscosity of chitlac solution in the presence of lysozyme.

3.2 Materials and Methods

3.2.1 Materials

Chitosan and sodium cyanoborohydride ($NaBH_3CN$) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Deionized MilliQ water (Millipore, MA, USA) was used to prepare all the aqueous solutions. Lysozyme (chicken egg white) was purchased from Sigma (St.Louis, MO, USA). All other chemicals were of analytical grade.

3.2.2 Methods

Chitosan Purification

Commercially available chitosan (Aldrich), highly deacetylated (89%) and of medium MW (about 350 kDa), was purified by isopropanol precipitation from an acidified aqueous solution (pH~4.5). The precipitation was repeated three times, and the precipitated air dried before dissolution in milliQ water (pH~4.5). Chitosan solution was then exhaustively dialyzed against milliQ water, filtered through 0.45μm Millipore filters and freeze-dried.

Chitlac Synthesis

The synthesis of lactose-modified chitosan (Chitlac) was performed according to the procedure reported elsewhere (Yalpani and Hall, 1984).

Briefly, chitosan (200 mg) was dissolved in 14 ml of a mixture 1:1 of methanol and acetic acid 1% (pH 4.5); 8 ml of the same methanol:acetic acid solution containing lactose (840 mg) and sodium cyanoborohydride (350 mg) were slowly added. The obtained solution was then incubated under

stirring at room temperature for 24 hours. At the end of the reaction 60 ml of water were added and the reaction mixture was dialyzed exhaustively against deionized water, filtered through 0.45 μm Millipore filters and the polymer recovered by freeze-drying.

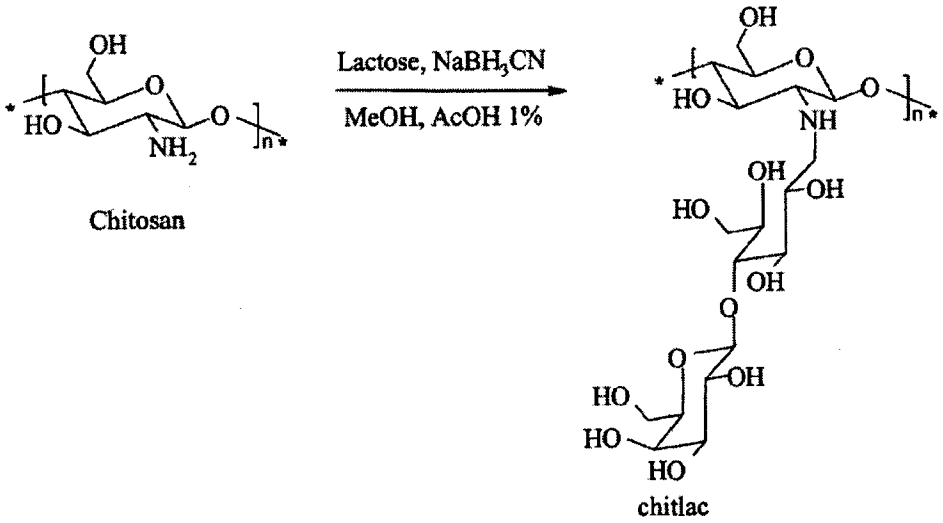


Fig. 3.1: Synthesis of chitlac via N-alkylation of chitosan with lactose (Donati et al., 2005).

Viscosity Measurements

Capillary viscosity measurements were performed at 25 °C by means of a Schott-Geräte AVS/G automatic apparatus using an Ubbelohde type viscometer.

The viscosity behaviour of macromolecular substances in solution is one of the most frequently used approaches for their characterization. From an experimental point of view, using a capillary viscometer, the viscosity of a solution is simply obtained by measuring the time employed by a given volume of liquid to flow under the gravity force through a given capillary.

The measured time interval, Δt, for a fixed geometry (length and diameter) of the capillary is dependent only from the density ρ and the viscosity η of the liquid, therefore a measurement of the flow time is a direct measurement of the liquid viscosity.

The relative viscosity η_{rel} is defined as (eq. 3.1):

$$\eta_{rel} = \frac{\eta_1}{\eta_0} = \frac{t_1 \rho_0}{t_0 \rho_1} \qquad \textit{eq. 3.1}$$

where indexes 1 and 0 are referred to the solution and to the solvent, respectively.

For sufficiently dilute solutions, $\rho_0 \cong \rho_1$, therefore (eq. 3.2):

$$\eta_{rel} = \frac{t_1}{t_0} \qquad \textit{eq. 3.2}$$

From simple time flow measurements the specific viscosity (η_{sp}) and the reduced specific viscosity (η_{sp}/c) of a polymer solution of concentration c can then be obtained (eq. 3.3 and 3.4):

$$\eta_{sp} = \eta_{rel} - 1 \qquad \textit{eq. 3.3}$$

$$\eta_{sp} / c = (\eta_{rel} - 1)/c \qquad \textit{eq. 3.4}$$

The limit of reduced specific viscosity for $c \to 0$ is the intrinsic viscosity $[\eta]$ a typical dynamic global property of a polymer chain.

Since the intrinsic viscosity $[\eta]$ is a limiting value at infinite dilution, it is a parameter which directly reflects molecular properties of the solute. Indeed, $[\eta]$ depends on size and shape of the investigated polymer, being directly proportional to its hydrodynamic volume: $[\eta]$ represents the specific volume (typically expressed as dl of solvent per gram of polymeric solute) that the chain occupies when swollen by solvent molecules, at infinite dilution.

For the polymers used in the present thesis, the intrinsic viscosity ($[\eta]$) values were determined by extrapolating the reduced specific viscosity (η_{sp}/c) values to zero concentration by the use of the Huggins equation (eq. 3.5) and by extrapolation of the reduced logarithm of the relative viscosity ($ln(\eta_{rel})/c$) by the use of Kraemer equation (eq. 3.6), respectively:

$$\frac{\eta_{sp}}{c} = [\eta] + k' \cdot [\eta]^2 \cdot c \qquad \textit{eq. 3.5}$$

$$\frac{ln(\eta_{rel})}{c} = [\eta] - k'' \cdot [\eta]^2 \cdot c \qquad \textit{eq. 3.6}$$

where k' and k'' are the Huggins and Kraemer constants, respectively.
A rather surprising generality in polymer field is that $[\eta]$ for a given polymer of molar mass M, under a fixed solvent condition (solvent species and temperature) follows a simple power law as:

$$[\eta] = kM^a \qquad \textit{eq. 3.7a}$$

For a mixture of chemically identical polymers, differing only for the molar mass, the experimentally determined average intrinsic viscosity of the mixture, $[\eta]_{exp}$, with:

$$[\eta]_{\exp} = \sum_i [\eta]_i \qquad \textit{eq. 3.7b}$$

it holds:

$$[\eta]_{\exp} = k \cdot (M_v)^a \qquad \textit{eq. 3.7c}$$

where M_v is the viscosity-average molar mass.

The relation between the intrinsic viscosity value $[\eta]$ and the polymer molar mass (M) in eq. 3.7a is referred to as the Mark-Houwink-Sakurada relation. k and a are given parameters for a specific solute-solvent system. The numerical values of these constants depending on both the nature of the polymer and the nature of the solvent, as well as on temperature.

The value of the coefficient a normally varies between 0.5 and 1. The value of this coefficient is related to the conformation of the polymer ranging from 0.5 to 0.8 for flexible polymers (coils), reaching value of $\cong$ 1 for polymers characterized by a high rigidity, whereas a values greater than 1 are expected for so called "rigid-rod" chain shape. Therefore the intrinsic viscosity has a direct correlation with the molecular weight of the polymer, through parameters that reflect chain conformation and rigidity.

Rheological Characterization

Steady-state Shear Viscosity

Polymeric fluids exhibit a variety of non-Newtonian rheological properties (Barnes et al., 1989; Bird et al., 1987).

The shear viscosity of these materials is often a non-linear function of the rate of shear. Indeed, polymeric liquids are viscoelastic materials in the sense that the stress experienced by a fluid particle depends upon the history of the deformation experienced by that particle. The elastic character of a given flow is measured by the dimensionless Weissenberg number $We = \lambda \dot{\gamma}$ where λ is a characteristic relaxation time (usually expressed in s) of the fluid, and $\dot{\gamma}$ is a characteristic shear rate of the flow(usually expressed in s^{-1}. While $We = 0$ for Newtonian fluids, it is of order 1 or 10 in many applications involving polymeric liquids.

In non-Newtonian fluids, the viscosity is generally found to decrease with increase in shear rate, giving rise to a phenomenon known as "shear-thinning" behaviour. For these materials the curve of viscosity against shear rate indicates that only in the limit of very low shear rates the viscosity is constant, whilst in the limit of very high shear rates the viscosity is again constant, but at a lower level. In the intermediate shear regime, viscosity decreases with increasing shear rate. These two extremes are known as the upper and lower Newtonian regions, and defines the two regions where the viscosity reaches constant values. The higher constant value is called "zero-shear viscosity" (η_0). Polymers are expected to show Newtonian behaviour as long as the rate of shear is low enough to allow the molecules to respond and the transient network of hydrated polymer chains to "reorganize"; when the velocity gradient is too large, the molecules are unable to keep up, and non-Newtonian behaviour results.

A model to predict the shape of flow curves was described by Cross (eq. 3.8):

$$\frac{\eta_0 - \eta}{\eta - \eta_\infty} = \left(K\dot{\gamma}\right)^m \qquad \textit{eq. 3.8}$$

Where η_0 and η_∞ refers to the asymptotic values of viscosity at very low and very high shear rates respectively, K is a constant parameter with the dimension of time and m is a dimensionless constant.
If certain approximations to the Cross model are assumed it is possible to introduce other viscosity models. For values of $\eta << \eta_0$ and $\eta >> \eta_\infty$ the Cross model reduces to (eq. 3.9):

$$\eta = \frac{\eta_0}{\left(K\dot{\gamma}\right)^m} \qquad \textit{eq. 3.9}$$

which with a redefinition of parameters can be written (eq. 3.10):

$$\eta = K_2 \dot{\gamma}^{n-1} \qquad \textit{eq. 3.10}$$

This is the "power law" model and n is called the power-law index. K_2 is called the "consistency".

Viscosity-concentration Dependence

A log-log plot of the Newtonian viscosity versus the polymer concentration highlights the presence of two regions, each characterized by a distinct viscosity-concentration dependence. These two regions are generally represented by two straight-line portions delimiting the "dilute" solution hydrodynamic behaviour from that occurring in the so called "semi-dilute" concentration regime. The intersection between the two segments is called the overlap concentration (C^*), the critical value at which hydrodynamic interactions between different chains begin to rise, resulting in the formation of entanglements with interpenetrated coil regions.

The principal difference in the theoretical approach to the two regimes involves the absence or the presence of chain entanglements. For concentration values higher than C^* the presence of intermolecular entanglements arising from coils overlap and interpenetration predominate the overall molecular motion of polymer. On the other hand for concentration values lower than C^* individual coiled molecules only very occasionally impinge and interpenetrate, thereby preserving a statistically fluctuating conformation independent from each other. The critical concentration (C^*) is then clearly dependent on molecular size and conformation.

Oscillatory shear viscosity

In dynamic experiment the material is subjected to a sinusoidal shear strain (eq. 3.11):

$$\gamma = \gamma_0 \sin(\varpi t) \qquad \textit{eq. 3.11}$$

where γ_0 is the shear strain amplitude, ω is the oscillation frequency (which can be also expressed as $2\pi f$ where f is the frequency in Hz) and t the time. The mechanical response, expressed as shear stress τ of viscoelastic materials, is intermediate between an ideal pure elastic solid (obeying to the Hooke's law) and an ideal pure viscous fluid (obeying to the Newton's law) and therefore is out of phase respect to the imposed deformation as expressed by (eq. 3.12):

$$\tau = G'(\omega)\gamma_0 \sin(\omega t) + G''(\omega)\gamma_0 \cos(\omega t) \qquad \textit{eq. 3.12}$$

where $G'(\omega)$ is the shear storage modulus and $G''(\omega)$ is the shear loss modulus. G' gives information about the elasticity or the energy stored in the material during deformation, whereas G'' describes the viscous character of the energy dissipated as heat (Ferry, 1970).

Rheological Measurements

Rheological measurements were performed on a HAAKE controlled stress rheometer (RS 150 Rheostress), with a cone-plate geometry (C60/1° Ti, gap 43 μm), at a temperature of 25 °C. The solutions of chitosan and chitlac were loaded on the plate of the rheometer and the flow properties of the samples were determined. Two replicate measurements were performed for each sample. Rheological tests were performed under continuous shear

conditions to determine steady viscosity values in the stress range 0.05–500 Pa.

Fig. 3.2: HAAKE Rheometer (RS 150 Rheostress).

Preparation of Polymer Solutions

Proper amounts of chitosan were dissolved in 0.25 M CH_3COOH/0.25 M CH_3COONa (pH 4.7) for 4 hours at room temperature. Chitlac solutions were prepared using either the acetate buffer (pH 4.7) or a physiological aqueous salt solution (0.14 M NaCl, 0.01 M Tris/HCl, pH = 7.4). Capillary viscosity measurements were performed on 0.45 μm Millipore filtered solutions with polymer concentrations ranging from 0.03 to 0.1 g/dL.

Acetate buffered (pH 4.7) chitosan and chitlac solutions with polymer concentration ranging from 0.5% to 5% (w/w) were used for rheological measurements.

Rheological characterization of chitlac was performed as well in physiological condition, at pH 7.4 and I=0.15 M. I is the ionic strength of the solution: I was given by 0.14 M NaCl and 0.01 M Tris/HCl. Ten chitlac solutions were in tested this case, with concentration ranging from 0.5% to 5% (w/w).

Chitlac Enzymatic Degradation

The kinetic of chitlac degradation with lysozyme was assessed by measuring time depencence viscosity using a Schott-Geräte AVS/G automatic apparatus and an Ubbelohde type viscometer. As the enzymatic cleavage of the polymer proceeded, the capillary viscosity of the system decreased.

A certain amount of chitlac was dissolved in PBS solution (pH 7.4) at room temperature. Lysozyme was dissolved in the same buffer. The solutions were clarified by filtering through a 0.45 μm Millipore filter and mixed in known volume ratio in order to obtain the desired final polymer and enzyme concentration.

Measurements were carried out at constant enzyme concentration (1000 U/ml) for variable polymer concentrations (0.14, 0.7 and 0.014 g/dl), as well as at constant polymer concentration (0.14 g/dl) for variable enzyme concentrations (100-1000 U/ml). Measuring temperature was 37 °C.

3.3 Results and Discussion

3.3.1 Chitlac Synthesis

In this study a highly deacetylated chitosan (89%) has been derivatized with lactose moieties via reductive amination. This chemical reaction took place between the aldehyde group of the disaccharide and the free amino groups present on the backbone of the polymer. The obtained polymer, chitlac, was characterized by a low residual positive charge on the chain (polycation), by high hydrophilicity (due to the presence of the disaccharidic moieties as side chains) and by a good solubility in aqueous solution and at physiological pH. The biological activities of this biopolymer in solution have been extensively investigated, pointing out its biocompatibility, its ability to induce chondrocyte aggregation and to stimulate the synthesis of extracellular matrix components (Donati et al., 2005).

3.3.2 Viscosity Measurements

As indicated in eq. 3.5 and 3.6, η_{sp}/C and $(\ln\eta_{rel})/C$ are expected to vary linearly with polymer concentration, the C=0 intercept of the both straight lines corresponding to the intrinsic viscosity ($[\eta]$). In Fig. 3.3 the reported η_{sp}/C and $(\ln\eta_{rel})/C$ experimental data obtained for chitosan in 0.25 M CH_3COOH/0.25 M CH_3COONa (pH 4.7) nicely followed the predicted Huggins (eq. 3.5) and Kraemer (eq. 3.6) concentration dependence, leading to a value of 3.9 dl/g for the chitosan intrinsic viscosity $[\eta]$. A molar mass of approximately 3.65 x 10^5 g/mol could then be estimated with Mark-Houwink-Sakurada, eq. 3.7 (Roberts and Domzy, 1982). Intrinsic viscosity of

synthesized chitlac (Fig. 3.4) in the same experimental conditions resulted 3.02 dl/g (calculated with both the Huggins and Kraemer equations).

Chitosan derivatization with lactose residues is not a degradative reaction and leaves the degree of polymerization of the polymer unchanged. Hence, the grafting of the lactose moieties results in an augmentation of the molecular weight of the chitlac in comparison with native chitosan. Despite this increase in molar mass the intrinsic viscosity [η] of chitlac is lower than the value of the parent chitosan, indicating a reduced value of the hydrodynamic volume-to-molar mass ratio. Having the intrinsic viscosity the dimension of a specific volume this finding, in turn, should correspond to a more "dense" coiled chain arrangement for chitlac with respect to the native chitosan. The reduced charge density accompanying lactose grafting likely favour the observed coiling ability increase. Chitosan grafting reaction, transforms primary amino groups into secondary groups, therby modifying the overall positive charge on the polymer chain, although in a way not easily quantifiable.

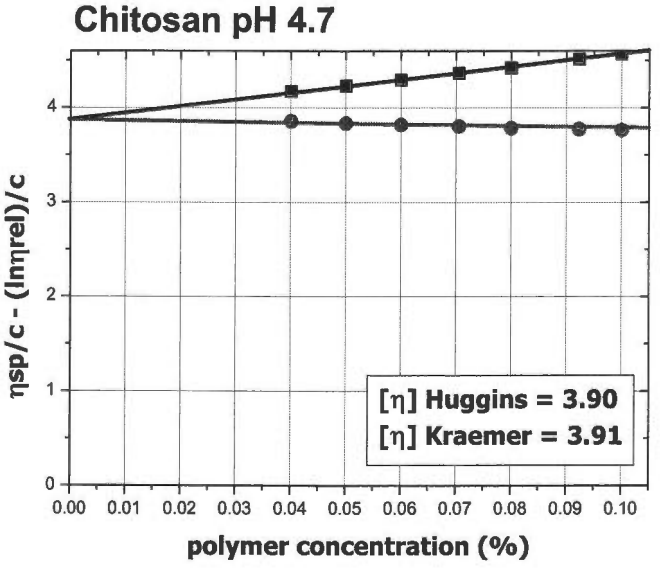


Fig. 3.3: Concentration dependence of η_{sp}/C and $\ln(\eta_{rel})/C$ for chitosan (pH 4.7).

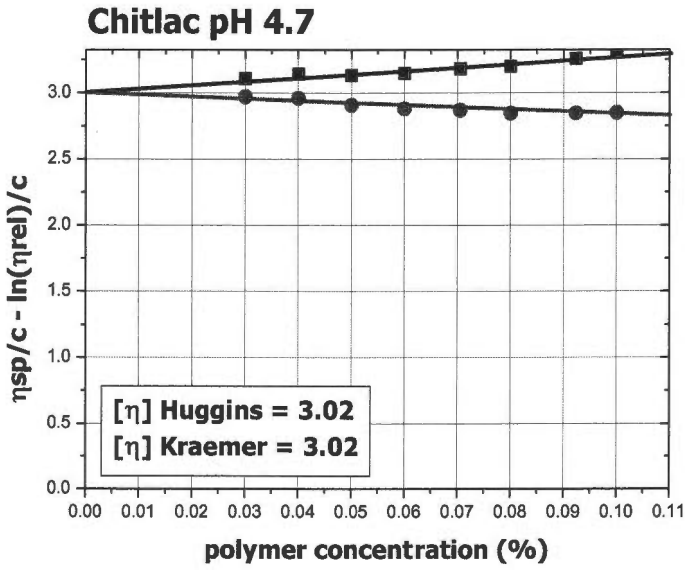


Fig. 3.4: Concentration dependence of η_{sp}/C and $\ln(\eta_{rel})/C$ for chitlac (pH 4.7).

3.3.3 Rheological Characterization

Rheological characterization of native chitosan and lactose-modified chitosan was performed in order to assess the influence of the residues grafted onto the polymer backbone on hydrodynamic behaviour of the biopolymer. Flow properties of the samples were evaluated through steady shear measurements to determine the viscosity of the polymer solutions as function of the shear rate. The concentration range was chosen in order to cover both the dilute and semi-dilute intervals, so to characterize polymer hydrodynamic behaviour in both regimes. To widen the otherwise limited concentration range achievable by the used rheometer, additional viscosity data in the dilute region were obtained using capillary viscometer.

Given the low solubility of chitosan the comparative study of chitosan and chitlac was carried out at pH 4.7, further investigations on chitlac were performed at pH 7.4, to reproduce physiological conditions needed for possible applications *in vivo*.

3.3.4 Chitosan and Chitlac Flow Curves pH 4.7

The shear rate dependence of viscosity was obtained for chitosan and chitlac (in 0.25 M CH_3COOH/0.25 M CH_3COONa) at pH 4.7 (Fig. 3.5 and 3.6) in the concentration range of 0.5-5% (w/w). Throughout the entire range, with the exclusion of the most diluted 0.5% (w/w) polymeric solution, two distinct viscosity behaviour regions were observed: the Newtonian flow region showing the constant zero-shear viscosity (η_0) at low shear rate and the power-law flow region showing the shear rate dependent apparent viscosity (η_{app}) at relatively higher shear rate. The shear rate interval at which viscosity was maintained constant covered roughly two orders of magnitude. There the rate of intermolecular disentanglements brought about by shear force exerted was nearly the same as that of entanglements newly formed (Graessley, 1974). The zero-shear viscosity has been frequently employed to study the structure-function relation of biopolymeric systems (Hwang and Kokini, 1991). Actually the magnitude of zero-shear viscosity is a macroscopic representation of the microstructural nature of biopolymers.

In contrast, the viscosity decreased with increasing shear rates in the power-law region, where the rate of disentanglements was higher than that for newly forming entanglements (Graessley, 1974). Increasing shear rate dependence of viscosity was observed with increasing polymer concentration. The explanation of this phenomenon came in terms of the degree of chain entanglements. As polymer concentration was increased, the freedom of movement of the individual chains became restricted due to the correspondingly increased number of entanglements (Graessley, 1974). This gave rise to an increase in time required to form new entanglements to replace those disrupted by the externally imposed deformation. Thus, the shear rate at which the Newtonian behaviour was lost progressively moved towards lower values with the increasing polymer concentrations, according to findings reported elsewhere (Morris et al., 1981).

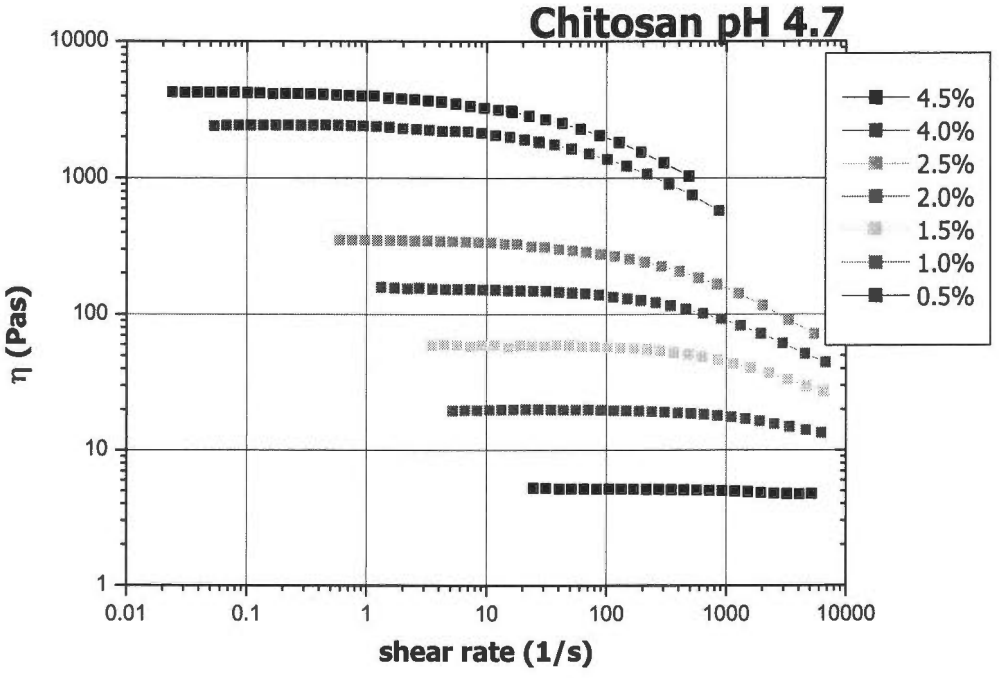


Fig. 3.5: Flow curves of solutions of chitosan at pH 4.7

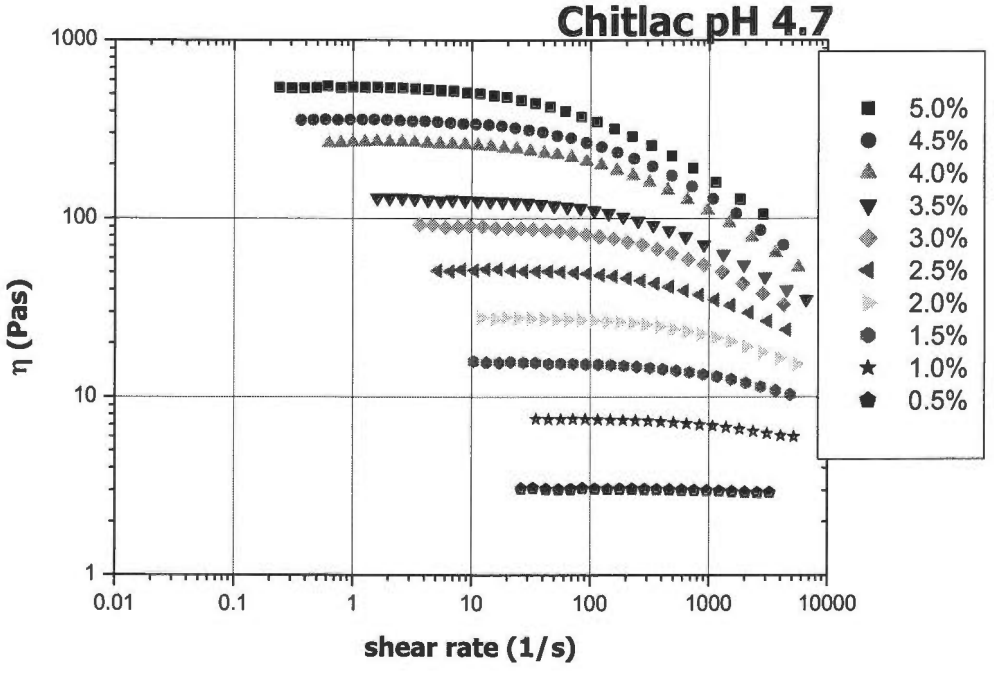


Fig. 3.6: Flow curves of solutions of chitlac at pH 4.7.

As described so far, the overall behaviour of the solutions of chitosan and chitlac was hence comparable, with an almost completely Newtonian behaviour for polymeric concentration of 0.5% (w/w) and the presence of the phenomenon of shear thinning for concentration higher than 1% (w/w). The main difference observed between chitosan and chitlac was the Newtonian viscosity value (η_0) for comparable concentrations. If, as an example, we consider the 4.5% (w/w) concentration (Fig. 3.7), there was almost one order of magnitude of difference between the chitosan sample and the chitlac sample. This situation can be accounted for by the decreased ability of chitlac to form a highly interpenetrated polymeric network. In line with the more dense coiling form, above deduced by intrinsic viscosity data, the presence of the lateral residues grafted on the polymer backbone seemed here to impede the chain to be extensively interacting through topological constrains with other polymeric chains.

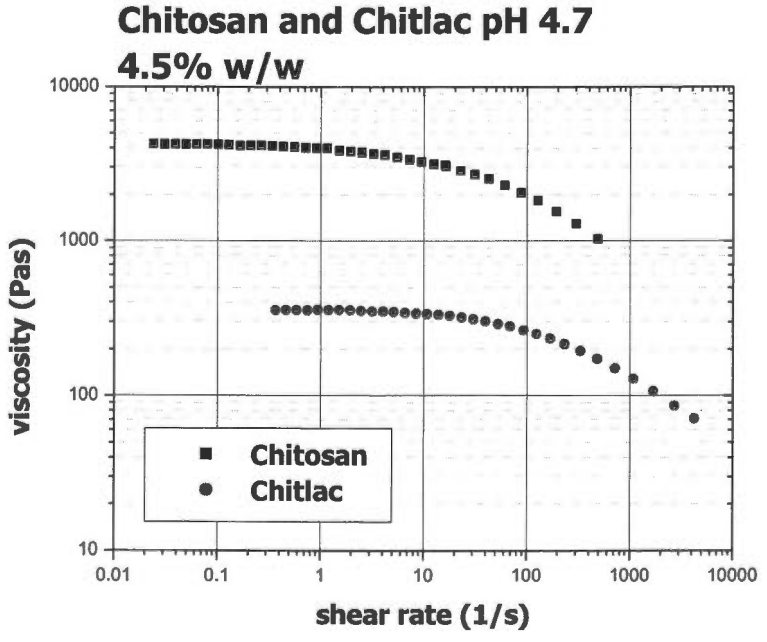


Fig. 3.7: Flow curves of solutions of chitosan and chitlac (pH 4.7, polymer concentration 4.5% w/w).

Direct comparison of the dependence of the Newtonian viscosity on the concentration of polymer solutions at pH 4.7 showed for both chitosan and chitlac the typical behaviour of polymers in good solvent (Fig. 3.8 and 3.9). Linear least square fitting of the data showed the presence of two different zones delimiting the dilute and semi-dilute concentration regimes of the polymer solution. The intersection between the two segments is the overlap concentration (C^*), the value at which hydrodynamic interactions between different chains begin to be seen, resulting in topological constrains and entanglements formation. The figures showed that C^* was determined to be approximately 1.17% for chitosan, while a value of 1.56% was found for chitlac. Intermolecular entanglements predominate the overall molecular motion of polymers at $C > C^*$, while individual molecules are statistically separated from other molecules at $C < C^*$. The critical concentration (C^*) is dependent on molecular size and conformation of a polymer: the higher molecular weight and the more rigid conformation, the lower C^*.

In the dilute region ($C < C^*$) Fig. 3.6 and 3.8 showed that the slope of the logarithmic viscosity dependence on $\ln C$ was 1.48 and 1.29 for chitosan and chitlac, respectively. These gradients are in fairly good agreement with the value range of 1.1-1.4 reported for other polysaccharides (Launay et al., 1986). The gradients in dilute solutions are almost independent of the degree of branching and the intrinsic viscosity. In contrast, the slope was 3.91 for chitosan and 3.25 for chitlac in the more concentrated region ($C > C^*$). A value close to 3.4 is found for the majority of semi-flexible polysaccharides with an exception of galactomannans showing 5.1 (Launay et al., 1986; Morris et al., 1981). The higher gradient value found for chitosan (3.91) with respect to chitlac (3.25) suggested that a higher rate of interconnections formation in the polymeric network belonged to the native, underivatized chitosan.

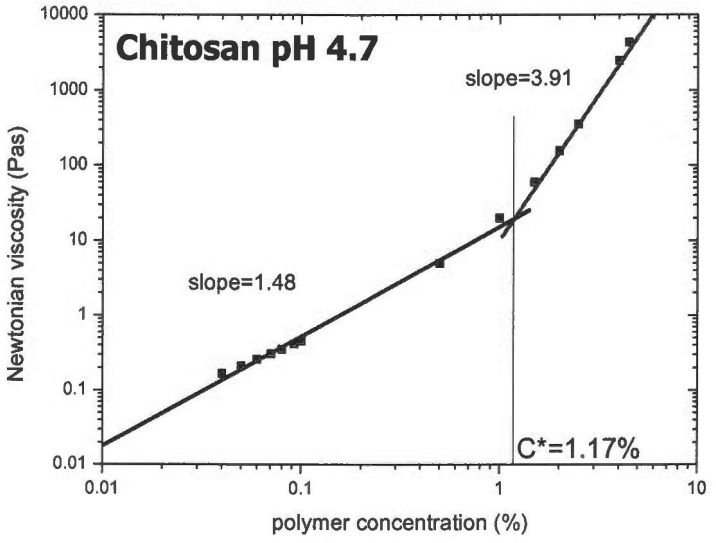


Fig. 3.8: Dependence of the Newtonian viscosity on the polymer concentration for solutions of chitosan at pH 4.7.

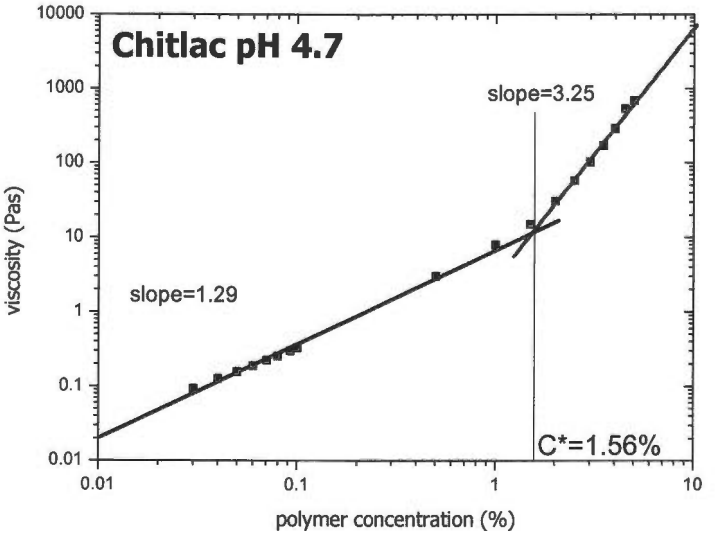


Fig. 3.9: Dependence of the Newtonian viscosity on the polymer concentration for solutions of chitlac at pH 4.7.

3.3.5 Chitlac Flow Curves pH 7.4

Rheological measurements were performed on chitlac solutions in physiological conditions (pH 7.4 and ionic strength 0.15 M), in order to mimic the environment in which the polymer should be used *in vivo*. The behaviour of the polymer at pH 7.4 (Fig. 3.10) was comparable to that observed at pH 4.7. The values of Newtonian viscosity were similar in both cases, showing a rather unperturbed hydrodynamic behaviour with pH variations.

Under these experimental conditions, chitlac solutions exhibited a typical shear thinning behaviour with a Newtonian plateau at low shear rates at concentrations higher than 1.5% w/w. When the shear rate exceeded the rate at which chains were able to relax, the structures remained distorted and being unable to recover the equilibrium network organization a drop of the viscosity resulted. For chitlac solution below 1.5% (w/w) the Newtonian behaviour covered the entire range of investigated shear-rates.

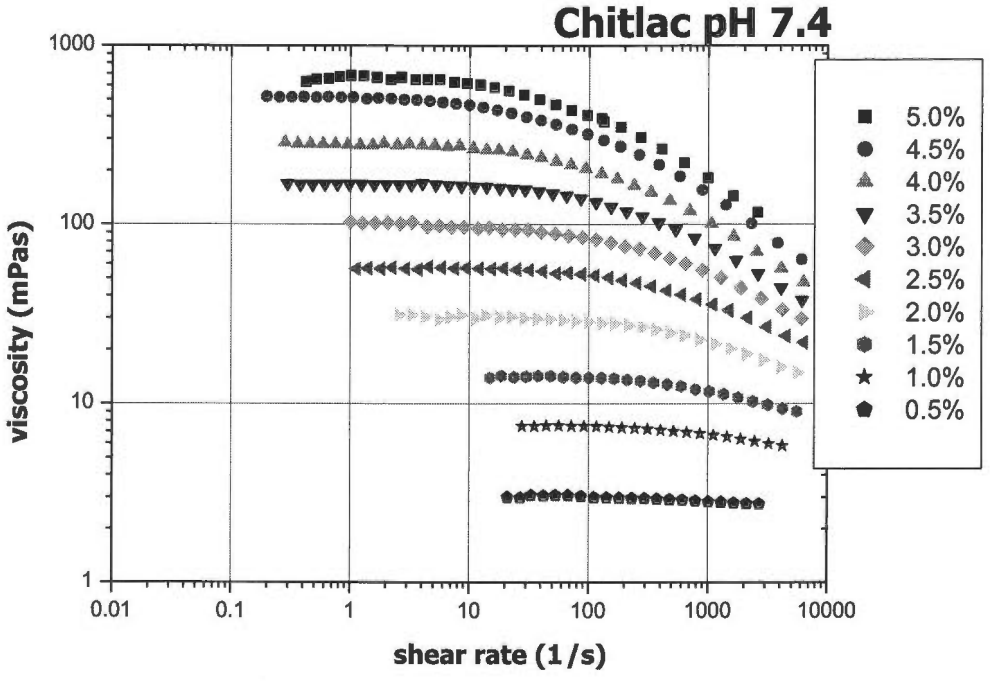


Fig. 3.10: Flow curves of solutions of chitlac at pH 7.4.

3.3.6 Chitlac Enzymatic Degradation

The time course of the enzymatic chitlac degradation of chitlac was followed by solution viscosity measurements. Fig. 3.11 and Fig 3.12 showed the decrease on time of the specificic reduced viscosity as a result of the β-1,4 N-acetylglucosamine linkage cleavages. The enzymatic hydrolytic reaction almost reduced by half the molar mass at the end of the time interval investigated (Fig. 3.11). More than three times of reduction of the initial molar mass was instead observed, after the same time period, using a higher concentration of enzyme (Fig. 3.12) . The zero-order model that assumes a degradation rate independent on substrate concentration but proportional to the enzyme concentration seemed here to likely apply. The degradation lead to a rapid viscosity decrease at short degradation times followed by a slower rate subsequently (Figs. 3.11 and 3.12). Assuming that a fast and a slow component were both contributing to the bond cleavage a bi-exponential function was used to fit the experimentally determined reduced specific viscosity data and the predominant contribution of the slow component was then deduced.

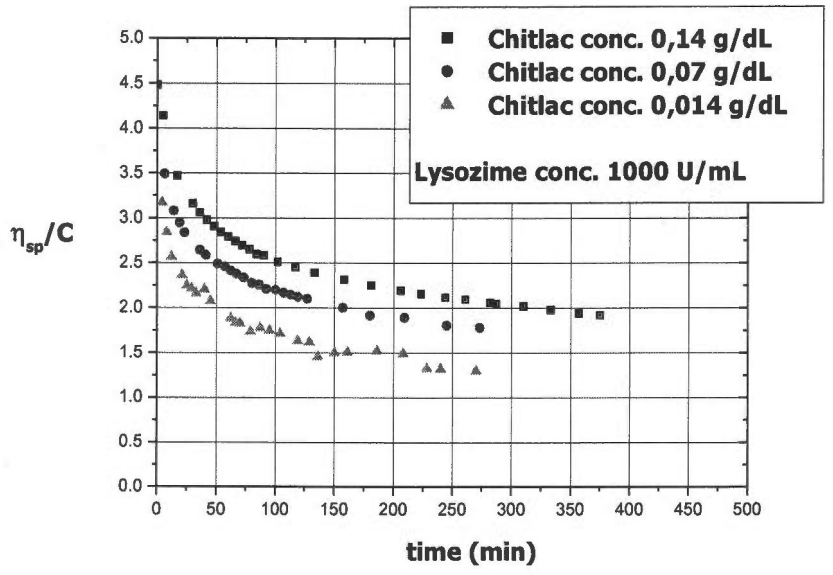


Fig. 3.11: Chitlac enzymatic degradation; dependence on the polymer concentration.

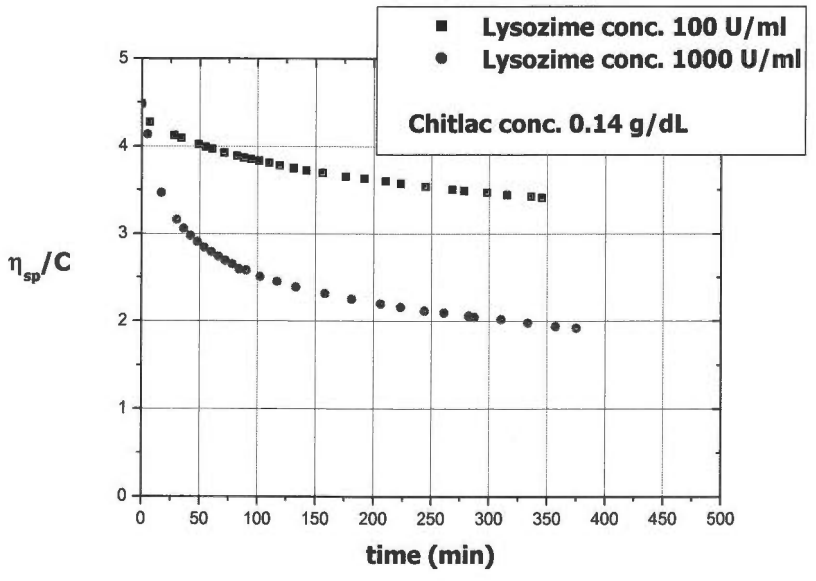


Fig. 3.12: Chitlac enzymatic degradation; dependence on the enzyme concentration.

3.4 Conclusions

In this chapter a physico-chemical characterization of the lactose-derivative of chitosan was accomplished and polymer properties compared with that observed for the parent polysaccharide. From the obtained intrinsic viscosity data it turned out that the observed reduced hydrodynamic volume-to-mass ratio resulting by lactose branching accounted for a more dense coiling conformation assumed by chitlac chains with respect to the parent chitosan.
Rheological studies further confirmed the above reported finding. As deduced from the displayed low Newtonian viscosity, chitlac showed a strongly decreased ability to coil interpenetration in comparison with the native chitosan. This low tendency to form hydrodynamic inter-chain interactions is further confirmed by the shift of the overlap concentration toward a higher value for chitlac than for chitosan. Beside the increase of the molecular weight, lactose branching favours a more hydrodynamically unperturbed coiled conformation of the chain.
In order to mimic the *in vivo* environment, chitlac rheological characterization was carried out also in physiological conditions.The comparable results obtained at the two investigated pH values (4.7 and 7.4) accounted for a hydrodynamic behaviour rather unperturbed by pH variations.
An important issue to be addressed was the lysozyme degradative action on the lactose-derived chitosan. Lysozyme is the main enzyme responsible for the degradation of chitosan *in vivo*, so the evaluation of the action of this enzyme on chitlac needed to be investigated. Data were interpreted assuming that a fast and a slow component were both contributing to the chain cutting. In order to discriminate the relative weight of the two components a bi-exponential function was used to fit the experimentally determined specific viscosity.

CHAPTER 4
Characterization of Blends of Chitlac and Hyaluronic Acid

4.1 Introduction

Hyaluronic acid (HA) is the molecule responsible for the rheological properties of synovial fluid, enabling it to act as a lubricant or shock-absorber in dependence of the forces exerted upon it (Mazzucco et al., 2002).
Viscosupplementation is the symptomatic treatment of osteoarthritis by intra-articular injection of exogenous HA or its derivatives. This therapy aims to restore both the physiological homeostasis of the pathologically altered joint and the normal hyaluronic acid metabolism (Kelly et al., 2004).
Rheological characterization of blends of chitlac and hyaluronic acid were performed to investigate the effect of the lactose-modified chitosan on the viscoelastic behaviour of the HA. These experiments were focused on a possible coupling of the viscoelastic properties of the two polymers with the already demonstrated biological activity of chitlac (Donati et al., 2005). A favourable interaction of the two polymers could be exploited in the optic of designing a new formulation to be used in a viscosupplementation protocol. The rheological measurements were carried out using hyaluronic acid of two different molecular weights, to relate the viscoelastic properties displayed by the HA-chitlac blends to the relative chain sizes of the involved polymers.

4.2 Materials and Methods

4.2.1 Materials

Chitosan and sodium cyanoborohydride ($NaBH_3CN$) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Hyaluronic acid of two different molecular weights was a gift from Genzyme (Cambridge, MA, USA).
Deionized MilliQ water (Millipore, MA, USA) was used to prepare all the aqueous solutions. All other chemicals were of analytical grade.

Acronym	Characteristic	Molecular weight
LMW HA	Low Molecular Weight Hyaluronic Acid	Approx. $2.5x10^5$ g/mol
HMW HA	High Molecular Weight Hyaluronic Acid	Approx. $1x10^6$ g/mol

Table 4.1. Different hyaluronic acid used in the experiments.

4.2.2 Methods

Preparation of Polymer Solutions

For HA molar mass determination by using capillary viscosity measurements hyaluronic acid of low molecular weight (LMW HA) or high molecular weight (HMW HA) was dissolved in in 0.14 M NaCl and 0.01 M Tris/HCl, pH 7.4 solution for 4 hours at room temperature. Measurements were performed covering the 0.03 to 0.1 % (w/w) concentration range on solutions previously clarified by filtering through a 0.45 μm Millipore filter before measurements.
HA-chitlac blends were prepared starting from pure (3% w/w) HA or chitlac solutions in 0.14 M NaCl and 0.01 M Tris/HCl, pH 7.4. Blends with a constant

total polymer concentration (3% w/w), used for rheological measurements, were obtained by mixing known weights of pure HA and chitlac solutions.

Viscosity Measurements

Reduced capillary viscosity was measured at 25 °C by means of a Schott-Geräte AVS/G automatic apparatus and an Ubbelohde type viscometer, as described in the Section 3.2.2. Measurements were performed solely on HA solutions in order to derive the molar mass of the employed samples.

Chitlac-HA Blends Rheological Characterization

Rheological measurements were performed on a stress controlled HAAKE rheometer (RS 150 Rheostress), with a cone-plate geometry (C60/1° Ti, gap 43 μm). All the measurements were performed at 25 °C.

Rheological tests were performed on blends of hyaluronic acid and chitlac under continuous shear conditions to determine steady viscosity values in the stress range 0.05–500 Pa, as well as under oscillatory shear conditions to individuate the extension of the linear viscoelasticity regime (stress sweep tests at 1 Hz) and to determine the mechanical spectra (frequency sweep).

The complex viscosity (η^*), the storage (G') and loss (G'') moduli of the binary polymer solutions were recorded in the frequency range 0.01 – 100 Hz (maximum strain < 10%). Two replicate measurements were performed for each sample.

4.3 Results and Discussion

4.3.1 Reduced Viscosity

The molecular weight of commercially available hyaluronic acid was determined by the capillary viscometry. Using Huggins equation (eq. 3.5) an intrinsic viscosity $[\eta]$ value of 5.99 dl/g for the low molecular weight (LMW HA) sample was obtained from data, as reported in Fig. 4.1, which compares well with that of 5.97 dl/g obtained by Kraemer equation (eq. 3.6). By using the proper Mark-Houwink parameters (a=0.81, k=2.63 x 10^{-4}, (Gamini et al., 1992)) a molar mass of approximately 2.5 x 10^5 g/mol was estimated. The intrinsic viscosity of HMW HA in the same experimental conditions (Fig. 4.2) was 19.75 dl/g (as obtained by the Huggins equation) and 20.15 dl/g (Kraemer equation), corresponding to a molecular weight of approximately 1 x 10^6 g/mol.

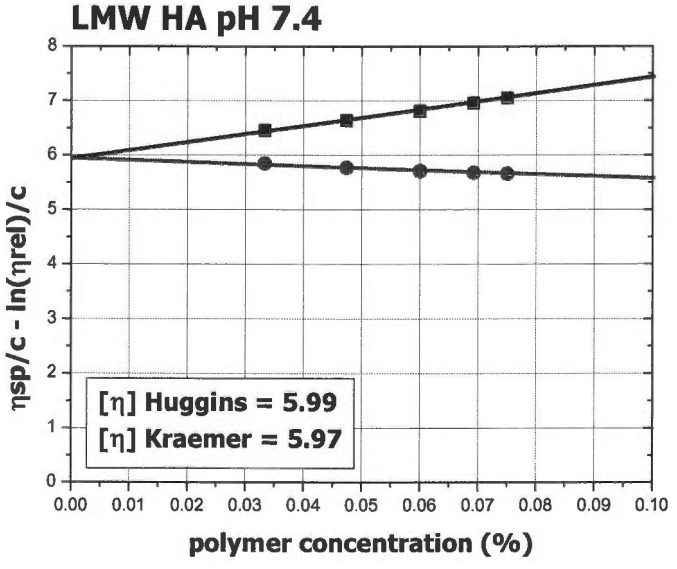


Fig. 4.1: Concentration dependence of η_{sp}/C and $\ln(\eta_{rel})/C$ for LMW HA (pH 7.4).

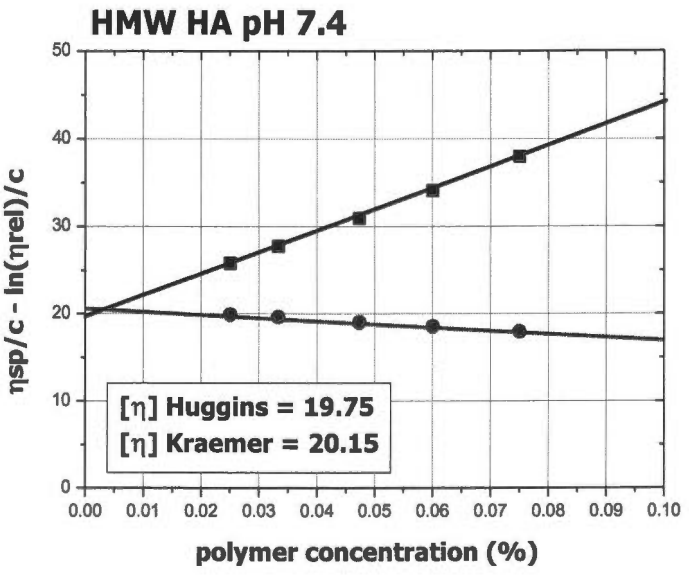


Fig. 4.2: Concentration dependence of η_{sp}/C and $\ln(\eta_{rel})/C$ for HMW HA (pH 7.4).

4.3.2 Chitlac-HA Blends

Once the rheological characterization of chitosan and chitlac has been carried out, and the hydrodynamic properties of the derivatized polymer have been evaluated, attention was focused on the hydrodynamic properties of chitlac-hyaluronan polymer blends.

Indeed, the assessment of the influence of chitlac on the well known viscoelastic properties of hyaluronan aqueous solutions (Gibbs et al., 1968), beside its great scientific interest, is of fundamental importance for application purposes, beside its great scientific interest. Since hyaluronan is the main component of all the preparations used for viscosupplementation (Kelly et al., 2004), the promising therapy for symptomatic treatment of osteoarthritis, a favourable interaction of the two polymers could be exploited aiming at designing new formulations in the field of viscosupplementation. The coupling of the rheological properties of hyaluronic acid with the already proven biological activity of chitlac towards

chondrocytes would give additional valuable properties to the polymer matrix for applications in the biomedical field.

To investigate the properties of the transient mixed-polymer network as a function of HA chain size two different molecular weights were considered for the hyaluronic acid, i.e. the above indicated samples LMW HA and HMW HA, respectively.

4.3.3 Characterization of Chitlac-LMW HA Blends

Flow Curves

In Fig 4.3 the steady-state shear flow curves for mixtures of chitlac and a low molecular weight hyaluronic acid (MW=2.5 x 10^5 g/mol) at total polymer concentration of 3% are reported.

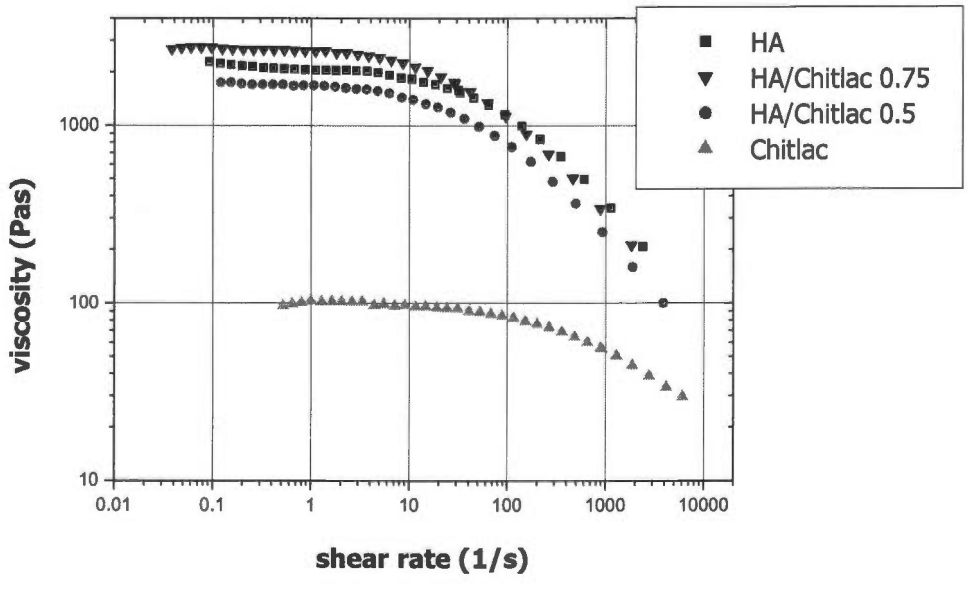


Fig. 4.3: Flow curves of blends of LMW HA and chitlac (pH 7.4), total concentration 3% w/w.

In this case, taking into account that HA has a repeating unit mass per unit length (m_0/L) of 408 g/mol*nm, the average chain contour length of the used HA sample (~620 nm) was comparable to that of chitlac (~1000 nm). The Newtonian viscosity measured for pure HA solution (3% w/w) was one order of magnitude higher than that observed for chitlac solution of identical concentration. Interestingly enough, HA/chitlac mixed solutions showed an initial viscosity increase for chitlac weight fraction up to 0.25, followed by a slight decrease on increasing the chitlac abundancy, recovering the viscosity observed for a pure 3% HA solution at a 1:1 polymer weight ratio (Fig. 4.4).

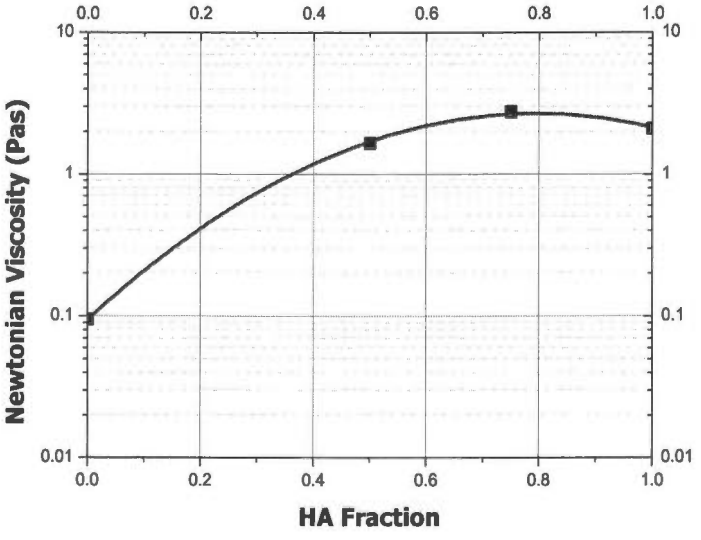


Fig. 4.4: Dependence of the Newtonian viscosity on the weight ratio in blends of LMW HA and chitlac (pH 7.4).

The further rising of the chitlac weight fraction up to 0.75 lead to phase separation, with the solution turning into an opaque suspension of polymeric aggregates. The above reported results suggested that the positively charged chitlac chains, establishing electrostatic interaction with (negatively charged) HA, likely acted as a sort of inter-chain bridging. A stabilizing effect

of the transient network then resulted, if the number of chitlac chains was kept sufficiently low as found for the HA-rich mixture.

On the contrary, when in the blend the number of chitlac chains in the blend closely approached that of HA, electrostatic interactions could occur across the whole polymer network and the high probability of extensive inter-chain bridging establishment lead to stable, water insoluble, macroscopic HA-chitlac aggregates.

Frequency Sweep

Frequency sweep tests in the frequency range 0.01 – 100Hz (maximum strain < 10%) were performed to evaluate the dependence of dynamic moduli G' (storage modulus) and G'' (loss modulus), of LMW HA-chitlac mixture, on the periodically varying applied stress frequency (mechanical spectrum).

As shown in Fig. 4.5 the viscoelastic spectrum obtained for a 3% HA solution was characterized by a loss modulus (G'') higher than the storage modulus (G') throughout almost the entire range of analyzed frequency. Only for frequencies above 20 Hz (crossover point), the elastic component became higher than the viscous one. For blends containing increasing chitlac fractions up to 50% of abundancy, the crossover point shifted towards lower frequencies (10 Hz) indicating an increase of the mixed-network elastic response. This finding, in agreement with the above discussed flow behaviour, further confirmed the higher stability of the HA-chitlac mixed network with respect to the transient network formed by a pure 3% HA solution, as a result of chitlac-mediated inter-chain bridgings established by favourable electrostatic interactions.

Taking into account that here both HA and chitlac shared comparable chain-lengths, one can figure out this more stable network as formed mainly by loosely hydrodynamically interacting chains, kept together by

electrostatically interacting (likely short) chain portions in the form of few and rather distant cross-linking sites.

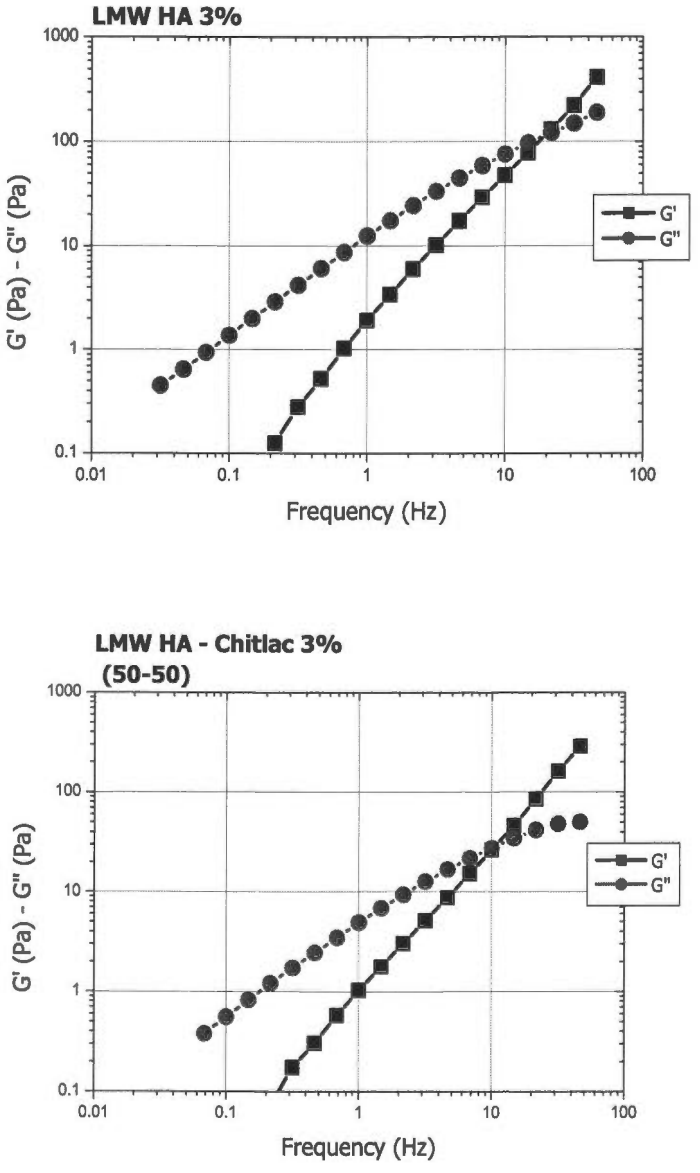


Fig.4,5: Frequency sweep measures of LMW HA solution, 3% w/w (top), and of a blend of HA/chitlac, ratio 1:1, total concentration 3% w/w (bottom).

4.3.4 Characterization of Chitlac-HMW HA Blends

A second set of experiments was carried out on mixtures of chitlac and a high molecular weight hyaluronic acid (HMW HA, MW=1 x 10^6 g/mol), a polymer with a chain contour length much higher than that of chitlac (i.e ~2400 nm for HMW HA, ~1000 nm for chitlac). The experimental conditions were the same as in the measurements previously performed with the LMW HA-chitlac mixtures (physiological condition, total polymer concentration 3% w/w and variable polymer ratio).

Flow Curves

Flow curves obtained with blends of HMW HA and lactose-modified chitosan also showed a shear-thinning behaviour (Fig. 4.6). The Newtonian viscosity of a 3% solution of HMW HA was extremely high, as expected from the large size of the HA polymeric chains.

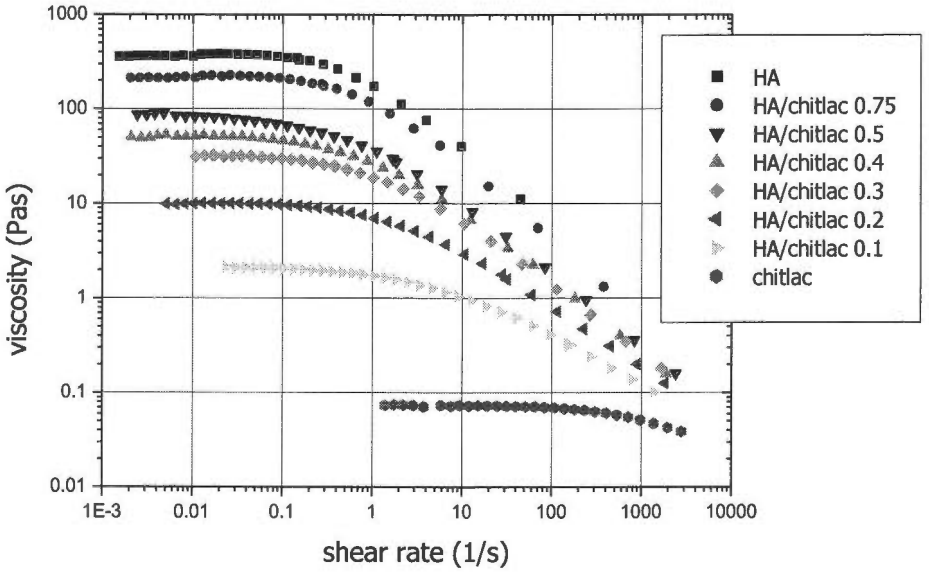


Fig. 4.6: Flow curves of blends of HMW HA-chitlac (pH 7.4), total concentration 3% w/w.

Flow curves obtained with these blends showed that the rheological behaviour of the mixtures was predominantly governed by the HMW HA component (Fig. 4.7).

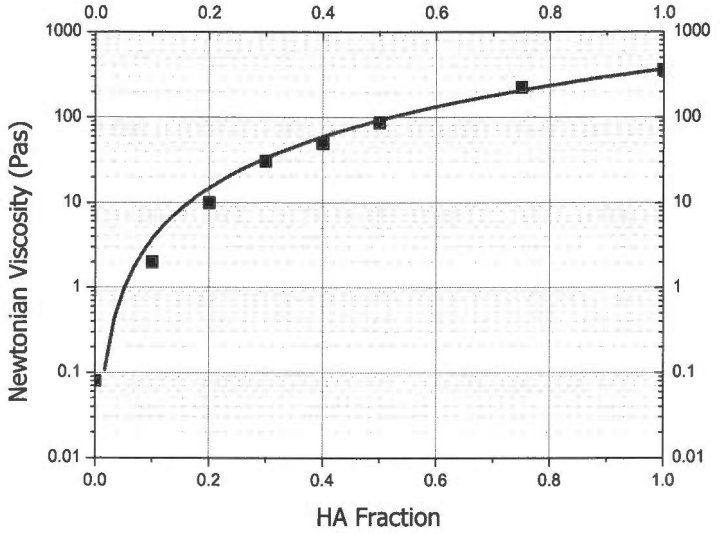


Fig. 4.7: Dependence of the Newtonian viscosity on the weight ratio in blends of HMW HA and chitlac (pH 7.4).

Indeed, only for HMW HA fraction lower than 0.3 the Newtonian viscosity abruptly dropped showing a decrease of more than one order of magnitude. Given the substantial difference between HA and chitlac chain size, the chitlac fraction had to be raised to significant values to observe a destabilization of the network formed by the molecules of HMW HA.

No phase separation took place over the entire range of polymer ratios examined. In the polymer mixtures here investigated the chitlac chains were rather short with respect to those of the HA sample. Here also chitlac-mediated electrostatic inter-chain interactions were likely to occur but their bridging ability was not as estensive as to perturbe the already stable, highly interpenetrated network of very long HA chains.

Frequency Sweep

Frequency sweep measurements were performed in order to evaluate the dependence of dynamic moduli G' (storage modulus) and G'' (loss modulus), of HMW HA-chitlac blends on the periodically varying frequency of the applied stress (mechanical spectrum).

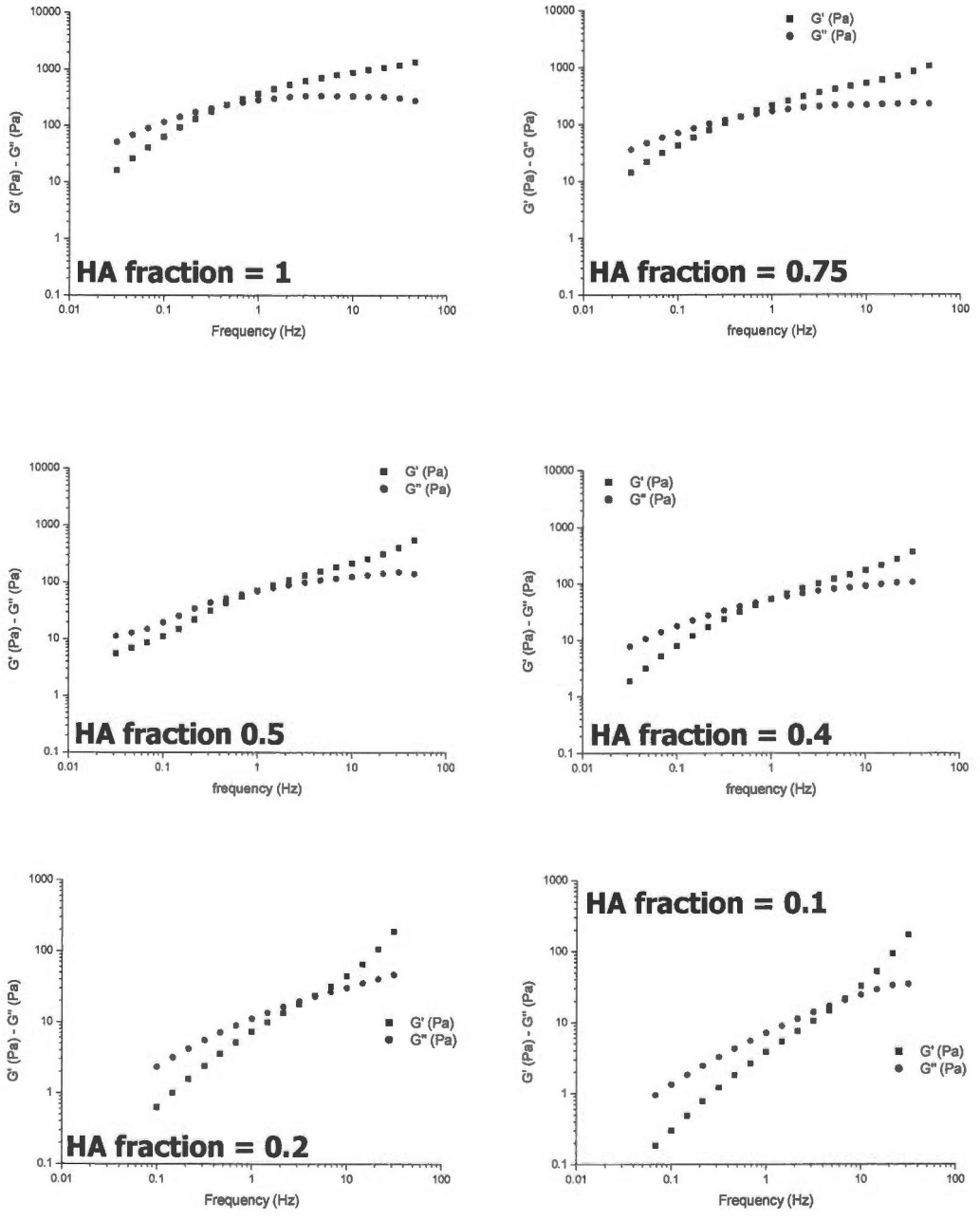


Fig.4,8: Frequency sweep measures of HMW HA solution, 3% (w/w), and of blends of HMW HA-chitlac, different ratios, total concentration 3% (w/w).

As shown in Fig. 4.8 the presence of chitlac had here an opposite effect compared with the LMW HA–chitlac blends. Blends containing up to 25% of chitlac displayed a dynamic response that, in terms of both elastic and viscous components, was comparable to that of a pure 3% solutions of HMW HA. However, upon increasing the chitlac polymer fraction, the cross-over point of the G' and G'' moduli shifted to higher frequencies, clearly indicating that the presence of chitlac at weight fractions > 0.5 drastically reduced the elastic response of the polymer matrix. Furthermore, a concomitant decrease on moduli magnitude at cross-over point was observed, demonstrating how the increasing chitlac fraction destabilized the HMW HA network.

4.4 Conclusions

In this chapter a rheological characterization of blends of chitlac and hyaluronic acid was performed, in order to assess the influence of the chitosan-derived polymer on the well known viscoelastic properties of hyaluronic acid (HA). Two different HA samples, each other differing on chain length, were used for blends.

Steady state shear viscosity data as well as oscillatory dynamic spectra obtained for blends containing chitlac and HA, both of comparable chain length (i.e.: LMW HA-chitlac mixtures), indicated that a stabilizing effect was exerted by the presence in the mixed polymer matrix of lactose-branched chitosan chains. Indeed, a synergistic effect on Newtonian viscosity as well as an increase in the elastic response were observed for these HA-chitlac transient networks, providing the chitlac weight fraction was sufficiently low. The higher stability of these chitlac/HA-riched mixed networks with respect to the transient network formed by a pure 3% w/w HA solution, likely accounted for chitlac-mediated inter-chain bridgings established by favourable electrostatic interactions. Apparently, for blends containing high chitlac weight fractions apparently the inter-chain bridging was so extensive as to lead to the formation of macroscopic, water insoluble aggregates.

Steady state shear viscosity as well as oscillatory dynamic spectra obtained for chitlac-HMW HA mixtures highlighted that the rheological behaviour of the mixtures was essentially governed by the high molar mass HA component. A substantial fraction of chitlac (≈ 0.5) was needed to reduce of one order of magnitude the Newtonian viscosity showed by a pure 3% w/w HA solution. These findings were confirmed by frequency sweep data, clearly indicating that the presence of chitlac at weight fractions > 0.5 drastically reduced the elastic response of the polymer matrix, as deduced from the high frequency shift of the G' and G'' cross-over point, as well as from the decreased moduli magnitude. For the chitlac-HMW HA blends, the

wide difference in contour chain length between HA and chitlac likely accounted for the high amount of chitlac required to reduce the viscoelastic properties of a HA-based transient network.

Here also chitlac-mediated electrostatic inter-chain interactions are likely occurring but their bridging extent can not be as extensive as to perturbe the already stable, highly interpenetrated network of very long HA chains.

From a biomedical point of view, the synergistic effect observed in the system chitlac-LMW HA could be exploited to upgrade the viscoelastic properties of LMW fractions of HA (that are *per se* devoid of significant viscoelastic properties of appealing commercial interest) with the bioactive chitlac component, in order to develop a new formulation to be used in viscosupplementation protocols. On the other hand, the system chitlac-HMW HA could be useful as well as it has been already proved that high molecular weight hyaluronan-based preparations works better as viscosupplementation agents (Bellamy et al., 2006). Moreover, the additional presence of a small fraction of chitlac could introduce bioactive properties without depleting the viscoelastic properties of HMW HA matrix.

CHAPTER 5
3D Scaffolds Based on Crosslinked Chitlac

5.1 Introduction

Chitosan has been used as a scaffolding material in articular cartilage tissue engineering (Nettles et al., 2002). Several *in vitro* studies yielded evidence of its potential value as a matrix to promote articular cartilage repair. It efficiently supports not only chondrogenic activities (Sechriest et al., 2000; Suh and Matthew, 2000), but also the *in vitro* expression of cartilage extracellular matrix proteins by human chondrocytes (Lahiji et al., 2000). It can also serve as a carrier for growth factors, and the polymer presents as well excellent biodegradability as well.

An effective strategy to produce a stable scaffold based on chitosan is the utilization of a crosslinking agent to covalently bind different polymeric chains (Berger et al., 2004b). Three different crosslinkers have been selected from literature to be used for the synthesis of chemically crosslinked chitlac-based scaffolds. The principal aim was to develop a stable three dimensional structure able to maintain the biological properties demonstrated by the polymer in solution (Donati et al., 2005) and therefore to be favourably applicable in cartilage tissue engineering. Several structures were synthesized and different techniques were used to characterize them. SEM was used to characterize the microstructure of the scaffolds, while water uptake and stability were assessed by equilibrium swelling experiments. The best scaffolds were then biologically tested, and primary cultures of porcine chondrocytes were seeded on them. The cell morphology, proliferation and matrix synthesis were evaluated.

5.2 Materials and Methods

5.2.1 Materials

Chitosan and sodium cyanoborohydride ($NaBH_3CN$) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The crosslinker agents 1,4 Butanediol Diglycidyl Ether (BDGE), Dimethyl Suberimidate (DMS) and Diethyl Squarate (DES) were purchased from Sigma (St.Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) culture medium, streptomycin, penicillin, foetal calf serum (FCS), trypsin/EDTA and PBS buffer were purchased from GIBCO-BRL (Grand Island, NY, USA). 12-plates and 24-plates multiwell were from Sarstedt (Newton, NC, USA). Deionized MilliQ water (Millipore, MA, USA) was used to prepare all the aqueous solutions. Type II collagenase was from Worthington Biochemical Corp. (USA). Ialuronidase and sodium cyanoborohydride were purchased from Sigma (St.Louis, MO, USA). Alamar Blue™ was purchased from Prodotti Gianni (Italy). All other chemicals were of analytical grade.

5.2.2 Methods

Scaffolds Preparation

Lyophilized Chitlac was dissolved in milliQ water, to obtain a 2% solution (20 mg/ml). 500 µl of 2% chitlac solution were poured in each well of a 24-plate multiwell, frozen for 24h at -20 °C and freeze-dried overnight. Different protocols were followed depending on the type of the crosslinker agent used. In all cases, after the crosslinking reaction scaffolds were thoroughly washed with milliQ water to remove any excess of reagents and unreacted

molecules. At least 4 crosslinked samples were prepared with each type of crosslinking agent.

Crosslinkers Used

Three different crosslinking agents were selected to be used to produce covalently crosslinked chitlac structures: 1,4 Butanediol Diglycidyl Ether (BDGE), Dimethyl Suberimidate (DMS) and Diethyl Squarate (DES).

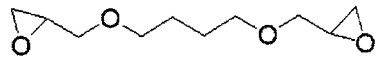
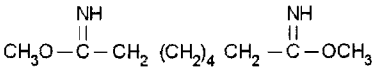
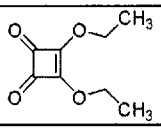
Crosslinker	*Acronym*	*Chemical Structure*	*References*
1,4 Butanediol Diglycidyl Ether	**BDGE**		(Subramanian and Lin, 2005)
Dimethyl Suberimidate	**DMS**		(Charulatha and Rajaram, 1997) (Charulatha and Rajaram, 2003)
Diethyl Squarate	**DES**		(de Nooy et al., 2000)

Table 5.1: Crosslinking agents used.

Preparation of Chitlac Crosslinked with BDGE

Freeze-dried chitlac scaffolds were incubated at 37 °C for 16 hours with isopropanol solutions containing different percentages of BDGE ranging from 0.5 to 4%. At the end of the reaction the samples were extensively washed with isopropanol, in order to remove any unreacted BDGE molecule. The BDGE-crosslinked chitlac scaffolds were then exhaustively washed with milliQ water and air-dried.

Preparation of Chitlac Crosslinked with DMS

Freeze-dried chitlac scaffolds were incubated at room temperature overnight with aqueous solution (pH 9) containing different DMS percentage in the range 0.5-4%. The DMS-crosslinked chitlac scaffolds were then exhaustively washed with high amount of milliQ water and air-dried.

Preparation of Chitlac Crosslinked with DES

Freeze-dried chitlac scaffolds were incubated at room temperature overnight with aqueous solution (pH 11) containing different DES percentage in the range 0.5-4%. The DES-crosslinked chitlac scaffolds were then exhaustively washed with high amount of milliQ water and air-dried.

Swelling Behaviour of the Scaffolds

In order to assess the swelling behaviour of the crosslinked scaffolds in PBS, the specimens were withdrawn from a PBS solution at different time intervals and their wet weights were determined after blotting with a filter paper to remove the surface water. The swelling ratio was calculated using the equation:

$$E_{sr}(\%) = \left(\frac{W_s - W_d}{W_d} \right) \times 100 \qquad \textit{eq. 5.1}$$

where E_{sr} is the amount of uptaken water (weight percent) by the polymer matrix, W_d and W_s are the weights of the samples in the dry and the swollen state, respectively.

Scanning Electron Microscopy (SEM)

The scanning electron microscope (SEM) is a type of electron microscope capable of producing high-resolution images of a sample surface. Due to the manner in which the image is created, SEM reproductions have a characteristic three-dimensional appearance and are useful for characterizing the surface structure of the sample. SEM has the capacity of magnifying features from 10 to 100,000X with a great depth of field and increased resolution, allowing the observation of magnified images of the specimen showing details not visible with a light microscope leading to a length resolution in the order of 25 Å. The SEM uses electrons instead of light (photons) to form an image, in fact a very finely focused beam of electrons is produced at the top of the microscope by heating a metallic filament. The electron beam follows a vertical path through the column of the microscope. It makes its way through electromagnetic lenses which focus and direct the beam down towards the sample. Once it hits the sample, other electrons (backscattered or secondary) are ejected from the sample. Detectors collect the secondary or backscattered electrons, and convert them to a signal that is sent to a viewing screen, producing an image. As the electron beam scans the specimen, providing topographical information. The imaging process of the SEM takes place when a cathode ray tube (CRT) is scanned simultaneously with the electron beam. The most common method of imaging utilizes secondary electrons. As the electron beam is scanned over the specimen, surface features in line of sight of the secondary electron detector will generate proportionally more electrons. The detector generates a signal that is proportional to the number of the electrons received as various surface features come under the electron beam. The intensity of the CRT beam is modulated proportionally to represent the magnitude of the signal arriving from the secondary electron detector. A picture is built up that represents the surface topographical features and are discerned by the effect that, for a "hill" on the surface, the side facing the detector will generate

more secondary electrons that are likely to arrive at the detector, and consequently, that side will appear brighter than the side that is not in the line of sight of the detector.

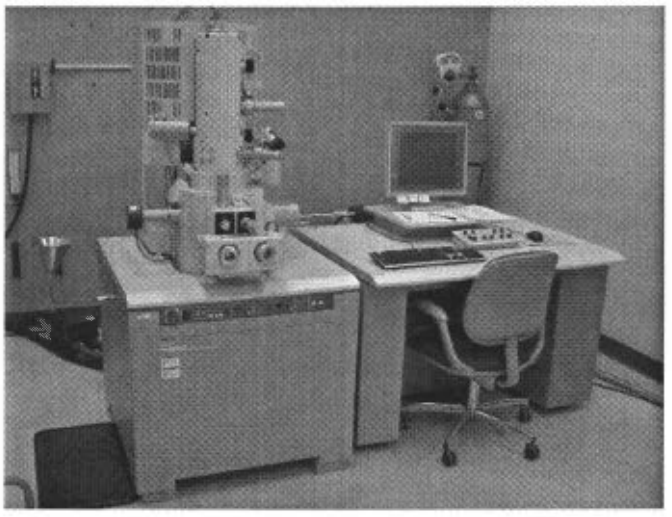


Fig. 5.1: Scanning electron microscope (S-4700 SEM, Hitachi High Technologies Corp.).

This technique has been used here to characterize the microstructure of the scaffolds produced via crosslinking of chitlac and the cell morphology of the chondrocytes seeded on the structures.

SEM of Scaffolds

Scaffolds were freeze-dried, split into two parts through the sagittal plane, mounted on an aluminium stub using double stick carbon tape and sputter coated with gold for 4 minutes. The outer surface and middle region of each sample were viewed using a scanning electron microscope (S-4700 SEM, Hitachi High Technologies Corp.). Digital images were acquired using the software of the microscope.

Isolation of Cartilage

Thin slices of articular cartilage were aseptically removed from the humeral proximal head of mature pigs within 2 hours from the sacrifice. Cells were then isolated by enzymatic digestion of the tissue as described (Grandolfo et al., 1993).

Chondrocyte Isolation

The joint was cleaned, and the exposed cartilage was washed in sterile phosphate buffered saline (PBS). Articular cartilage from the humeral proximal head was dissected using a sterile surgical scalpel. The pieces of tissue were collected in sterile Petri-dishes containing hyaluronidase (270 U/ml; Sigma, I-S type), penicillin (500 U/ml; Sigma) and streptomycin (500 U/ml; Sigma). The tissue was chopped into fine pieces and moved in a solution containing type II collagenase (250 U/ml; Worthington; ratio: 10 ml solution to 1 g tissue) in PBS, in presence of penicillin and streptomycin (same concentration as above). Enzymatic digestion of the tissue was carried on for 15 hours at 37 °C in a Budnoff chamber under stirring (80 rpm). The digestion solution was filtered with a sterile filter for plankton (pore size 20 μm). The resulting cell suspension was centrifuged at 1000 rpm for 10 minutes. The supernatant solution was aspirated and sterile PBS was added to the centrifuge tubes to wash the cells and remove residual collagenase. The cells were then suspended in 1-2 ml of complete DMEM and the cell pellet was broken by repeated pipetting using a 5 ml pipette. After a uniform cell suspension was obtained, cells were counted in a Thoma chamber and cultured at the desired concentration.

Chondrocyte Culture

Chondrocytes isolated from articular cartilage were transferred in a 75 ml flask with 10 ml of DMEM medium supplemented with 2% glutamine, 500 U/ml penicillin, 500 mg/ml streptomycin and 10% FCS and cultured in a humidified incubator (37 °C, 5% CO_2).

Cell Seeding into Scaffolds

A cell suspension was obtained by enzymatic digestion with trypsin EDTA in PBS. The cells were washed by centrifugation (1200 rpm) and diluted to a concentration of 3.5 x 10^5 cells per ml in the appropriate culture medium. Before cell-seeding, scaffolds were arranged in separate wells of 12-well plates and sterilized with a cycle (15 minutes) of UV irradiation on each side of the structure.

The cell suspension was pipetted through each scaffold (1 ml per scaffold) in order to foster cell attachment and cultured in a humidified incubator (37 °C, 5% CO_2).

Cell-seeded Scaffolds Culture

Seeded scaffolds were transferred to 12-well non-tissue culture treated plates. One scaffold was placed in each well containing 1 ml DMEM medium. Three times per week, medium was removed and replaced with fresh culture medium. Cell-seeded scaffolds were cultured for 6 weeks in a humidified incubator (37 °C, 5% CO_2).

SEM of Cell-seeded Scaffolds

Cell-seeded scaffolds were washed with PBS (3X), fixed in 2.5% (*v/v*) glutaraldehyde solution in PBS at 4 °C for 3 hours, before washing in PBS.

Samples were then dehydrated through an increasing series of ethanol (70% [*v/v*], 95% [*v/v*] and 100% [*v/v*]). After dehydration in 100% ethanol, a critical point dryer was used to avoid the introduction of surface tension artefacts. Finally the scaffolds were cut, mounted on an aluminium stub using double stick carbon tape. Samples were then introduced into the chamber of the sputter coater, coated with gold for 4 minutes and viewed under the SEM microscope (S-4700 SEM, Hitachi High Technologies Corp.). Digital images were acquired using the software of the microscope.

Alamar Blue™ Assay for Cell Proliferation

The Alamar Blue™ assay is based on the detection of metabolic activity of cells (Fields and Lancaster, 1993). The assay reagent, Alamar Blue™, contains a reduction-oxidation (REDOX) indicator (resazurin). The metabolic activity of cells, conferring reducing properties to the medium induces a chemical reduction of the reagent, leading to the formation of resorufin, a pink fluorescent product (O'Brien et al., 2000). Decreased fluorescence levels are indicative of a decrease in the synthetic rates of cells and therefore suggestive of cells being cultured in a less favourable environment and having a lower relative viability compared to cells that show higher fluorescence levels when incubated with Alamar Blue™.

Calibration Curve for Alamar Blue™ Assay

A cell number titration assay was performed in order to determine a correlation between the number of cells seeded in each well and the fluorescence level.

Chondrocytes were plated on a 12-well plate, in the range of 5×10^3 to 2×10^5 cells/well in 1ml DMEM medium. The cells were cultured overnight in a humidified incubator (37 °C, 5% CO_2) to let the cells adhere to the plate.

The wells were washed twice with PBS and incubated with 1 ml of 10% Alamar Blue™ in DMEM culture medium for 4 hours in darkness within the humidified incubator (37 °C, 5% CO_2). Following gentle agitation, the solution was removed from each well. The samples were diluted 1:1 with DMEM culture medium and transferred into a quartz cuvette of 1 cm of optical path before the fluorescence measurement.

The fluorescence intensity, with an excitation wavelength of 530 nm, was measured in the 500 to 700 nm range, amply covering the emission spectrum of Alamar Blue™ dye. A Fluorimeter Perkin Elmer LS50B was used, the measurements performed at 25°C using a thermostated cell holder. As a control, 1 ml of 10% Alamar Blue™ in DMEM culture medium without cells was incubated for 4 hours and analyzed as described above.

After correction for the control readings, fluorescence intensity values measured at 590 nm were plotted as a function of cell number and the calibration curve obtained by linear least-square fitting.

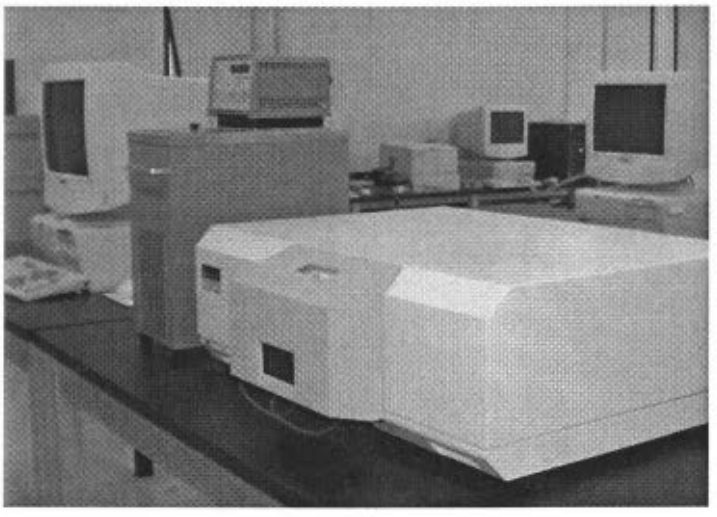


Fig. 5.2: Perkin Elmer LS50B Luminescence Spectrometer.

Cell proliferation on Cell-seeded Scaffolds

Cell-seeded scaffolds were transferred to a 12-well plate, washed twice with PBS and incubated with 1 ml of 10% Alamar Blue™ in DMEM culture medium for 4 hours in darkness within a humidified incubator (37 °C, 5% CO_2). Following gentle agitation, 1 ml was removed from each well. The samples were diluted 1:1 with DMEM culture medium and transferred in a quartz cuvette of 1 cm of optical path before the assessment of the fluorescence.
Using an excitation wavelength of 530 nm, fluorescence intensity readings were collected in the 500 to 700 nm range. As a control, scaffolds without cells were incubated for 4 hours with 1 ml of 10% Alamar Blue™ in DMEM culture medium and analysed as described above.

5.3 Results and Discussion

5.3.1 Scaffolds Production

Three different crosslinking agents have been used to produce chitlac-based scaffolds, in all cases exploiting as the crosslinking sites the primary amino groups remaining after chitosan derivatization.

One of the physico-chemical properties shown by chitlac in comparison with native chitosan is the complete solubility at physiological pH. It is therefore of fundamental importance the introduction of covalent crosslinks to obtain a stable network of chitlac chains. The final purpose of a 3D chitlac-based scaffold is its application in a physiological environment in the hydrated state. Therefore, it is mandatory that the introduction of covalent crosslinks give rise to a stable network of covalently linked chitlac chains.

The reticulation of the chitlac network, beside creating a stable structure, should preserve the bioactive lactose moieties available for the cells eventually seeded on the structure, stimulating them to synthesize the extracellular matrix components typical of the cartilage.

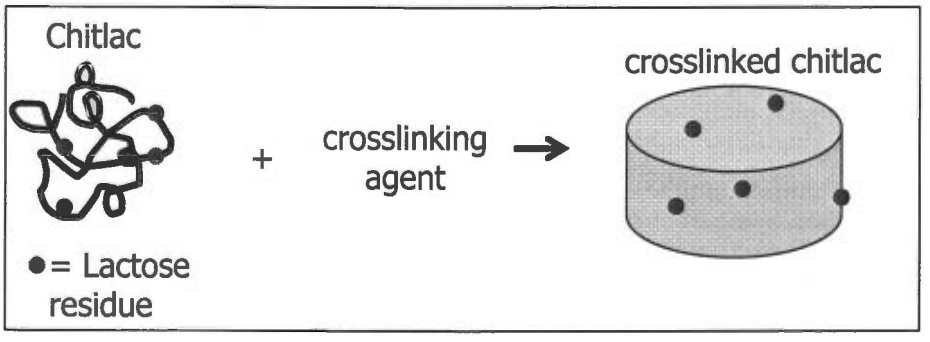


Fig. 5.3: Scheme of the crosslinking of chitlac.

1,4 Butanediol Diglycidyl Ether (BDGE), Dimethyl Suberimidate (DMS) and Diethyl Squarate (DES) have already been used in literature as crosslinker of

different polymers, among which also chitosan, for the production of scaffolds in protocols of tissue engineering (Charulatha and Rajaram, 2003; Charulatha and Rajaram, 1997; Subramanian and Lin, 2005; de Nooy et al., 2000).

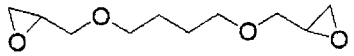
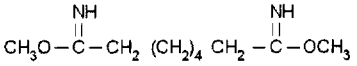
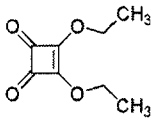
Crosslinker	*Acronym*	*Chemical Structure*
1,4 Butanediol Diglycidyl Ether	**BDGE**	
Dimethyl Suberimidate	**DMS**	
Diethyl Squarate	**DES**	

Table 5.2: Crosslinkers Used.

5.3.2 Preliminary Characterization by SEM

The first characterization of the obtained structures has been carried out using SEM. It was therefore possible to assess the microstructure of the scaffolds, with respect to the overall porosity, the pore size and the interconnectivity of the pores. The crosslinked structures were compared with a scaffold prepared by freeze-drying a not-crosslinked solution of chitlac used as reference material. The following pictures showed two progressive magnification of the same portion of the scaffolds (100x on the upper part of the picture, 400x on the lower), in order to focus the aspect of the microstructure at two different levels of organization.

The SEM analysis of not-crosslinked chitlac scaffold (Fig. 5.4), obtained by freeze-drying a 2% aqueous solution of chitlac, showed a highly porous structure, with interconnected and randomly oriented pores.

The drawbacks of this structure were related to the lack of covalent crosslinks being the scaffold actually a "frozen" transient network, whose structure would be preserved only in the dry state.

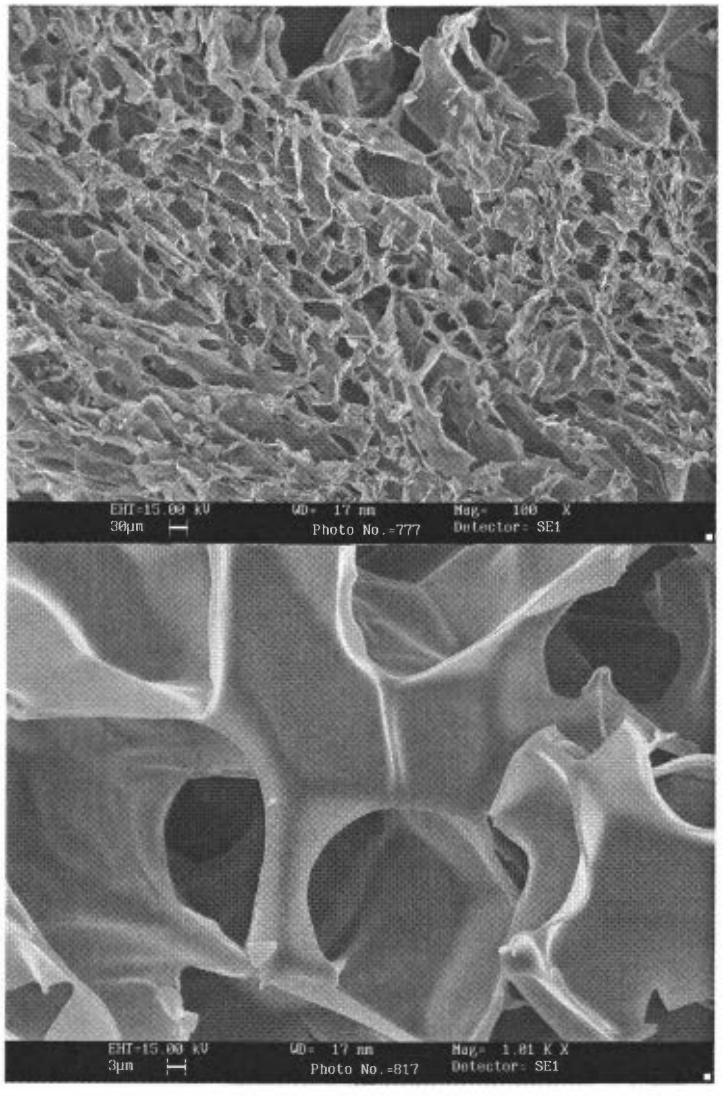


Fig. 5.4: Freeze-dried not-crosslinked chitlac scaffold (2%).

The structures produced with 1,4 butanediol diglycidyl ether (BDGE) as crosslinking agent (Fig. 5.5) showed a tightly packed surface, while images taken from a transversal plane of the cylindrical samples presented a very regular structure, characterized by high porosity and by the presence of interconnected pores. The average pore size was 100 μm, inside the range suggested as optimum for the infiltration and attachment of chondrocytes (Agrawal and Ray, 2001).

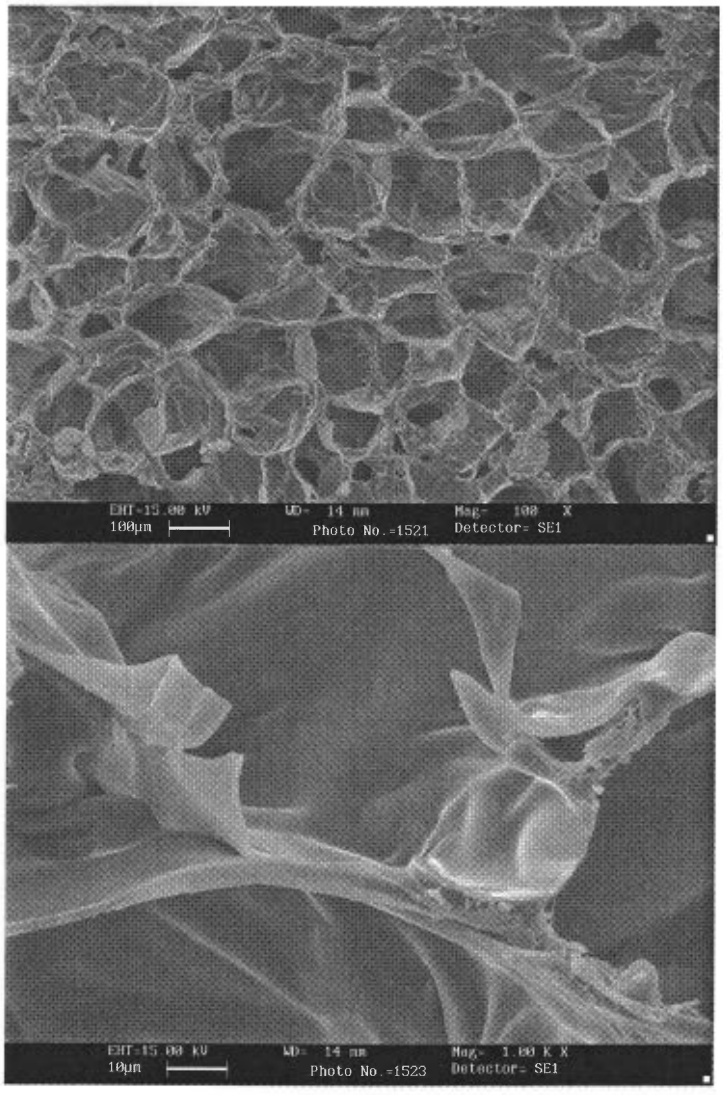


Fig. 5.5: Scaffold of chitlac 2%, crosslinked with BDGE 1%.

Dimethyl suberimidate (DMS) crosslinked chitlac scaffolds (Fig. 5.6) were characterized by an extremely regular texture throughout the entire samples. Porosity of the structure was definitely high as was the interconnectivity of the pores. Pore size ranged from 20 to 50 μm.

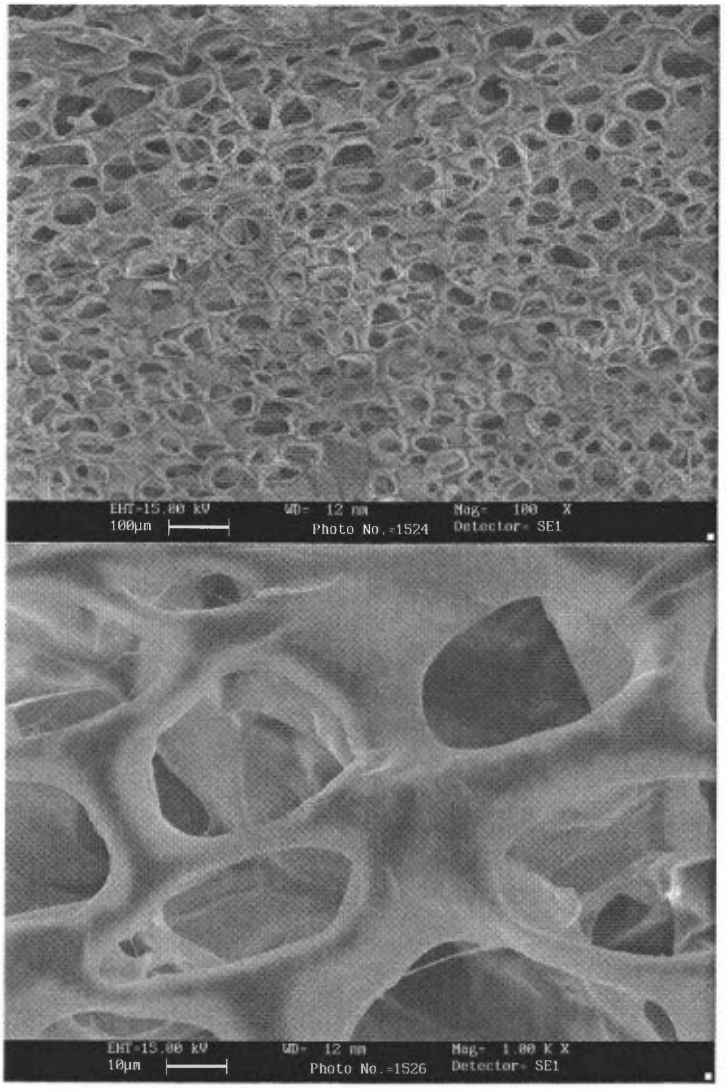


Fig. 5.6: Scaffold of chitlac 2%, crosslinked with DMS 0.5%.

Diethyl squarate (DES) has been already used in literature as crosslinker of chitosan. The observed structure (Fig. 5.7) was highly variable throughout the different parts of the samples, and the main consequence was a different pattern of porosity and pore size. The lack of a uniform pattern in the texture of the DES crosslinked scaffolds made it difficult to identify average pore size and general features of the samples.

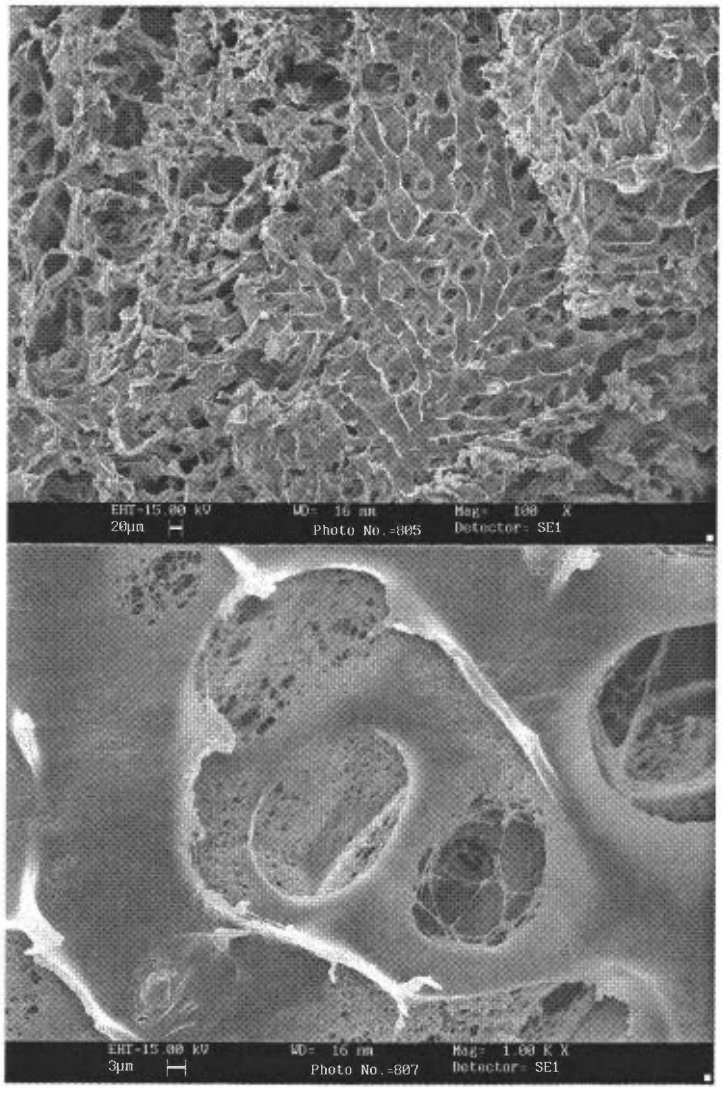


Fig. 5.7: Scaffold of chitlac 2%, crosslinked with DES 1%.

The preliminary characterization of the different samples with SEM allowed to visualize the main characteristics of the structures obtained with the different crosslinkers used (Table 5.3). These experiments were fundamental to assess the microstructural features of the scaffolds and to define the protocol of crosslinking of the structures themselves. The structures produced with the use of BDGE showed pore size range inside that suggested (Agrawal and Ray, 2001) as optimum for cell infiltration and attachment. The DMS crosslinked scaffolds were characterized by the highest porosity and pore interconnectivity. However, its pore size would be perhaps too low to be used as support matrix for cell delivery, if a final purpose is its application in cartilage tissue repair protocols.

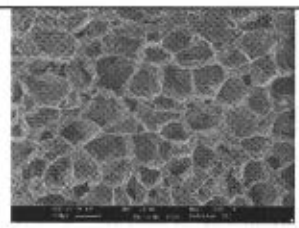
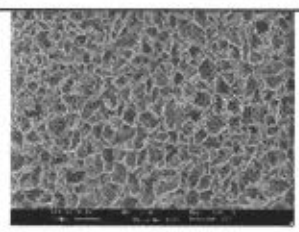
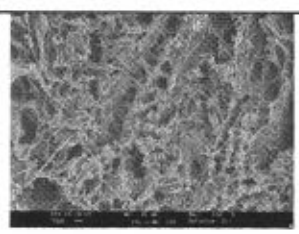
Crosslinker	***SEM***	***Pore Size***	***Interconnectivity***
BDGE		100 µm	low
DMS		20-50 µm	high
DES		variable	low

Table 5.3. Summary of the preliminary SEM characterization of the crosslinked scaffolds.

5.3.3 Swelling Properties

A further set of experiments was performed to assess the stability as well as the ability of water uptaking of the synthesized 3D structures (Fig. 5.8). In order to mimic the conditions of a physiological environment these measurements were carried out in phosphate buffer solution (PBS) at pH 7.4. It is of paramount importance for a scaffold intended to be used in tissue engineering protocol to maintain its shape and mechanical stability for a time long enough to let the cells seeded on it synthesize a sufficient amount of extracellular matrix components. Indeed, specific biological and mechanical properties of scaffolds are required if to restore the physiological functionality of the damaged cartilage is the final purpose.

The preliminary results of these experiments (not reported) showed that the scaffolds produced using DMS and DES as crosslinker were not stable enough with re-hydration. These scaffolds, whose structure characterization (SEM) in the dry state produced promising results, were therefore discarded for the use in biological tests. The main issue was that the structures were unable to retain their shape when immersed in the PBS solution with a consequent dissolution of the loosely crosslinked polymer in a time not compatible with any application.

Attention was focused on the scaffolds produced by crosslinking chitlac with BDGE. These structures exhibited the ability of uptaking a great amount of aqueous medium, while maintaining their shape and a certain mechanical stability.

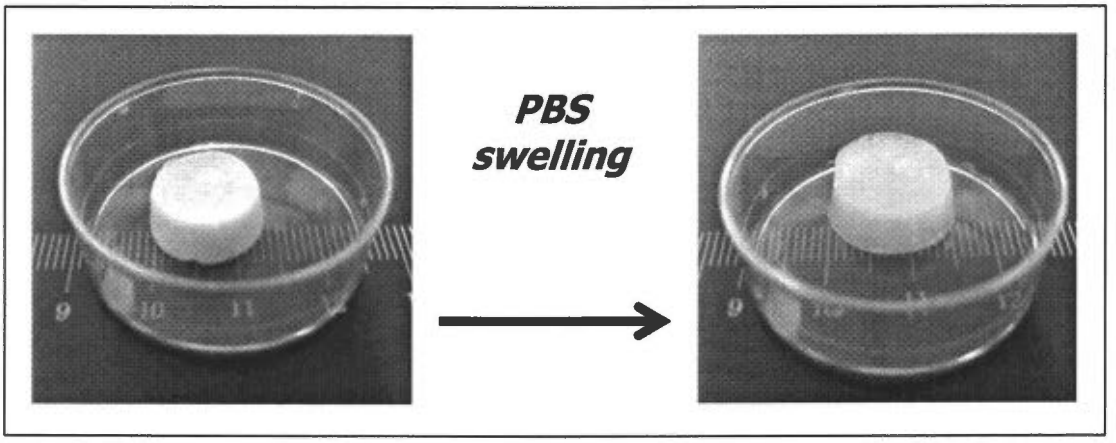


Fig. 5.8: Pictures of dry and wet scaffold made of chitlac crosslinked with BDGE (4% of crosslinker). Swelling in PBS.

In order to improve mechanical properties of the scaffolds, it was decided to increase crosslinking density by increasing the concentration of the crosslinking agent. Hence, swelling experiments were performed on structures obtained by using 2% and 4% of BDGE. The time dependent swelling behaviours of BDGE-crosslinked chitlac scaffolds in PBS (pH 7.4) at room temperature are given in Figure 5.9 and Figure 5.10.

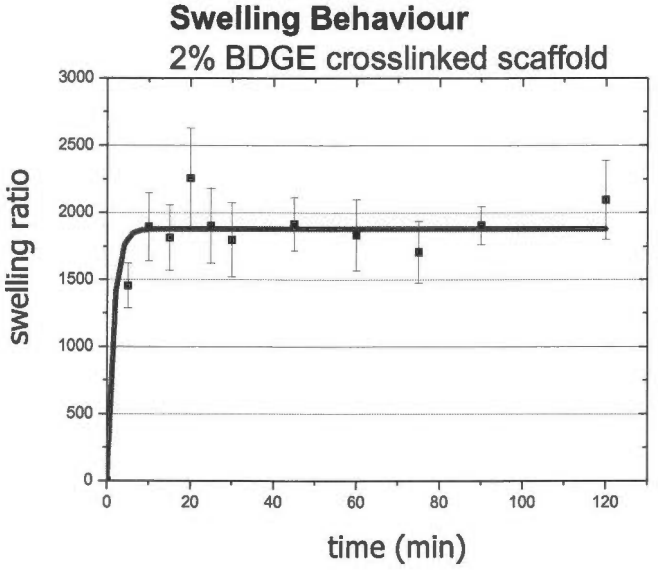


Fig. 5.9: Swelling behaviour of 2% BDGE-crosslinked scaffolds.

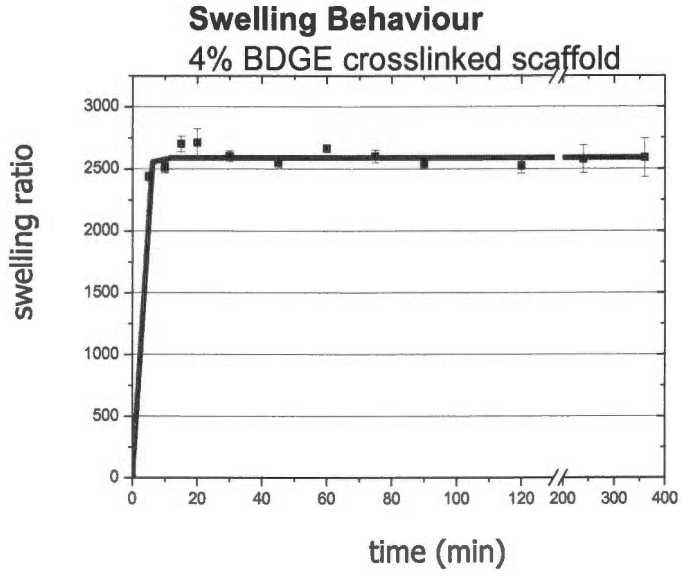


Fig. 5.10: Swelling behaviour of 4% BDGE-crosslinked scaffolds.

Swelling behaviour data are the average of three trials. The reticulated structures showed to uptake a rather high amount of solvent reaching an equilibrium swelling ratios of 1880% and 2590% for the structures crosslinked with 2% and 4% of BDGE, respectively. These results were rather unusual, since generally an increase of the number of inter-chain linkages is expected to occur on increasing crosslinker concentration. The increased rigidity of the network reduces its swelling ability. The swelling equilibrium was reached very quickly for both the crosslinker concentrations, less than 20 minutes were necessary for the wet weight to get stable. Standard deviation was extremely low for the 4% BDGE samples, in comparison with the results obtained with 2%, indicating a higher variability for the samples produced with the lower amount of crosslinker. Since the scaffolds were already highly porous, water uptake did not significantly affect the volume of the scaffolds.

Having these scaffolds shown a high water uptaking ability as well as a satisfactory chemical stability a further characterization by SEM was performed (Fig. 5.11). These analysis were undertaken to assess the possible consequences of the use of a larger amount of BDGE on the overall porosity and on the pore size of the 3D structures.

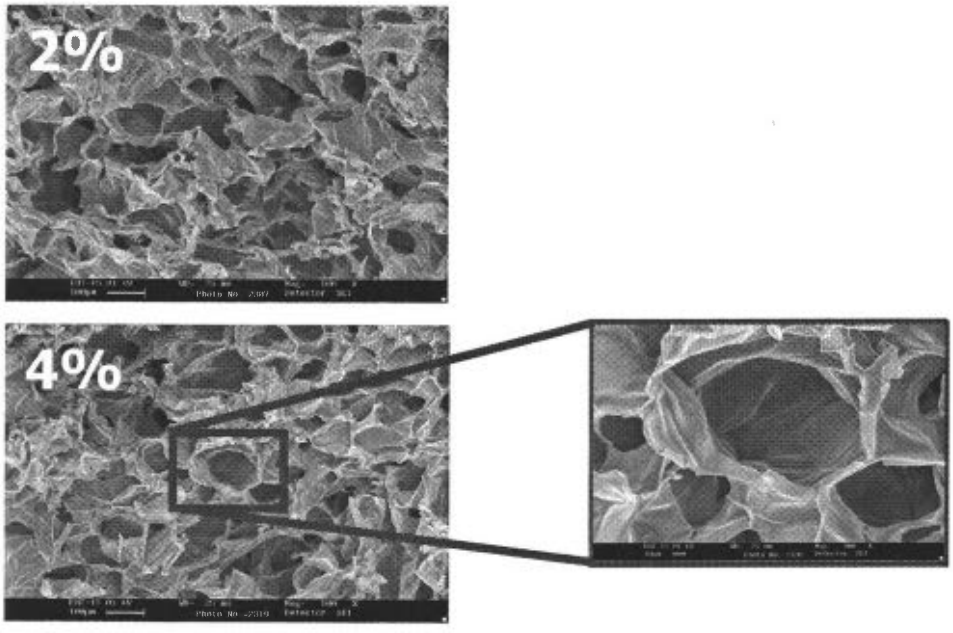


Fig.5.11: SEM of chitlac scaffolds crosslinked with BDGE (2% of crosslinker, 100x and 4% of crosslinker, 100x; in the box on the right: particular of a pore, 4% of crosslinker, 400x).

A porous structure was in all cases ascertained, whatever the concentration of the crosslinker used (2% or 4%). It has been shown that the pore size was more variable than previously found, ranging from 20 to 100 μm, but a satisfactory degree of pore interconnectivity was maintained. An additional observation was that the global appearance of the structure using a crosslinker concentration of 4% was more regular.

5.3.4 Chondrocyte-seeded Scaffolds

Following the results obtained from the swelling experiments and SEM characterization, scaffolds obtained by crosslinking of chitlac with 4% BDGE were chosen as testing material for cell seeding and further biological investigation.

The effects of the scaffolds on primary cultures of porcine chondrocytes is of fundamental importance to assess their biocompatibility *in vitro* as well as to verify that the bioactive stimuli already exhibited by the polymer in solution (Donati et al., 2005) is here maintained.

Chondrocyte-seeded scaffolds were cultured for a period of 6 weeks. At defined time intervals the cell proliferation was assessed with the Alamar Blue™ method whereas the matrix synthesis and cell morphology was investigated with the SEM.

5.3.5 SEM Analysis of Chondrocyte-seeded Scaffolds

The scaffolds were analyzed after fixation with 2.5% glutaraldheyde at different time intervals, at 1, 3 and 15 days after cell seeding.

Day 1 (Fig. 5.12)

Cells were attached to the surface of the polymeric structure. It was possible to observe cellular processes interacting with the chitlac scaffold. The cell morphology was rounded, as expected from completely differentiated chondrocytes. The cells were not spread on the surface, so it was possible to exclude any dedifferentiation of the cells towards a fibroblast-like phenotype. After only 24 hours from the seeding cell aggregates were present.

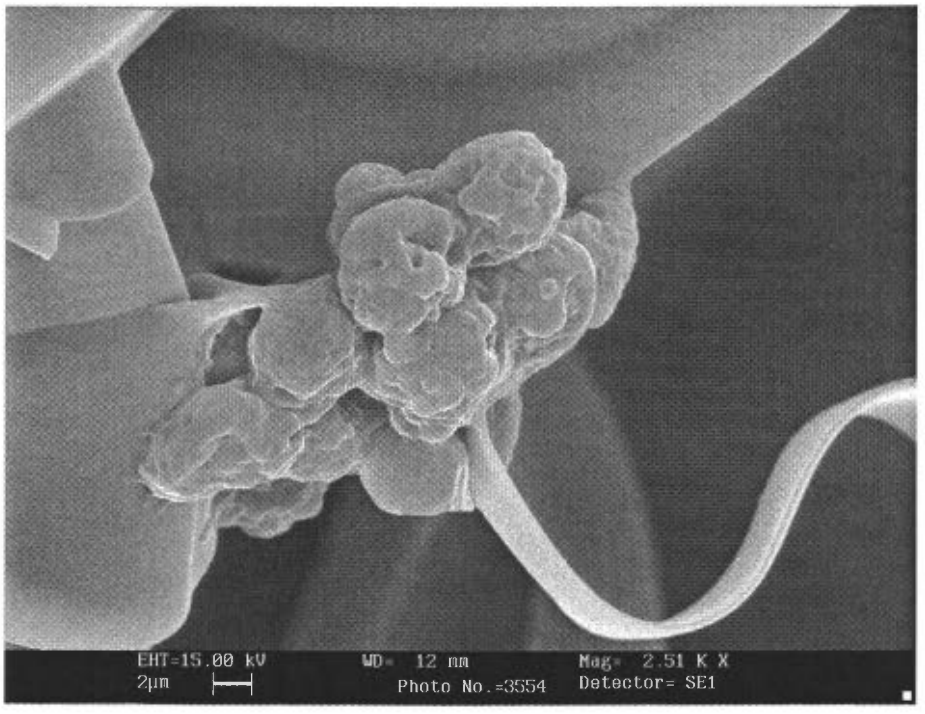


Fig. 5.12: chondrocyte-seeded BDGE-crosslinked chitlac scaffold, 1 day after seeding.

As demonstrated previously by our group (Donati et al., 2005) lactose-modified chitosan was capable to induce cell aggregation. This feature was observed on the three dimensional scaffolds as well, clearly suggesting the maintenance of specific bioactivity also after the BDGE-crosslinking procedure.

Day 3 (Fig. 5.13)

After 3 days of culture, cells were still attached on the scaffolds, the presence of aggregates was observed and it was possible to identify the synthesis of newly formed fibres surrounding the aggregates.

These 3D scaffolds turned then out to be able to maintain the differentiation of the chondrocytes (round morphology) and to trigger the synthesis of components of the extracellular matrix.

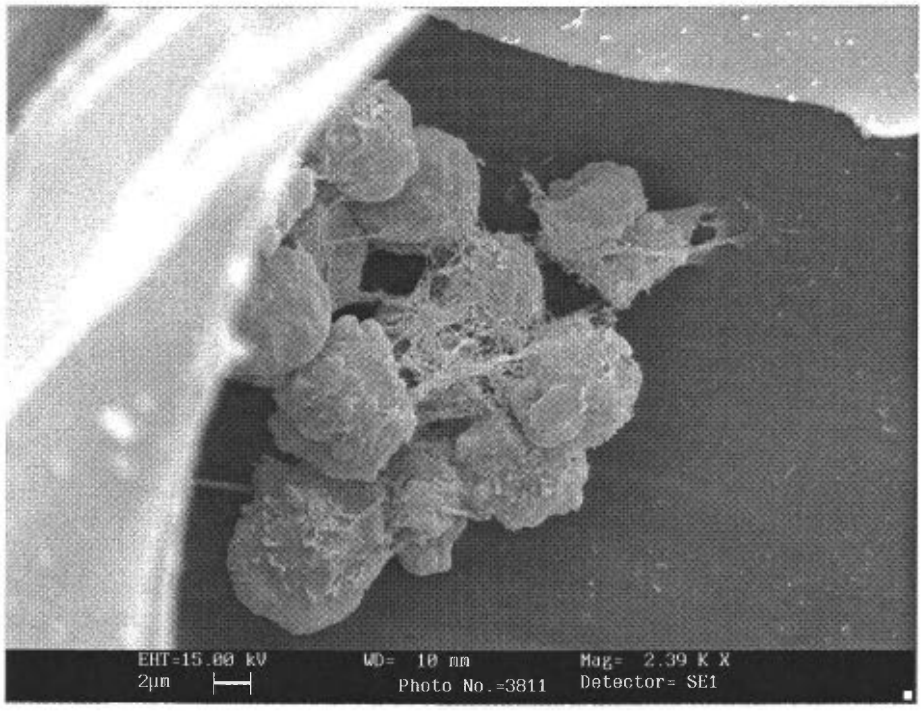


Fig. 5.13: chondrocyte-seeded BDGE-crosslinked chitlac scaffold, 3 days after seeding.

Day 15 (Fig. 5.14)

The size of the cell aggregates was increased after 15 days of culture, as it was increased the amount of the fibres surrounding the cells.

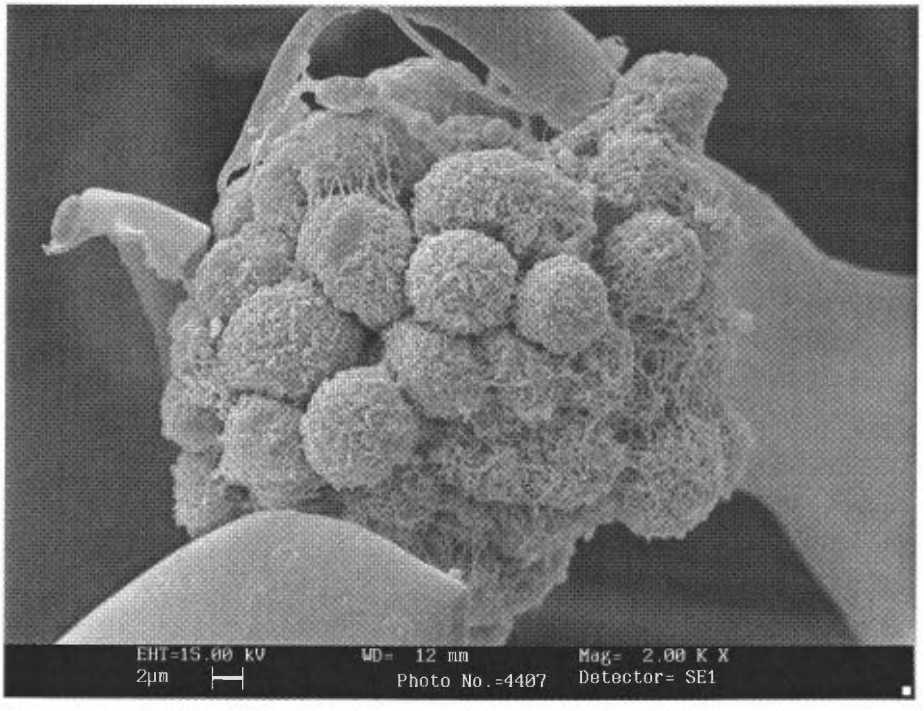


Fig. 5.14: chondrocyte-seeded BDGE-crosslinked chitlac scaffold, 15 days after seeding.

At this time point a mesh of fibres is completely surrounding the cells attached to the scaffold. A large extent of newly produced matrix was present over each cell aggregate.

In conclusion, SEM demonstrated that the BDGE-crosslinked chitlac scaffolds had many positive features as structures to be used in cartilage tissue engineering protocols:

- The polymer used as building block and the three dimensional microstructure were able to maintain the differentiation of the chondrocytes seeded on the scaffolds. There were no fibroblast-like cells spread on the surface of the construct.
- Chitlac maintains the capacity of inducing chondrocyte aggregation, as it was previously demonstrated for the polymer dissolved in solution and hence it was possible to identify, 24 hours from the seeding, the presence of large aggregates whose size increased with time culture.
- Cells seeded on the scaffolds were stimulated to synthesize components of the extracellular matrix. 3 days after seeding it was already possible to identify fibres surrounding the cell aggregates; 15 days after the seeding the cells were completely surrounded by a dense mesh of newly synthesized matrix.

5.3.6 Cell Proliferation

To perform cell proliferation assay on the scaffolds, it was essential to determine a calibration curve to correlate the fluorescence value resulting from the reduction of the Alamar Blue™ reagent with the related number of cells. A known number of chondrocytes, ranging from 5×10^3 to 2×10^5 was plated and analyzed with the Alamar Blue™. The measured fluorescence

intensity values were linearly least-square fitted and the calibration curve obtained (Fig. 5.15).

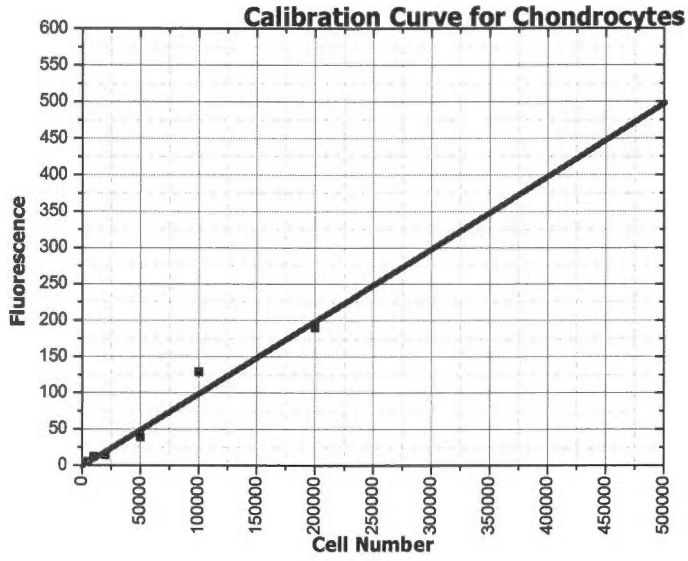


Fig. 5.15: Calibration Curve for chodrocytes with the Alamar Blue™.

Making use of the above reported curve, it was easy to determine the exact number of cells present on the scaffolds as a function of time culture.
The scaffold were seeded with 3.5×10^5 chondrocytes and the proliferation was assessed after 3 days, 11 days, 22 days and 44 days.
The fluorescence value obtained on the first measurement at day 3 after seeding corresponded to a number of cells of 1.2×10^5. Unfortunately, this data showed that the seeding protocol adopted was not completely efficient, resulting in a partial delivery of the initially seeded cells on the scaffold. In fact, a large amount of cells growing attached to the bottom of the well, away from the scaffolds could be easily visualized by optical microscope (data not shown). Then, to avoid any interference from the cells growing without being attached to the scaffolds, the structures were moved in a new multi-well plate before each Alamar Blue™ measurement.

In the following 3 weeks an increase in the cell number on the scaffolds was observed, with the total number of chondrocytes reaching 2.65 x 10^5. The last analysis, performed 6 weeks after the seeding, showed a substantially unchanged number on cells on the scaffolds.

In conclusion, a better seeding protocol should be prepared, in order to deliver the desired number of cells on the scaffold, avoiding them to fall away from the structure or pushing them on it.

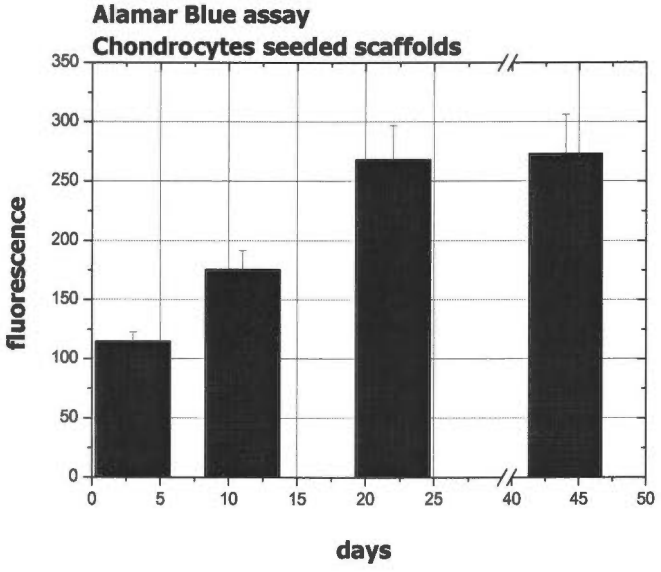


Fig. 5.16. Cell proliferation on chondrocyte-seeded scaffolds.

Days	***Fluorescence***	***Cell Number***
3	115	120000
11	175	175000
22	268	265000
44	273	270000

Table 5.4: Cell number from Alamar Blue™ assay.

5.4 Conclusions

In this chapter, three different crosslinking agents were evaluated as crosslinking agents in order to produce three-dimensional scaffolds based on lactose-modified chitosan.

The structures synthesized using BDGE, DMS and DES were initially characterized with SEM in order to evaluate the microstructure of the scaffolds, with respect to the overall porosity, pore size and pore interconnectivity. The scaffolds produced with BDGE had an average pore size of 100 μm, those crosslinked with DMS a pore size ranging from 20 to 50 μm and a high porosity and interconnectivity. DES-crosslinked structures showed a more variable structure, with a random pattern of porosity and pore size. A set of swelling experiments was performed to assess the fluid uptake and the stability of the scaffolds.

Although swelling results were rather unusual, the 3D structures produced with BDGE were the only ones stable enough to retain their shape and size with re-hydration. Having ascertained the swelling properties as well as chemical stability of the BDGE-cross linked scaffolds, attention was focused on this particular scaffold to carry out the biological tests.

Primary cultures of porcine chondrocytes were seeded on the scaffolds and cultured for 6 weeks. Cell morphology and matrix synthesis were assessed with the SEM while the cell proliferation was investigated by the Alamar Blue™ method.

SEM analysis showed that the BDGE-crosslinked chitlac scaffolds were able to maintain the chondrocyte phenotype, as demonstrated by the spherical shape of the cells attached on the structures. The formation of cell aggregates was demonstrated after 24 hours from the seeding as it was possible to identify the presence of newly formed fibres surrounding the

aggregates (from day 3 of culture), with the formation of a mesh of matrix inglobating the cells at day 15 after seeding.

Alamar Blue™ assay disclosed the low initial efficiency of the seeding method used. However, Alamar Blue™ assay clearly showed that the cell number increased in the first three weeks of cultures and then remained stable for the following three weeks.

In conclusion, the BDGE-crosslinked chitlac scaffold turned out to be a very promising biomaterial that coupling biocompatibility with specific biological activity could likely be used in protocols for cartilage tissue engineering. Further characterization of the produced matrix is needed in order to qualitatively, as well as quantitatively, determine the synthesizing activity of the chondrocytes.

CHAPTER 6
Summary and Conclusions

The overall objective of this study was the utilization of a lactose-modified chitosan (chitlac) in cartilage tissue engineering. Chitosan is a cationic polymer that has been extensively used in biomedical sciences, mainly for its several disclosed properties: it is biocompatible, biodegradable, easy to manufacture and of low cost.

Many chitosan-based scaffolds have been tested *in vitro* as delivery systems for chondrocytes, to be applied in cartilage tissue engineering protocols.

As far as the lactose-modified chitosan (chitlac) is concerned, it has been shown that, as solubilized polymer, it is able to maintain chondrocyte differentiation and to induce cell aggregation, resulting in the formation of nodules of considerable size. Moreover, the ability of the polymer to trigger the synthesis of specific markers (type II collagen and aggrecan) of ECM of the articular cartilage tissue has been reported as well.

The first aim of this work, was to investigate the hydrodynamic properties of the lactose-modified polymer in comparison with those of the native chitosan. From the intrinsic viscosity determination and rheology data it turned out that lactose branching accounted for a more dense coiling conformation assumed by chitlac chains with respect to the parent chitosan.

The influence of chitlac on the viscoelastic properties o hyaluronic acid (HA) was also addressed. HA is the polymer responsible for the viscoelastic properties of the synovial fluid and the main agent in viscosupplementation formulation.

Steady state shear viscosity data as well as oscillatory dynamic spectra obtained for blends containing chitlac and HA, both of comparable chain length (chitlac-LMW HA mixtures), indicated that a stabilizing effect was exerted by the presence in the mixed polymer matrix of lactose-branched

chains. Indeed, an unexpected synergistic effect on Newtonian viscosity as well as an increase in the elastic response were observed for these HA-chitlac transient networks. Rheological measurements for chitlac-HMW HA mixtures highlighted that the rheological behaviour of the mixtures was essentially governed by the high molar mass HA component. For the chitlac-HMW HA blends, the wide difference in contour chain length between HA and chitlac likely accounted for the high amount of chitlac required to reduce the viscoelastic properties of a HA-based transient network.

3D chitlac-based scaffolds were prepared, their chemical stability as well as biocompatibility and bioactivity toward chondrocyte cells investigated. The prepared scaffolds were able to maintain the bioactive properties shown by the polymer in solution. Indeed, they were able to maintain chondrocyte differentiation, to induce cell aggregation and to stimulate extracellular matrix synthesis.

As shown here chitlac displays rather interesting properties which could be exploited in cartilage tissue engineering protocols. Its hydrodynamic behaviour and intrinsic bioactivity, coupled with the already demonstrated viscoelastic properties of hyaluronic acid, would be of extreme interest for the development of new formulation for viscosupplementation treatment of osteoarthritis.

The preliminary results from the physico-chemical and biological characterization of crosslinked scaffolds based on chitlac are promising for a future application in the field of cartilage regeneration.

Further development of these scaffolds would be represented by the preparation of chitlac-based interpenetrated networks, where the additional polymer (for instance hyaluronic acid) would favourably contribute in terms of mechanical and bioactivity properties.

Reference List

Agrawal,C.M. and Ray,R.B. (2001). Biodegradable polymeric scaffolds for musculoskeletal tissue engineering. J. Biomed. Mater. Res. *55*, 141-150.

Aimin,C., Chunlin,H., Juliang,B., Tinyin,Z., and Zhichao,D. (1999). Antibiotic loaded chitosan bar. An in vitro, in vivo study of a possible treatment for osteomyelitis. Clin. Orthop. Relat Res. 239-247.

Alberts,B., Johnson,A., Lewis,J., Raff,M., Roberts,K., and Walter,P. (2002). *Molecular Biology of the Cell.*

Alsalameh,S., Amin,R., Gemba,T., and Lotz,M. (2004). Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. Arthritis Rheum. *50*, 1522-1532.

Athanasiou,K.A., Shah,A.R., Hernandez,R.J., and LeBaron,R.G. (2001). Basic science of articular cartilage repair. Clin. Sports Med. *20*, 223-247.

Aufderheide,A.C. and Athanasiou,K.A. (2004). Mechanical stimulation toward tissue engineering of the knee meniscus. Ann. Biomed. Eng *32*, 1161-1174.

Awad,H.A., Wickham,M.Q., Leddy,H.A., Gimble,J.M., and Guilak,F. (2004). Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials *25*, 3211-3222.

Balazs,E.A. and Denlinger,J.L. (1993). Viscosupplementation: a new concept in the treatment of osteoarthritis. J. Rheumatol. Suppl *39*, 3-9.

Ballantyne,B. and Jordan,S.L. (2001). Toxicological, medical and industrial hygiene aspects of glutaraldehyde with particular reference to its biocidal use in cold sterilization procedures. J. Appl. Toxicol. *21*, 131-151.

Barbero,A., Grogan,S., Schafer,D., Heberer,M., Mainil-Varlet,P., and Martin,I. (2004). Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. Osteoarthritis. Cartilage. *12*, 476-484.

Barnes,H.A., Hutton,J.F., and Walters,K. (1989). An Introduction to Rheology. Elsevier, Amsterdam).

Barondes,S.H., Castronovo,V., Cooper,D.N., Cummings,R.D., Drickamer,K., Feizi,T., Gitt,M.A., Hirabayashi,J., Hughes,C., Kasai,K., and . (1994). Galectins: a family of animal beta-galactoside-binding lectins. Cell *76*, 597-598.

Beauchamp,R.O., Jr., St Clair,M.B., Fennell,T.R., Clarke,D.O., Morgan,K.T., and Kari,F.W. (1992). A critical review of the toxicology of glutaraldehyde. Crit Rev. Toxicol. *22*, 143-174.

Beena,M.S., Chandy,T., and Sharma,C.P. (1995). Heparin immobilized chitosan--poly ethylene glycol interpenetrating network: antithrombogenicity. Artif. Cells Blood Substit. Immobil. Biotechnol. *23*, 175-192.

Bellamy,N., Campbell,J., Robinson,V., Gee,T., Bourne,R., and Wells,G. (2006). Viscosupplementation for the treatment of osteoarthritis of the knee. Cochrane. Database. Syst. Rev. CD005321.

Benjamin,M. and Ralphs,J.R. (2004). Biology of fibrocartilage cells. Int. Rev. Cytol. *233*, 1-45.

Berger,J., Reist,M., Mayer,J.M., Felt,O., and Gurny,R. (2004a). Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. Eur. J. Pharm. Biopharm. *57*, 35-52.

Berger,J., Reist,M., Mayer,J.M., Felt,O., Peppas,N.A., and Gurny,R. (2004b). Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. Eur. J. Pharm. Biopharm. *57*, 19-34.

Bhattarai,N., Ramay,H.R., Gunn,J., Matsen,F.A., and Zhang,M. (2005). PEG-grafted chitosan as an injectable thermosensitive hydrogel for sustained protein release. J. Control Release *103*, 609-624.

Bird,R.B., Armstrong,R.C., and Hassager,O. (1987). Dynamics of Polymeric Liquids Vol. 1: Fluid Mechanics. John Wiley, New York).

Bonaventure,J., Kadhom,N., Cohen-Solal,L., Ng,K.H., Bourguignon,J., Lasselin,C., and Freisinger,P. (1994). Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp. Cell Res. *212*, 97-104.

Bonzani,I.C., George,J.H., and Stevens,M.M. (2006). Novel materials for bone and cartilage regeneration. Curr. Opin. Chem. Biol. *10*, 568-575.

Brittberg,M., Lindahl,A., Nilsson,A., Ohlsson,C., Isaksson,O., and Peterson,L. (1994). Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N. Engl. J Med. *331*, 889-895.

Brooks,P.M. (2002). Impact of osteoarthritis on individuals and society: how much disability? Social consequences and health economic implications. Curr. Opin. Rheumatol. *14*, 573-577.

Brun,P., Abatangelo,G., Radice,M., Zacchi,V., Guidolin,D., Daga,G.D., and Cortivo,R. (1999). Chondrocyte aggregation and reorganization into three-dimensional scaffolds. J. Biomed. Mater. Res. *46*, 337-346.

Bryant,S.J. and Anseth,K.S. (2001). The effects of scaffold thickness on tissue engineered cartilage in photocrosslinked poly(ethylene oxide) hydrogels. Biomaterials *22*, 619-626.

Buckwalter,J.A. and Mankin,H.J. (1998a). Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. Instr. Course Lect. *47*, 487-504.

Buckwalter,J.A. and Mankin,H.J. (1998b). Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr. Course Lect. *47*, 477-486.

Buckwalter,J.A. and Martin,J.A. (2006). Osteoarthritis. Adv. Drug Deliv. Rev. *58*, 150-167.

Buranapanitkit,B., Srinilta,V., Ingviga,N., Oungbho,K., Geater,A., and Ovatlarnporn,C. (2004). The efficacy of a hydroxyapatite composite as a biodegradable antibiotic delivery system. Clin. Orthop. Relat Res. 244-252.

Buschmann,M.D., Gluzband,Y.A., Grodzinsky,A.J., Kimura,J.H., and Hunziker,E.B. (1992). Chondrocytes in agarose culture synthesize a mechanically functional extracellular matrix. J Orthop. Res. *10*, 745-758.

Cao,Y., Rodriguez,A., Vacanti,M., Ibarra,C., Arevalo,C., and Vacanti,C.A. (1998). Comparative study of the use of poly(glycolic acid), calcium alginate and pluronics in the engineering of autologous porcine cartilage. J. Biomater. Sci. Polym. Ed *9*, 475-487.

Caplan,A.I. and Bruder,S.P. (2001). Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol. Med. *7*, 259-264.

Carney,S.L. and Muir,H. (1988). The structure and function of cartilage proteoglycans. Physiol Rev. *68*, 858-910.

Carranza-Bencano,A., Perez-Tinao,M., Ballesteros-Vazquez,P., rmas-Padron,J.R., Hevia-Alonso,A., and Crespo,F.M. (1999). Comparative study of the reconstruction of articular cartilage defects with free costal perichondrial grafts and free tibial periosteal grafts: An experimental study on rabbits. Calcified Tissue International *65*, 402-407.

Carver,S.E. and Heath,C.A. (1999). Increasing extracellular matrix production in regenerating cartilage with intermittent physiological pressure. Biotechnol. Bioeng. *62*, 166-174.

Charulatha,V. and Rajaram,A. (1997). Crosslinking density and resorption of dimethyl suberimidate-treated collagen. J. Biomed. Mater. Res. *36*, 478-486.

Charulatha,V. and Rajaram,A. (2003). Influence of different crosslinking treatments on the physical properties of collagen membranes. Biomaterials *24*, 759-767.

Chen,F.S., Frenkel,S.R., and Di Cesare,P.E. (1999). Repair of articular cartilage defects: part I. Basic Science of cartilage healing. Am. J. Orthop. *28*, 31-33.

Chenite,A., Chaput,C., Wang,D., Combes,C., Buschmann,M.D., Hoemann,C.D., Leroux,J.C., Atkinson,B.L., Binette,F., and Selmani,A. (2000). Novel injectable neutral solutions of chitosan form biodegradable gels in situ. Biomaterials *21*, 2155-2161.

Chow,K.S. and Khor,E. (2000). Novel fabrication of open-pore chitin matrixes. Biomacromolecules. *1*, 61-67.

Chua,K.H., Aminuddin,B.S., Fuzina,N.H., and Ruszymah,B.H. (2004). Interaction between insulin-like growth factor-1 with other growth factors in serum depleted culture medium for human cartilage engineering. Med. J Malaysia *59 Suppl B*, 7-8.

Conrozier,T. and Vignon,E. (2005). Is there evidence to support the inclusion of viscosupplementation in the treatment paradigm for patients with hip osteoarthritis? Clin. Exp. Rheumatol. *23*, 711-716.

Cooper,D.N. and Barondes,S.H. (1999). God must love galectins; he made so many of them. Glycobiology *9*, 979-984.

Costain,D.J., Kennedy,R., Ciona,C., McAlister,V.C., and Lee,T.D. (1997). Prevention of postsurgical adhesions with N,O-carboxymethyl chitosan: examination of the most efficacious preparation and the effect of N,O-carboxymethyl chitosan on postsurgical healing. Surgery *121*, 314-319.

Cui,Y.L., Qi,A.D., Liu,W.G., Wang,X.H., Wang,H., Ma,D.M., and Yao,K.D. (2003). Biomimetic surface modification of poly(L-lactic acid) with chitosan and its effects on articular chondrocytes in vitro. Biomaterials *24*, 3859-3868.

Czitrom,A.A., Langer,F., McKee,N., and Gross,A.E. (1986). Bone and cartilage allotransplantation. A review of 14 years of research and clinical studies. Clin. Orthop. Relat Res. 141-145.

Davidson,D., Blanc,A., Filion,D., Wang,H., Plut,P., Pfeffer,G., Buschmann,M.D., and Henderson,J.E. (2005). Fibroblast growth factor (FGF) 18 signals through FGF receptor 3 to promote chondrogenesis. J Biol. Chem. *280*, 20509-20515.

De Angelis,A.A., Capitani,D., and Crescenzi,V. (1998). Synthesis and C-13 CP-MAS NMR characterization of a new chitosan-based polymeric network. Macromolecules *31*, 1595-1601.

de Nooy,A.E.J., Rori,V., Masci,G., Dentini,M., and Crescenzi,V. (2000). Synthesis and preliminary characterisation of charged derivatives and hydrogels from scleroglucan. Carbohydrate Research *324*, 116-126.

Deasy,B.M., Qu-Peterson,Z., Greenberger,J.S., and Huard,J. (2002). Mechanisms of muscle stem cell expansion with cytokines. Stem Cells *20*, 50-60.

Denkbas,E.B., Seyyal,M., and Piskin,E. (1999). 5-fluorouracil loaded chitosan microspheres for chemoembolization. J. Microencapsul. *16*, 741-749.

Di Martino,A., Sittinger,M., and Risbud,M.V. (2005). Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. Biomaterials *26*, 5983-5990.

Diduch,D.R., Jordan,L.C., Mierisch,C.M., and Balian,G. (2000). Marrow stromal cells embedded in alginate for repair of osteochondral defects. Arthroscopy *16*, 571-577.

Donati,I., Stredanska,S., Silvestrini,G., Vetere,A., Marcon,P., Marsich,E., Mozetic,P., Gamini,A., Paoletti,S., and Vittur,F. (2005). The aggregation of pig articular chondrocyte and synthesis of extracellular matrix by a lactose-modified chitosan. Biomaterials *26*, 987-998.

Dong,Y., Qiu,W., Ruan,Y., Wu,Y., Wang,M., and Xu,C. (2001). Influence of molecular weight on critical concentration of chitosan/formic acid liquid crystalline solution. Polym. J *33*, 387-389.

Dowthwaite,G.P., Bishop,J.C., Redman,S.N., Khan,I.M., Rooney,P., Evans,D.J.R., Haughton,L., Bayram,Z., Boyer,S., Thomson,B., Wolfe,M.S., and Archer,C.W. (2004). The surface of articular cartilage contains a progenitor cell population. Journal of Cell Science *117*, 889-897.

Draget,K.I. (1996). Associating phenomena in highly acetylated chitosan gels. J Polymer Gels and Networks *2*, 143-151.

Elders,M.J. (2000). The increasing impact of arthritis on public health. J Rheumatol. Suppl *60*, 6-8.

Felson,D.T. (1988). Epidemiology of hip and knee osteoarthritis. Epidemiol. Rev. *10*, 1-28.

Felson,D.T. (1990). The epidemiology of knee osteoarthritis: results from the Framingham Osteoarthritis Study. Semin. Arthritis Rheum. *20*, 42-50.

Felson,D.T., Zhang,Y., Hannan,M.T., Naimark,A., Weissman,B.N., Aliabadi,P., and Levy,D. (1995). The incidence and natural history of knee osteoarthritis in the elderly. The Framingham Osteoarthritis Study. Arthritis Rheum. *38*, 1500-1505.

Felt,O., Furrer,P., Mayer,J.M., Plazonnet,B., Buri,P., and Gurny,R. (1999). Topical use of chitosan in ophthalmology: tolerance assessment and evaluation of precorneal retention. Int. J. Pharm. *180*, 185-193.

Ferry,J.D. (1970). Viscoelastic Properties of Polymers. John Wiley, New York).

Fields,R.D. and Lancaster,M.V. (1993). Dual-attribute continuous monitoring of cell proliferation/cytotoxicity. Am. Biotechnol. Lab *11*, 48-50.

Fortier,L.A., Nixon,A.J., Mohammed,H.O., and Lust,G. (1997). Altered biological activity of equine chondrocytes cultured in a three-dimensional fibrin matrix and supplemented with transforming growth factor beta-1. Am. J Vet. Res. *58*, 66-70.

Fragonas,E., Valente,M., Pozzi-Mucelli,M., Toffanin,R., Rizzo,R., Silvestri,F., and Vittur,F. (2000). Articular cartilage repair in rabbits by using suspensions of allogenic chondrocytes in alginate. Biomaterials *21*, 795-801.

Freed,L.E., Grande,D.A., Lingbin,Z., Emmanual,J., Marquis,J.C., and Langer,R. (1994a). Joint resurfacing using allograft chondrocytes and synthetic biodegradable polymer scaffolds. J Biomed. Mater. Res. *28*, 891-899.

Freed,L.E., Hollander,A.P., Martin,I., Barry,J.R., Langer,R., and Vunjak-Novakovic,G. (1998). Chondrogenesis in a cell-polymer-bioreactor system. Exp. Cell Res. *240*, 58-65.

Freed,L.E., Marquis,J.C., Nohria,A., Emmanual,J., Mikos,A.G., and Langer,R. (1993). Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers. J Biomed. Mater. Res. *27*, 11-23.

Freed,L.E., Martin,I., and Vunjak-Novakovic,G. (1999). Frontiers in tissue engineering. In vitro modulation of chondrogenesis. Clin. Orthop. Relat Res. S46-S58.

Freed,L.E., Vunjak-Novakovic,G., Biron,R.J., Eagles,D.B., Lesnoy,D.C., Barlow,S.K., and Langer,R. (1994b). Biodegradable polymer scaffolds for tissue engineering. Biotechnology (N. Y.) *12*, 689-693.

Frenkel,S.R., Toolan,B., Menche,D., Pitman,M.I., and Pachence,J.M. (1997). Chondrocyte transplantation using a collagen bilayer matrix for cartilage repair. J Bone Joint Surg. Br. *79*, 831-836.

Friedman,M.J., Berasi,C.C., Fox,J.M., Del,P.W., Snyder,S.J., and Ferkel,R.D. (1984). Preliminary results with abrasion arthroplasty in the osteoarthritic knee. Clin. Orthop. Relat Res. 200-205.

Fujisato,T., Sajiki,T., Liu,Q., and Ikada,Y. (1996). Effect of basic fibroblast growth factor on cartilage regeneration in chondrocyte-seeded collagen sponge scaffold. Biomaterials *17*, 155-162.

Gamini,A., Paoletti,S., and Zanetti,F. (1992). Chain Rigidity of Polyuronates: Static Light Scattering of Aqueous Solutions of Hyaluronate and Alginate. In Laser Light Scattering in Biochemistry, Royal Society of Chemistry, Cambridge), pp. 294-311.

Geng,L., Feng,W., Hutmacher,D.W., Wong,Y.S., Loh,H.T., and Fuh,J.Y.H. (2005). Direct writing of chitosan scaffolds using a robotic system. Rapid Prototyping Journal *11*, 90-97.

Gibbs,D.A., Merrill,E.W., Smith,K.A., and Balazs,E.A. (1968). Rheology of hyaluronic acid. Biopolymers *6*, 777-791.

Gobin,A.S., Froude,V.E., and Mathur,A.B. (2005). Structural and mechanical characteristics of silk fibroin and chitosan blend scaffolds for tissue regeneration. J. Biomed. Mater. Res. A *74*, 465-473.

Goldberg,V.M. and Buckwalter,J.A. (2005). Hyaluronans in the treatment of osteoarthritis of the knee: evidence for disease-modifying activity. Osteoarthritis. Cartilage. *13*, 216-224.

Graessley,W.W. (1974). The entanglement concept in polymer rheology. Adv. Polym. Sci. *16*, 1-179.

Grande,D.A., Halberstadt,C., Naughton,G., Schwartz,R., and Manji,R. (1997). Evaluation of matrix scaffolds for tissue engineering of articular cartilage grafts. J. Biomed. Mater. Res. *34*, 211-220.

Grandolfo,M., D'Andrea,P., Paoletti,S., Martina,M., Silvestrini,G., Bonucci,E., and Vittur,F. (1993). Culture and differentiation of chondrocytes entrapped in alginate gels. Calcif. Tissue Int. *52*, 42-48.

Gray,M.L., Pizzanelli,A.M., Grodzinsky,A.J., and Lee,R.C. (1988). Mechanical and physiochemical determinants of the chondrocyte biosynthetic response. J Orthop. Res. *6*, 777-792.

Guilak,F. and Mow,V.C. (2000). The mechanical environment of the chondrocyte: a biphasic finite element model of cell-matrix interactions in articular cartilage. J. Biomech. *33*, 1663-1673.

Haisch,A., Loch,A., David,J., Pruss,A., Hansen,R., and Sittinger,M. (2000). Preparation of a pure autologous biodegradable fibrin matrix for tissue engineering. Med. Biol. Eng Comput. *38*, 686-689.

Hangody,L., Feczko,P., Bartha,L., Bodo,G., and Kish,G. (2001). Mosaicplasty for the treatment of articular defects of the knee and ankle. Clinical Orthopaedics and Related Research S328-S336.

Hangody,L., Rathonyi,G.K., Duska,Z., Vasarhelyi,G., Fules,P., and Modis,L. (2004). Autologous osteochondral mosaicplasty. Surgical technique. J Bone Joint Surg. Am. *86-A Suppl 1*, 65-72.

Hardingham,T.E. (1979). The role of link-protein in the structure of cartilage proteoglycan aggregates. Biochem. J. *177*, 237-247.

Hasler,E.M., Herzog,W., Wu,J.Z., Muller,W., and Wyss,U. (1999). Articular cartilage biomechanics: theoretical models, material properties, and biosynthetic response. Crit Rev. Biomed. Eng *27*, 415-488.

Hayashi,T., Abe,E., and Jasin,H.E. (1996). Fibronectin synthesis in superficial and deep layers of normal articular cartilage. Arthritis Rheum. *39*, 567-573.

Heath,C. and Magari,C. (1996). Mini-review: Mechanical factors affecting cartilage regeneration *in vitro.* Biotechnology and Bioengineering *50*, 430-437.

Hendrickson,D.A., Nixon,A.J., Grande,D.A., Todhunter,R.J., Minor,R.M., Erb,H., and Lust,G. (1994). Chondrocyte-fibrin matrix transplants for resurfacing extensive articular cartilage defects. J Orthop. Res. *12*, 485-497.

Hooper,K.A., Macon,N.D., and Kohn,J. (1998). Comparative histological evaluation of new tyrosine-derived polymers and poly (L-lactic acid) as a function of polymer degradation. J Biomed. Mater. Res. *41*, 443-454.

Hsu,S.H., Whu,S.W., Hsieh,S.C., Tsai,C.L., Chen,D.C., and Tan,T.S. (2004). Evaluation of chitosan-alginate-hyaluronate complexes modified by an RGD-containing protein as tissue-engineering scaffolds for cartilage regeneration. Artif. Organs *28*, 693-703.

Hu,Q., Li,B., Wang,M., and Shen,J. (2004). Preparation and characterization of biodegradable chitosan/hydroxyapatite nanocomposite rods via in situ hybridization: a potential material as internal fixation of bone fracture. Biomaterials *25*, 779-785.

Huang,J.I., Kazmi,N., Durbhakula,M.M., Hering,T.M., Yoo,J.U., and Johnstone,B. (2005). Chondrogenic potential of progenitor cells derived from human bone marrow and adipose tissue: a patient-matched comparison. J Orthop. Res. *23*, 1383-1389.

Huckle,J., Dootson,G., Medcalf,N., McTaggart,S., Wright,E., Carter,A., Schreiber,R., Kirby,B., Dunkelman,N., Stevenson,S., Riley,S., Davisson,T., and Ratcliffe,A. (2003). Differentiated chondrocytes for cartilage tissue engineering. Novartis. Found. Symp. *249*, 103-112.

Hunt,S.A., Jazrawi,L.M., and Sherman,O.H. (2002). Arthroscopic management of osteoarthritis of the knee. J Am. Acad. Orthop. Surg. *10*, 356-363.

Hunter,W. (1743). Of the structure and disease of articulating cartilages. Philos Trans R Soc London *42*, 514-521.

Hutmacher,D.W. (2000). Scaffolds in tissue engineering bone and cartilage. Biomaterials *21*, 2529-2543.

Hwang,J.W. and Kokini,J.L. (1991). Structure and rheological function of side branches of carbohydrate polymers. J Texture Stud *22*, 123-167.

Indrawattana,N., Chen,G., Tadokoro,M., Shann,L.H., Ohgushi,H., Tateishi,T., Tanaka,J., and Bunyaratvej,A. (2004). Growth factor combination for chondrogenic induction from human mesenchymal stem cell. Biochem. Biophys. Res. Commun. *320*, 914-919.

Jackson,D.W., Lalor,P.A., Aberman,H.M., and Simon,T.M. (2001). Spontaneous repair of full-thickness defects of articular cartilage in a goat model. A preliminary study. J. Bone Joint Surg. Am. *83-A*, 53-64.

Jackson,D.W. and Simon,T.M. (1999). Tissue engineering principles in orthopaedic surgery. Clin. Orthop. Relat Res. S31-S45.

Jin,J., Song,M., and Hourston,D.J. (2004). Novel chitosan-based films cross-linked by genipin with improved physical properties. Biomacromolecules. *5*, 162-168.

Johnstone,B., Hering,T.M., Caplan,A.I., Goldberg,V.M., and Yoo,J.U. (1998). In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp. Cell Res. *238*, 265-272.

Kafienah,W., Jakob,M., Demarteau,O., Frazer,A., Barker,M.D., Martin,I., and Hollander,A.P. (2002). Three-dimensional tissue engineering of hyaline cartilage: comparison of adult nasal and articular chondrocytes. Tissue Eng *8*, 817-826.

Kawamura,S., Wakitani,S., Kimura,T., Maeda,A., Caplan,A.I., Shino,K., and Ochi,T. (1998). Articular cartilage repair. Rabbit experiments with a collagen gel-biomatrix and chondrocytes cultured in it. Acta Orthop. Scand. *69*, 56-62.

Kelly,M.A., Kurzweil,P.R., and Moskowitz,R.W. (2004). Intra-articular hyaluronans in knee osteoarthritis: rationale and practical considerations. Am. J Orthop. *33*, 15-22.

Khalid,M.N., Agnely,F., Yagoubi,N., Grossiord,J.L., and Couarraze,G. (2002). Water state characterization, swelling behavior, thermal and mechanical properties of chitosan based networks. Eur. J. Pharm. Sci. *15*, 425-432.

Kiani,C., Chen,L., Wu,Y.J., Yee,A.J., and Yang,B.B. (2002). Structure and function of aggrecan. Cell Res. *12*, 19-32.

Kirchner,M. and Marshall,D. (2006). A double-blind randomized controlled trial comparing alternate forms of high molecular weight hyaluronan for the treatment of osteoarthritis of the knee. Osteoarthritis. Cartilage. *14*, 154-162.

Klug,S., Merker,H.J., and Jackh,R. (2001). Effects of ethylene glycol and metabolites on in vitro development of rat embryos during organogenesis. Toxicol. In Vitro *15*, 635-642.

Knaul,J.Z., Hudson,S.M., and Creber,K.A.M. (1999). Crosslinking of chitosan fibers with dialdehydes: Proposal of a new reaction mechanism. Journal of Polymer Science Part B-Polymer Physics *37*, 1079-1094.

Kotze,A.F., Luessen,H.L., de Boer,A.G., Verhoef,J.C., and Junginger,H.E. (1999). Chitosan for enhanced intestinal permeability: prospects for derivatives soluble in neutral and basic environments. Eur. J. Pharm. Sci. *7*, 145-151.

Koyano,T., Minoura,N., Nagura,M., and Kobayashi,K. (1998). Attachment and growth of cultured fibroblast cells on PVA/chitosan-blended hydrogels. J. Biomed. Mater. Res. *39*, 486-490.

Kubota,N., Tatsumoto,N., Sano,T., and Toya,K. (2000). A simple preparation of half N-acetylated chitosan highly soluble in water and aqueous organic solvents. Carbohydr. Res. *324*, 268-274.

Kujawa,M.J. and Caplan,A.I. (1986). Hyaluronic acid bonded to cell-culture surfaces stimulates chondrogenesis in stage 24 limb mesenchyme cell cultures. Dev. Biol. *114*, 504-518.

Kuo,C.K., Li,W.J., Mauck,R.L., and Tuan,R.S. (2006). Cartilage tissue engineering: its potential and uses. Curr. Opin. Rheumatol. *18*, 64-73.

Kuo,C.K. and Tuan,R.S. (2003). Tissue engineering with mesenchymal stem cells. IEEE Eng Med. Biol. Mag. *22*, 51-56.

Lahiji,A., Sohrabi,A., Hungerford,D.S., and Frondoza,C.G. (2000). Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. J. Biomed. Mater. Res. *51*, 586-595.

Langer,R. and Vacanti,J.P. (1993). Tissue engineering. Science *260*, 920-926.

Launay,B., Doublier,J.L., and Cuvelier,G. (1986). Flow properties of aqueous solutions and idspersions of polysaccharides. In Functional properties of food macromolecules, Elsevier Applied Science Publishers, New York), pp. 12-19.

Lawrence,R.C., Helmick,C.G., Arnett,F.C., Deyo,R.A., Felson,D.T., Giannini,E.H., Heyse,S.P., Hirsch,R., Hochberg,M.C., Hunder,G.G., Liang,M.H., Pillemer,S.R., Steen,V.D., and Wolfe,F. (1998). Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. Arthritis Rheum. *41*, 778-799.

LeBaron,R.G. and Athanasiou,K.A. (2000). Ex vivo synthesis of articular cartilage. Biomaterials *21*, 2575-2587.

Lee,J.E., Kim,K.E., Kwon,I.C., Ahn,H.J., Lee,S.H., Cho,H., Kim,H.J., Seong,S.C., and Lee,M.C. (2004a). Effects of the controlled-released TGF-beta 1 from chitosan microspheres on chondrocytes cultured in a collagen/chitosan/glycosaminoglycan scaffold. Biomaterials *25*, 4163-4173.

Lee,J.E., Kim,S.E., Kwon,I.C., Ahn,H.J., Cho,H., Lee,S.H., Kim,H.J., Seong,S.C., and Lee,M.C. (2004b). Effects of a chitosan scaffold containing TGF-beta1 encapsulated chitosan microspheres on in vitro chondrocyte culture. Artif. Organs *28*, 829-839.

Lee,S.H., Kim,B.S., Kim,S.H., Kang,S.W., and Kim,Y.H. (2004c). Thermally produced biodegradable scaffolds for cartilage tissue engineering. Macromol. Biosci. *4*, 802-810.

Leung,H.W. (2001). Ecotoxicology of glutaraldehyde: review of environmental fate and effects studies. Ecotoxicol. Environ. Saf *49*, 26-39.

Li,W.J., Tuli,R., Okafor,C., Derfoul,A., Danielson,K.G., Hall,D.J., and Tuan,R.S. (2005). A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. Biomaterials *26*, 599-609.

Livesley,P.J., Doherty,M., Needoff,M., and Moulton,A. (1991). Arthroscopic lavage of osteoarthritic knees. J Bone Joint Surg. Br. *73*, 922-926.

Lo,G.H., LaValley,M., McAlindon,T., and Felson,D.T. (2003). Intra-articular hyaluronic acid in treatment of knee osteoarthritis: a meta-analysis. JAMA *290*, 3115-3121.

Loeser,R.F. (1993). Integrin-mediated attachment of articular chondrocytes to extracellular matrix proteins. Arthritis Rheum. *36*, 1103-1110.

Lohmander,L.S. (2003). Tissue engineering of cartilage: do we need it, can we do it, is it good and can we prove it? Novartis. Found. Symp. *249*, 2-10.

Lonner,J.H. (2004). Patellofemoral arthroplasty: pros, cons, and design considerations. Clin. Orthop. Relat Res. 158-165.

Madihally,S.V. and Matthew,H.W. (1999). Porous chitosan scaffolds for tissue engineering. Biomaterials *20*, 1133-1142.

Mao,J.S., Cui,Y.L., Wang,X.H., Sun,Y., Yin,Y.J., Zhao,H.M., and De,Y.K. (2004). A preliminary study on chitosan and gelatin polyelectrolyte complex cytocompatibility by cell cycle and apoptosis analysis. Biomaterials *25*, 3973-3981.

Marcon,P., Marsich,E., Vetere,A., Mozetic,P., Campa,C., Donati,I., Vittur,F., Gamini,A., and Paoletti,S. (2005). The role of Galectin-1 in the interaction between chondrocytes and a lactose-modified chitosan. Biomaterials *26*, 4975-4984.

Mazzucco,D., McKinley,G., Scott,R.D., and Spector,M. (2002). Rheology of joint fluid in total knee arthroplasty patients. J Orthop. Res. *20*, 1157-1163.

McDevitt,C.A. and Webber,R.J. (1990). The ultrastructure and biochemistry of meniscal cartilage. Clin. Orthop. Relat Res. 8-18.

Mi,F.L., Kuan,C.Y., Shyu,S.S., Lee,S.T., and Chang,S.F. (2000). The study of gelation kinetics and chain-relaxation properties of glutaraldehyde-cross-linked chitosan gel and their effects on microspheres preparation and drug release. Carbohydrate Polymers *41*, 389-396.

Mi,F.L., Tan,Y.C., Liang,H.F., and Sung,H.W. (2002). In vivo biocompatibility and degradability of a novel injectable-chitosan-based implant. Biomaterials *23*, 181-191.

Modawal,A., Ferrer,M., Choi,H.K., and Castle,J.A. (2005). Hyaluronic acid injections relieve knee pain. J. Fam. Pract. *54*, 758-767.

Monteiro,O.A., Jr. and Airoldi,C. (1999). Some studies of crosslinking chitosan-glutaraldehyde interaction in a homogeneous system. Int. J. Biol. Macromol. *26*, 119-128.

Morris,E.R., Cutler,A.N., Ross-Murphy,D.A., Rees,D.A., and Price,J. (1981). Concentration and shear rate dependence of viscosity in random coil polysaccharide solutions. Carbohydr. Polym *1*, 5-21.

Mort,J.S. and Billington,C.J. (2001). Articular cartilage and changes in arthritis: matrix degradation. Arthritis Res. *3*, 337-341.

Murata-Kamiya,N., Kamiya,H., Kaji,H., and Kasai,H. (1997). Mutational specificity of glyoxal, a product of DNA oxidation, in the lacI gene of wild-type Escherichia coli W3110. Mutat. Res. *377*, 255-262.

Muzzarelli,R.A. (1997). Human enzymatic activities related to the therapeutic administration of chitin derivatives. Cell Mol. Life Sci. *53*, 131-140.

Muzzarelli,R.A., Tanfani,F., Emanuelli,M., Pace,D.P., Chiurazzi,E., and Piani,M. (1984). Sulfated N-(carboxymethyl)chitosans: novel blood anticoagulants. Carbohydr. Res. *126*, 225-231.

Nakano,T., Dodd,C.M., and Scott,P.G. (1997). Glycosaminoglycans and proteoglycans from different zones of the porcine knee meniscus. J. Orthop. Res. *15*, 213-220.

Nehrer,S., Breinan,H.A., Ramappa,A., Shortkroff,S., Young,G., Minas,T., Sledge,C.B., Yannas,I.V., and Spector,M. (1997). Canine chondrocytes seeded in type I and type II collagen implants investigated in vitro. J. Biomed. Mater. Res. *38*, 95-104.

Nesic,D., Whiteside,R., Brittberg,M., Wendt,D., Martin,I., and Mainil-Varlet,P. (2006). Cartilage tissue engineering for degenerative joint disease. Adv. Drug Deliv. Rev. *58*, 300-322.

Nettles,D.L., Elder,S.H., and Gilbert,J.A. (2002). Potential use of chitosan as a cell scaffold material for cartilage tissue engineering. Tissue Eng *8*, 1009-1016.

Neustadt,D., Caldwell,J., Bell,M., Wade,J., and Gimbel,J. (2005). Clinical effects of intraarticular injection of high molecular weight hyaluronan (Orthovisc) in osteoarthritis of the knee: a randomized, controlled, multicenter trial. J Rheumatol. *32*, 1928-1936.

Neustadt,D.H. (2006). Intra-articular injections for osteoarthritis of the knee. Cleve. Clin. J Med. *73*, 897-4, 906.

Newman,A.P. (1998). Articular cartilage repair. Am. J. Sports Med. *26*, 309-324.

O'Brien,J., Wilson,I., Orton,T., and Pognan,F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. *267*, 5421-5426.

O'Driscoll,S.W. (1999). Articular cartilage regeneration using periosteum. Clin. Orthop. Relat Res. S186-S203.

Pagnano,M. and Westrich,G. (2005). Successful nonoperative management of chronic osteoarthritis pain of the knee: safety and efficacy of retreatment with intra-articular hyaluronans. Osteoarthritis. Cartilage. *13*, 751-761.

Paige,K.T., Cima,L.G., Yaremchuk,M.J., Vacanti,J.P., and Vacanti,C.A. (1995). Injectable cartilage. Plast. Reconstr. Surg. *96*, 1390-1398.

Park,Y., Sugimoto,M., Watrin,A., Chiquet,M., and Hunziker,E.B. (2005). BMP-2 induces the expression of chondrocyte-specific genes in bovine synovium-derived progenitor cells cultured in three-dimensional alginate hydrogel. Osteoarthritis. Cartilage. *13*, 527-536.

Patashnik,S., Rabinovich,L., and Golomb,G. (1997). Preparation and evaluation of chitosan microspheres containing bisphosphonates. J. Drug Target *4*, 371-380.

Patel,V.R. and Amiji,M.M. (1996). Preparation and characterization of freeze-dried chitosan-poly(ethylene oxide) hydrogels for site-specific antibiotic delivery in the stomach. Pharm. Res. *13*, 588-593.

Pavesio,A., Abatangelo,G., Borrione,A., Brocchetta,D., Hollander,A.P., Kon,E., Torasso,F., Zanasi,S., and Marcacci,M. (2003). Hyaluronan-based scaffolds (Hyalograft C) in the treatment of knee cartilage defects: preliminary clinical findings. Novartis. Found. Symp. *249*, 203-217.

Peppas,N.A., Bures,P., Leobandung,W., and Ichikawa,H. (2000). Hydrogels in pharmaceutical formulations. Eur. J Pharm. Biopharm. *50*, 27-46.

Pool,A.R. (2001). Arthritis and Allied Conditions: A Textbook of Rheumatology. Lippincott Williams and Wilkins), pp. 226-284.

Ramkumar,R. and Podder,S.K. (2000). Elucidation of the mechanism of interaction of sheep spleen galectin-1 with splenocytes and its role in cell-matrix adhesion. J Mol. Recognit. *13*, 299-309.

Redman,S.N., Oldfield,S.F., and Archer,C.W. (2005). Current strategies for articular cartilage repair. Eur. Cell Mater. *9*, 23-32.

Reginster,J.Y. (2002). The prevalence and burden of arthritis. Rheumatology. (Oxford) *41 Supp 1*, 3-6.

Risbud,M., Hardikar,A., and Bhonde,R. (2000). Chitosan-polyvinyl pyrrolidone hydrogels as candidate for islet immunoisolation: in vitro biocompatibility evaluation. Cell Transplant. *9*, 25-31.

Risbud,M., Ringe,J., Bhonde,R., and Sittinger,M. (2001). In vitro expression of cartilage-specific markers by chondrocytes on a biocompatible hydrogel: implications for engineering cartilage tissue. Cell Transplant. *10*, 755-763.

Risbud,M.V. and Bhonde,R.R. (2000). Polyacrylamide-chitosan hydrogels: in vitro biocompatibility and sustained antibiotic release studies. Drug Deliv. *7*, 69-75.

Roberts,G.A.F. and Domzy,J.G. (1982). Determination for the viscometric constants for chitosan. Int. J. Biol. Macromol. *4*, 374-377.

Rossi,S., Sandri,G., Ferrari,F., Bonferoni,M.C., and Caramella,C. (2003). Buccal delivery of acyclovir from films based on chitosan and polyacrylic acid. Pharm. Dev. Technol. *8*, 199-208.

Saris,D.B., Mukherjee,N., Berglund,L.J., Schultz,F.M., An,K.N., and O'Driscoll,S.W. (2000). Dynamic pressure transmission through agarose gels. Tissue Eng *6*, 531-537.

Sechriest,V.F., Miao,Y.J., Niyibizi,C., Westerhausen-Larson,A., Matthew,H.W., Evans,C.H., Fu,F.H., and Suh,J.K. (2000). GAG-augmented polysaccharide hydrogel: a novel biocompatible and biodegradable material to support chondrogenesis. J. Biomed. Mater. Res. *49*, 534-541.

Serafini-Fracassini,A. and Smith,J.W. (1974). The Structure and Biochemistry of Cartilage. Churchill Livingstone, London,).

Shahabeddin,L., Berthod,F., Damour,O., and Collombel,C. (1990). Characterization of skin reconstructed on a chitosan-cross-linked collagen-glycosaminoglycan matrix. Skin Pharmacol. *3*, 107-114.

Shapiro,F., Koide,S., and Glimcher,M.J. (1993). Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. J. Bone Joint Surg. Am. *75*, 532-553.

Solchaga,L.A., Dennis,J.E., Goldberg,V.M., and Caplan,A.I. (1999). Hyaluronic acid-based polymers as cell carriers for tissue-engineered repair of bone and cartilage. J. Orthop. Res. *17*, 205-213.

Song,J., Suh,C.H., Park,Y.B., Lee,S.H., Yoo,N.C., Lee,J.D., Kim,K.H., and Lee,S.K. (2001). A phase I/IIa study on intra-articular injection of holmium-166-chitosan complex for the treatment of knee synovitis of rheumatoid arthritis. Eur. J. Nucl. Med. *28*, 489-497.

Song,L., Baksh,D., and Tuan,R.S. (2004). Mesenchymal stem cell-based cartilage tissue engineering: cells, scaffold and biology. Cytotherapy. *6*, 596-601.

Sorlier,P., Denuziere,A., Viton,C., and Domard,A. (2001). Relation between the degree of acetylation and the electrostatic properties of chitin and chitosan. Biomacromolecules. *2*, 765-772.

Stevens,M.M., Marini,R.P., Martin,I., Langer,R., and Prasad,S., V (2004). FGF-2 enhances TGF-beta1-induced periosteal chondrogenesis. J Orthop. Res. *22*, 1114-1119.

Subramanian,A. and Lin,H.Y. (2005). Crosslinked chitosan: its physical properties and the effects of matrix stiffness on chondrocyte cell morphology and proliferation. J. Biomed. Mater. Res. A *75*, 742-753.

Subramanian,A., Lin,H.Y., Vu,D., and Larsen,G. (2004). Synthesis and evaluation of scaffolds prepared from chitosan fibers for potential use in cartilage tissue engineering. Biomed. Sci. Instrum. *40*, 117-122.

Suh,J.K. and Matthew,H.W. (2000). Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials *21*, 2589-2598.

Sung,H.W., Liang,I.L., Chen,C.N., Huang,R.N., and Liang,H.F. (2001). Stability of a biological tissue fixed with a naturally occurring crosslinking agent (genipin). J. Biomed. Mater. Res. *55*, 538-546.

Temenoff,J.S. and Mikos,A.G. (2000a). Injectable biodegradable materials for orthopedic tissue engineering. Biomaterials *21*, 2405-2412.

Temenoff,J.S. and Mikos,A.G. (2000b). Review: tissue engineering for regeneration of articular cartilage. Biomaterials *21*, 431-440.

Tomford,W.W., Springfield,D.S., and Mankin,H.J. (1992). Fresh and frozen articular cartilage allografts. Orthopedics *15*, 1183-1188.

Tuzlakoglu,K., Alves,C.M., Mano,J.F., and Reis,R.L. (2004). Production and characterization of chitosan fibers and 3-D fiber mesh scaffolds for tissue engineering applications. Macromol. Biosci. *4*, 811-819.

Vacanti,C.A., Langer,R., Schloo,B., and Vacanti,J.P. (1991). Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation. Plast. Reconstr. Surg. *88*, 753-759.

Van den Brule,F.A., Buicu,C., Baldet,M., Sobel,M.E., Cooper,D.N., Marschal,P., and Castronovo,V. (1995). Galectin-1 modulates human melanoma cell adhesion to laminin. Biochem. Biophys. Res. Commun. *209*, 760-767.

Vandevord,P.J., Matthew,H.W., DeSilva,S.P., Mayton,L., Wu,B., and Wooley,P.H. (2002). Evaluation of the biocompatibility of a chitosan scaffold in mice. J. Biomed. Mater. Res. *59*, 585-590.

varez-Lorenzo,C., Concheiro,A., Dubovik,A.S., Grinberg,N.V., Burova,T.V., and Grinberg,V.Y. (2005). Temperature-sensitive chitosan-poly(N-isopropylacrylamide) interpenetrated networks with enhanced loading capacity and controlled release properties. J. Control Release *102*, 629-641.

von der Mark,K. and Kuhl,U. (1985). Laminin and its receptor. Biochim. Biophys. Acta *823*, 147-160.

Vunjak-Novakovic,G. (2003). The fundamentals of tissue engineering: scaffolds and bioreactors. Novartis. Found. Symp. *249*, 34-46.

Wang,C.T., Lin,J., Chang,C.J., Lin,Y.T., and Hou,S.M. (2004). Therapeutic effects of hyaluronic acid on osteoarthritis of the knee. A meta-analysis of randomized controlled trials. J Bone Joint Surg. Am. *86-A*, 538-545.

Wasano,K. and Hirakawa,Y. (1997). Recombinant galectin-1 recognizes mucin and epithelial cell surface glycocalyces of gastrointestinal tract. J Histochem. Cytochem. *45*, 275-283.

WHO. World; World Health Report Archives 1995-2000. 2001.
Ref Type: Report

Wong,M., Siegrist,M., Wang,X., and Hunziker,E. (2001). Development of mechanically stable alginate/chondrocyte constructs: effects of guluronic acid content and matrix synthesis. J Orthop. Res. *19*, 493-499.

Woodfield,T.B., Bezemer,J.M., Pieper,J.S., van Blitterswijk,C.A., and Riesle,J. (2002). Scaffolds for tissue engineering of cartilage. Crit Rev. Eukaryot. Gene Expr. *12*, 209-236.

Worster,A.A., Brower-Toland,B.D., Fortier,L.A., Bent,S.J., Williams,J., and Nixon,A.J. (2001). Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix. J Orthop. Res. *19*, 738-749.

Yagi,K., Michibayashi,N., Kurikawa,N., Nakashima,Y., Mizoguchi,T., Harada,A., Higashiyama,S., Muranaka,H., and Kawase,M. (1997). Effectiveness of fructose-modified chitosan as a scaffold for hepatocyte attachment. Biol. Pharm. Bull. *20*, 1290-1294.

Yalpani,M. and Hall,M.D. (1984). Some chemical and analytical aspects of polysaccharide modifications. 3. Formation of branched-chain, soluble chitosan derivatives. Macromolecules *17*, 272-281.

Yamada,K., Chen,T., Kumar,G., Vesnovsky,O., Topoleski,L.D., and Payne,G.F. (2000). Chitosan based water-resistant adhesive. Analogy to mussel glue. Biomacromolecules. *1*, 252-258.

Yang,T.C., Chou,C.C., and Li,C.F. (2005). Antibacterial activity of N-alkylated disaccharide chitosan derivatives. Int. J. Food Microbiol. *97*, 237-245.

Zhang,Y., Cheng,X., Wang,J., Wang,Y., Shi,B., Huang,C., Yang,X., and Liu,T. (2006). Novel chitosan/collagen scaffold containing transforming growth factor-beta1 DNA for periodontal tissue engineering. Biochem. Biophys. Res. Commun. *344*, 362-369.

Zhang,Y. and Zhang,M. (2004). Cell growth and function on calcium phosphate reinforced chitosan scaffolds. J. Mater. Sci. Mater. Med. *15*, 255-260.

Zhao,F., Yin,Y., Lu,W.W., Leong,J.C., Zhang,W., Zhang,J., Zhang,M., and Yao,K. (2002). Preparation and histological evaluation of biomimetic three-dimensional hydroxyapatite/chitosan-gelatin network composite scaffolds. Biomaterials *23*, 3227-3234.

Appendix

Publications in Peer-reviewed Journals

Synergistic effect on semi-ilute arginate/lactose-modiifed chitosan (chitlac) solutions
Donati I, Haug IJ, Scarpa T, Borgogna M, Draget K, Sjåk-Bræk G and Paoletti S
Biomacromolecules (2007), *8*, 977-62.

A comparative rheological study of aqueous salt solutions of chitosan and chitlac, a lactose-modified chitosan.
Scarpa T, Lapasin R, Paoletti S and Gamini A.
in preparation

3D scaffolds for tissue engineering: synthesis, characterization and biological properties of a cross-linked lactose-modified chitosan.
Scarpa T, Semeraro S, Paoletti S and Gamini A.
in preparation

Oral Presentation

Oral presentation at the International workshop on Applications of Chitosan in Medical Sciences, Venice, Italy, Jan 2007.
Characterization of Crosslinked Scaffolds Based on Lactose-Modified Chitosan.
Scarpa T, Gamini A and Paoletti S.

Poster Presentations

Poster presentation at 19th European Conference on Biomaterials, ESB2005, Sorrento, Italy, Set 2005.
Characterisation of the Physico-Chemical Properties of Lactose-Modified Chitosan.
Scarpa T, O'Halloran D, Donati I, Gamini A, Paoletti S and Pandit A.

Poster presentation at 20th European Conference on Biomaterials, ESB2006, Nantes, France, Set 2006.
Rheological Characterisation of Blends of Hyaluronic Acid and Lactose-Modified Chitosan

Scarpa T, Gamini A, Paoletti S and Pandit A.

Poster presentation at 9th CIB National Congress, CNB9, Torino, Italy, Set 2006.
Characterization of Crosslinked Scaffolds Based on Lactose-Modified Chitosan.
Scarpa T, Gamini A and Paoletti S.

Poster presentation at the International workshop on Applications of Chitosan in Medical Sciences, Venice, Italy, Jan 2007.
Rheological Characterisation of Blends of Hyaluronic Acid and Lactose-Modified Chitosan
Scarpa T, Gamini A, Paoletti S and Pandit A.

Synergistic Effects in Semidilute Mixed Solutions of Alginate and Lactose-Modified Chitosan (Chitlac)

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The present study specifically aimed at preparing and characterizing semidilute binary polymer mixtures of alginate and chitlac which might find an application in the field of cell encapsulation. A polyanion, alginate, and a polycation, a lactose-modified chitosan, were mixed under physiological conditions (pH 7.4 and NaCl 0.15) and at a semidilute concentration avoiding associative phase separation. The mutual solubility was found to be dependent on the charge screening effect of the added NaCl salt, being prevented below 0.05 M NaCl. A comparison with the behavior of the polyanion (alginate) under the same experimental conditions revealed that both the viscosity and the relaxation times of the binary polymer solutions are strongly affected by the presence of the polycation. In particular, the occurrence of electrostatic interactions between the two oppositely charged polysaccharides led to a synergistic effect on the zero-shear viscosity of the solution, which showed a 4.2-fold increase with respect to that of the main component of the solution, i.e., alginate. Moreover, the relaxation time, calculated as the reciprocal of the critical share rate, markedly increased upon reducing the alginate fraction in the binary polysaccharide solution. However, the formation of the soluble complexes and the synergistic effect are reduced upon increasing the concentration of the 1:1 electrolyte. By containing a gel-forming polyanion (alginate, e.g., with Ca^{2+} ions) and a bioactive polycation (chitlac, bearing a β-linked D-galactose), the present system can be regarded as a first step toward the development of biologically active scaffold from polysaccharide mixtures.

Introduction

Polysaccharides have been extensively used for a wide variety of applications encompassing food science (as thickeners, stabilizers, fat substitute, and taste release systems) and bio-(medical)technology (articular viscosupplementation[1−4] and cell therapy[5−7] above all). In particular, the latter has gained an increasing attention in the scientific community, and numerous applications of alginate, hyaluronan, and chitosan for tissue engineering and cell encapsulation have been proposed.[8,9]

Besides the interest elicited by natural polyelectrolytes when considered singularly, it is generally agreed that their mixing could lead to complex biomimetic systems resembling the biological milieu of entangled macromolecules, commonly termed as Extracellular Matrix (ECM), that embeds cells providing for adhesion and proper biomechanical signaling. Moreover, the simultaneous presence of different polysaccharides is expected to induce notable variation of the overall physicochemical properties of the system. In fact, it has been established that the solution properties of biopolymer mixtures can be quite different from those of the pure components,[10,11] due to their synergistic interactions.[12−17]

Alginate and chitosan are a polyanion and a polycation, respectively, easily isolated from natural sources. The former polysaccharide is produced by marine brown algae and bacteria and consists of 1→4 linked β-D-mannuronic acid and α-L-guluronic acid arranged in different types of block-wise patterns. At variance, chitosan is obtained from chemical deacetylation of chitin, the main component of the exoskeleton of crustaceans, and is composed of 1→4 linked β-D-glucosamine units with some residual 2-acetamido-2-deoxy-β-D-glucosamine groups randomly distributed along the polymer chain. When considering the possibility of preparing solutions containing both such oppositely charged polysaccharides, it should be kept in mind that the use of pH values above ~6 is prevented by the precipitation of highly deacetylated chitosans.[18] For a similar reason (gelation/precipitation of alginate), the use of pH values lower than ~3 has to be avoided. This implies that when alginate and chitosan solutions are mixed, the contemporaneous presence of negative charges on the polyanion (alginate) and positive charges on the polycation (chitosan) leads to associative phase separation which cannot be suppressed by adding 1:1 electrolytes, as usual for mixtures of oppositely charged polysaccharides.[19] In fact, the intrinsic lack of mutual solubility between these two polysaccharides has been exploited to reinforce and to control the permeability of calcium alginate capsules in the field of cell microencapsulation.[20,21] The latter has emerged as a feasible approach for the production of insulin-producing biorectors for the treatment of type I-diabetes.[22]

However, the analysis of the main features of the two polysaccharides as such might sprout the idea that a soluble combination of the two systems could be beneficial for many applications. In fact, alginate has been extensively studied for its ability to form stable gels, under cell-friendly conditions, when in contact with solutions containing the proper divalent cation (such as calcium).[7,23] On the other hand, chitosan is a biodegradable biopolymer with interesting antimicrobial and hemostatic properties.[24] Moreover, the presence of amino

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groups on the polymer backbone renders highly deacetylated chitosans particularly appealing candidates for the development of a bioactive biomaterial through the insertion of cell specific ligands such as oligosaccharide sequences. As an example, the introduction of β-linked D-galactose moieties (as the non-reducing end in lactitol) on the chitosan backbone led to an engineered polysaccharide, named chitlac, granted of both biological significance[25,26] and complete solubility at neutral pH.

Prompted by these considerations, the possibility of preparing binary polymer solutions by using the two oppositely charged polysaccharides, namely alginate and chitlac, was explored. In the present paper, the flow properties of the binary polymer solutions were analyzed by means of a rheological approach and the results compared with those displayed by the single polymeric components considered separately. In particular, the presence of a synergistic effect on the viscosity and on the relaxation time of the binary polymer solution, as a consequence of the inter-polysaccharide electrostatic interactions, was assessed.

Materials and Methods

Sodium alginate isolated from *Laminaria hyperborea* stipe, LF 10/60 (F_G = 0.69; F_{GG} = 0.56; Mw = 1.3 × 10^5), was provided by FMC Biopolymers (Norway). Chitlac (1-Deoxylactit-1-ylchitosan (CAS registry number 85941-43-1), Mw approximately 1.5 × 10^6) sample was prepared according to the procedure reported elsewhere.[25] *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) sodium salt (HEPES) and poly-L-lysine (PLL) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium chloride was from Aldrich Chemical Co. (Milwaukee, WI).

Binary Polymer Solution. The preparation of the binary polymer solution was accomplished by mixing different amounts of a 1% (w/V) solution of chitlac with a 3% (w/V) solution of alginate under vigorous stirring. Binary polysaccharide mixtures containing different alginate weight fractions (Ψ_{Alg} refers to the binary polymer composition) were therefore obtained. The polymer solutions were prepared in the presence of 0.15 M sodium chloride maintaining a constant HEPES concentration (10 mM, pH 7.4). In addition, the mixing of the alginate and chitlac solution was also attempted at pH 5.5 in the presence of NaCl (0.15 M) and at pH 7.4 without additional 1:1 electrolyte.

Photon Transmission Intensity (Transmittance) Measurements. The transmittance of the binary polymer mixtures containing alginate and chitlac was measured at different wavelength values (namely 480 nm, 520 nm, and 600 nm, respectively) with a Cary4E UV-visible spectrophotometer. For comparison, the transmittance of each of the two oppositely charged polysaccharides was determined.

^{1}H NMR Spectroscopy. The ^{1}H NMR spectra were recorded at 90 °C with a Bruker Advance DPX 400 spectrometer with the chemical shifts expressed in ppm downfield from the signal of 3-(trimethylsilyl)-propansulfonate. The binary polymer solution, at a total concentration of 0.2 g/L, was treated following the procedure reported by Grasdalen,[27] and the pH was adjusted to approximately 7 prior to analysis.

Viscosity Measurements. Reduced capillary viscosity was measured at 25 °C by means of a Schott-Geräte AVS/G automatic apparatus and an Ubbelohde type viscometer. For the polysaccharides used in the present study, the intrinsic viscosity ([η]) values were determined by analyzing the polymer concentration dependence of the reduced specific viscosity (η_{sp}/c) and of the reduced logarithm of the relative viscosity ($\ln \eta_{rel}/c$) by use of the Huggins (eq 1) and Kraemer (eq 2) equations, respectively:

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c \qquad (1)$$

$$\frac{\ln(\eta_{rel})}{c} = [\eta] - k''[\eta]^2 c \qquad (2)$$

where k' and k'' are the Huggins and Kraemer constants, respectively.

Rheological Measurements. Rheological measurements were performed on a Stress-Tech general-purpose rheometer (Reologica instruments AB, 22363 Lund, Sweden) using a cone-plate geometry (50 mm radius, 1°). The solutions of alginate, chitlac, and the different binary polymer mixtures were loaded on the plate of the rheometer, and the viscosities at different shear rates were obtained by means of steady-state measurements. Two replicate measurements were performed for each sample. The complex viscosity (η^*) and the storage (G′) and loss (G″) moduli of the binary polymer solutions were recorded in the frequency range 0.01−50 Hz (maximum strain <3%). All the measurements were performed at 25 °C.

Results and Discussion

(a) Binary Polymer Solutions from Oppositely Charged Polysaccharides. In order to study the behavior of a semidilute binary polymer mixture of alginate and chitlac, a 2% (w/V) solution with an alginate weight fraction (ψ_{Alg}) of 0.75 was considered.[28] First, the possibility of preparing such a mixture containing the two oppositely charged polysaccharides under semidilute conditions avoiding extended coacervation/precipitation was assessed. As reported in Figure 1a (column A), a complete miscibility between alginate and chitlac was achieved under physiological conditions, i.e., in the presence of NaCl 0.15 M and at pH 7.4. Moreover, it was noted that both neutral pH and the presence of a sufficient amount of added salt (NaCl) are required to guarantee the mutual solubility of the oppositely charged polysaccharides. In fact, the transmittance of the binary mixture dropped when the pH of the solution was lowered to 5.5 (Figure 1a column B): it can be reasonably correlated with the simultaneous increase of charge density on the chitlac component of the mixture and the decrease on the alginate one. Similarly a decrease of the concentration of the added salt in the binary polymer mixture induced an associative phase separation (Figure 1a column C) due to (i) a net entropic gain by the counterions of the polyelectrolytes upon phase separation and (ii) a reduction of the charge screening effect between the polyelectrolyte chains (i.e., increasing the Debye length, κ^{-1}, defined as the distance over which the electrostatic field of an ion extends with appreciable strength, being $\kappa^2 \propto I$). The use of pH = 7.4 and the presence of a 1:1 electrolyte at 0.15 M concentration did not, *per se*, imply the mutual miscibility of oppositely charged polyelectrolytes. In fact, Figure 1a (column D) reports the decrease in transmittance detected upon replacing chitlac with poly-L-lysine (PLL) in the binary polymer solution. This result was not surprising, since the polycation PLL has been extensively reported to stabilize calcium-alginate gel beads through the formation of coacervates on the surface of the negatively charged bead in the so-called "coating" process.[29,30] Therefore, an active role of the bulky *N*-lactit-1-yl groups of chitlac in hampering polyanion−polycation extended associations at neutral pH can be preliminarily proposed.

The effect of 1:1 electrolytes on the solubility of the binary mixture was further assessed by reducing the overall concentration of NaCl in the solution. In all the cases reported, the reduction of NaCl was compensated by the addition of a nonionic solute (mannitol) to attain isoosmolar conditions. As seen from Figure 1b (column C), a minimum concentration of 0.05 M of NaCl is required to allow for the mutual solubility of the two polysaccharides.

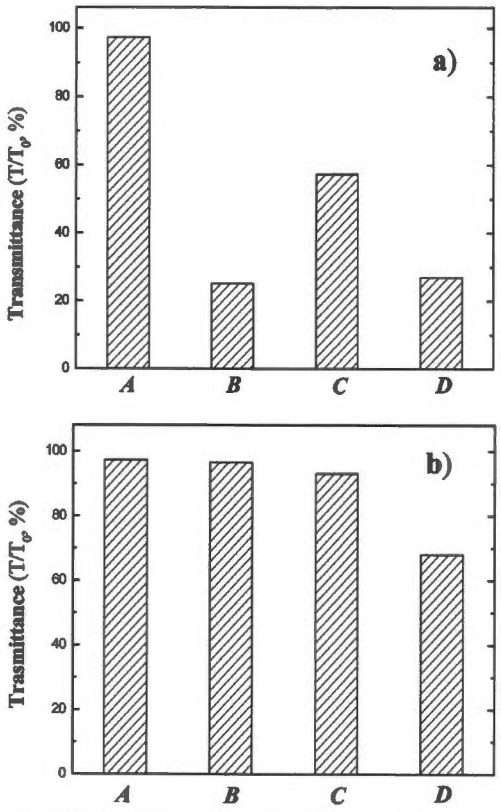

Figure 1. (a) Relative transmittance (%, 600 nm) of a binary polymer solution containing alginate (1.5%) and chitlac (0.5%) in (A) NaCl 0.15 M, HEPES 10 mM, pH 7.4; (B) NaCl 0.15 M, pH 5.5; (C) HEPES 10 mM pH 7.4; (D) relative transmittance (%, 600 nm) of a binary polymer mixture containing alginate (1.5%) and poly-L-lysine (0.5%) in NaCl 0.15 M, HEPES 10 mM, pH 7.4. (b) Relative transmittance (%) of a binary polymer solution containing alginate (1.5%) and chitlac (0.5%) in (A) NaCl 0.15 M, HEPES 10 mM, pH 7.4; (B) NaCl 0.1 M, mannitol 0.1 M, HEPES 10 mM, pH 7.4; (C) NaCl 0.05 M, Mannitol 0.2 M, HEPES 10 mM, pH 7.4; (D) NaCl 0.025 M, Mannitol 0.25 M, HEPES 10 mM, pH 7.4. (T_0 = trasmittance of the alginate solution considered separately).

The binary solution containing both alginate and chitlac was characterized by means of ^{1}H NMR analysis (Figure 2b) which revealed, in addition to the signals assigned to the polyanion (Figure 2a), the presence of a doublet centered at around 4.55 ppm, arising from the side-chain galactose moieties on chitlac. It is noteworthy that the NMR signals of the anomeric protons of alginate in the binary polymer mixture sample have not been altered, under the experimental conditions used for the analysis, by the presence of the polycation. In fact, the diadic composition calculated for the alginate in the binary polymer mixture (F_G = 0.67; F_{GG} = 0.55) correlates very well with pure alginate sample (F_G = 0.69; F_{GG} = 0.56). Since the anomeric protons of the in-chain sugar residues of chitlac are shifted upfield at pH 7.4 (spectrum not reported), no interference of such signals in the anomeric region of the spectrum was detected.

(b) Alginate Solutions in the Semidilute Regime. An analysis of the main rheological properties of semidilute alginate solutions has been considered as preliminary to the investigation of any synergistic interaction of alginate with chitlac in the polymer mixture. To this end, the specific viscosity of alginate solutions was measured under the same conditions used for the preparation of the binary polymer mixture (i.e., NaCl 0.15M; HEPES 10 mM; pH 7.4). Figure 3 reports the dependence of η_{sp} on the coil overlap factor, $C_{Alg}[\eta]$, for alginate in both dilute

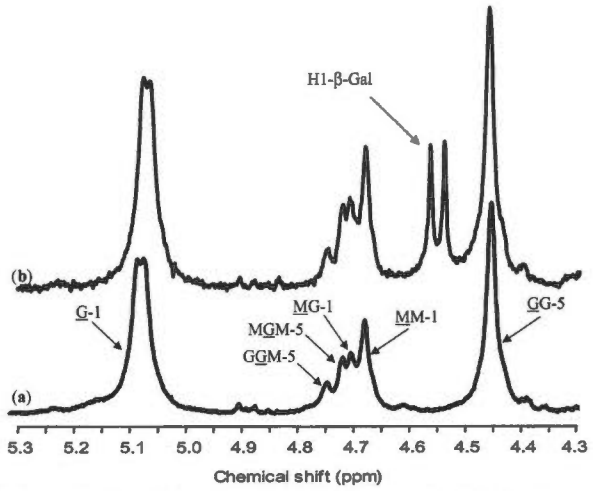

Figure 2. ^{1}H NMR spectra of (a) alginate and of (b) the binary polymer mixture of alginate (1.5%) and chitlac (0.5%). The additional peak at around 4.55 in part b can be assigned to the anomeric proton of the β-galactose moieties in chitlac.

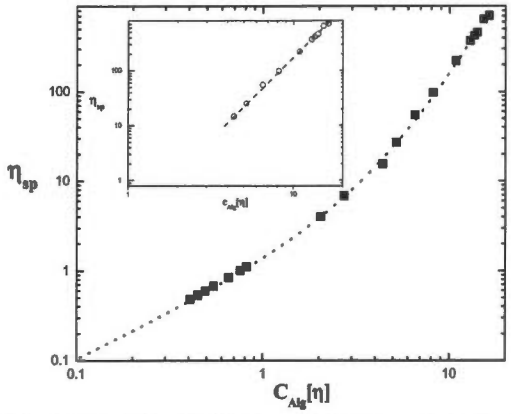

Figure 3. (■) Dependence of the specific viscosity on the coil overlap factor for alginate in NaCl 0.15 M, HEPES 10 mM, pH 7.4. (····) Theoretical dependence calculated on the basis of eq 4 ($[\eta]_{Alg}$ = 5.44dL/g; k' = 0.353 as determined from eq 1 in dilute conditions; c = C_{alg}). Inset: magnification of the main figure in the semidilute regime. The linear regression (- -) of the experimental data in the latter concentration regime gave R^2 = 0.987 and a slope of 3.1.

and semidilute regimes. C_{Alg} is the specific concentration of alginate, expressed in the units of $([\eta])^{-1}$. The measurements of η_{sp} have been performed with a capillary viscometer for $C_{Alg}[\eta]$ values below 4, while a rotational rheometer (steady-state conditions) was used for solutions with a higher polymer concentration. In the latter case, the zero-shear viscosity, $\eta_{\dot{\gamma}=0}$, was extrapolated from the $\eta - \dot{\gamma}$ dependence by means of the so-called Cross equation.[31,32]

In the semidilute condition, i.e., for a coil overlap factor higher than 4,[33] the specific viscosity of the polysaccharide solutions was found to scale with the polymer concentration according to $\eta_{sp} \propto C_{Alg}^{3.1}$ (Figure 3 inset). The latter value is somewhat lower than expected for a semidilute entangled system, i.e. $\eta_{sp} \propto c^{15/4}$, but it is in good agreement with the value already reported in literature, i.e., ~3.3,[33] thus supporting the validity of the experimental data. In the same concentration regime, the relaxation time, τ_{relax} ($\tau_{relax} = 1/\dot{\gamma}_{cr}$ where $\dot{\gamma}_{cr} = 0.8\dot{\gamma}_0$[34]) scaled as $\tau_{relax} \propto C_{Alg}^{1.8}$, thus substantially in good agreement with the value expected for a semidilute entangled

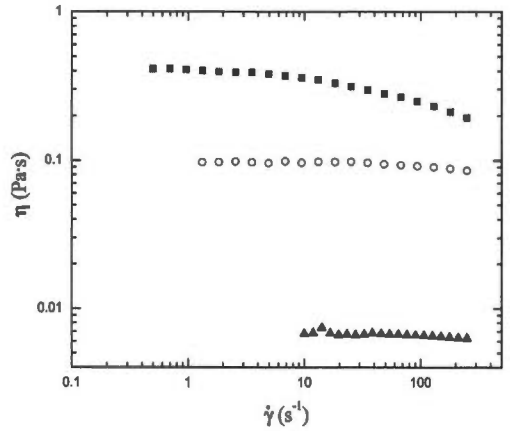

Figure 4. Dependence of the viscosity (η) on the shear rate ($\dot{\gamma}$) for (O) alginate (1.5%), (▲) chitlac (0.5%) and (■) their binary mixture (final concentration: alginate 1.5% and chitlac 0.5%; $\psi_{Alg} = 0.75$) in NaCl 0.15 M, HEPES 10 mM, pH 7.4.

system, i.e. $\tau \approx c^{3/2}$. For sake of comparison, a scaling law of $\tau_{relax} \propto c^{2.2}$ was found in the case of hyaluronan.[35]

It has been reported that in the case of hyaluronan[36] the experimental trend of the specific viscosity can be reproduced by means of the four-term equation resulting from the truncated form of the exponential expression derived by Martin:

$$\eta_{sp} = ([\eta]c)e^{k'[\eta]c} \tag{3}$$

which reads

$$\eta_{sp} = c[\eta] + k'(c[\eta])^2 + \frac{(k')^2}{2!}(c[\eta])^3 + \frac{(k')^3}{3!}(c[\eta])^4 \tag{4}$$

where $[\eta]$ and k' are the intrinsic viscosity and the Huggins constant, respectively, of the polysaccharide, and c is the polymer specific concentration. Along this line, eq 4 was used to calculate the $\eta_{sp} - (C_{Alg}[\eta])$ relationship for alginate under the above-reported experimental conditions. A good agreement between the experimental data and the theoretical curve (Figure 3) was obtained, hence demonstrating the feasibility of this approach.

(c) Addition of Chitlac: Synergistic Effect on Viscosity. The possibility of preparing binary polymer solutions containing a polyanion (alginate) and a polycation (chitlac) does not *per se* imply the complete lack of interaction between the two oppositely charged macromolecular species. On the contrary, the viscosity of a binary polymer solution (alginate weight fraction, $\psi_{Alg} = 0.75$: alginate concentration = 1.5%(w/V), chitlac concentration 0.5% (w/V)) (Figure 4) is about 4.2-fold higher than that exhibited by the main component of the mixture when considered separately, i.e., the 1.5% alginate solution. Taking into consideration the rather low viscosity displayed by a 0.5% solution of chitlac, it seems safe to allocate this synergistic effect on the viscosity of the binary system to the presence of interactions of electrostatic origin between the two polysaccharides. The latter statement is confirmed by considering the zero-shear viscosity of the same binary polymer solution in the presence of a higher concentration of added salt, namely NaCl 0.75 M, where the ratio between the viscosity of the binary system and that of the main component of the mixture, i.e., alginate, decreases from 4.2 to 3.1 (not reported). Once more, the increase of sodium chloride screened the electrostatic interactions between alginate and chitlac and reduced the entropy gain driving the formation of inter-polyelectrolyte complexes.

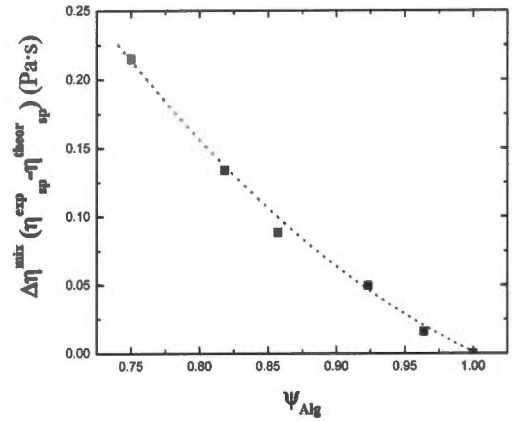

Figure 5. Dependence on the alginate weight fraction (ψ_{Alg}) of the difference between the experimentally measured (η_{sp}^{exp}) and theoretically calculated (η_{sp}^{theor}) specific viscosities of the binary polymer solution of alginate and chitlac. The latter was calculated on the basis of eq 5 assuming no interaction between the two polymeric components of the mixture ($[\eta]_{chitlac} = 3.49$dL/g; $K' = 0.488$ as determined from eq 1 in dilute conditions). Solvent: NaCl 0.15 M, HEPES 10 mM, pH 7.4. The line has been drawn to guide the eye.

In view of the very good agreement between the experimental data and the theoretical predictions (as calculated from eq 4) achieved for the viscosity of alginate solutions in semidilute conditions (see Figure 3), a similar approach was used to predict the specific viscosity of the binary mixture of alginate and chitlac, η_{sp}^{theor}, on the basis of the relative concentration of the two polysaccharides:

$$\eta_{sp}^{theor} = [\eta]^{theor}(C_{Alg} + C_{chit})e^{k^{theor}[\eta]^{theor}(C_{Alg}+C_{chit})} \tag{5}$$

where

$$[\eta]^{theor} = \sum_i \Phi_i[\eta]_i \tag{6}$$

Φ_i being the weight fraction of the i-th components of the mixture (alginate or chitlac) and $[\eta]_i$ its intrinsic viscosity.

Under the assumption that no interaction is occurring between the two oppositely charged polysaccharides, the Huggins cross-coefficient reads:

$$k^{theor} = \sum_i \Phi_i k_i \tag{7}$$

with k_i the Huggins coefficient of the i-th component of the mixture considered separately in the same solvent and at the same temperature.

Figure 5 reports the dependence of the difference between the experimental, η_{sp}^{exp}, and the theoretical zero-shear viscosity, η_{sp}^{theor}, on the alginate weight fraction in the case of binary polymer mixtures of alginate and chitlac. It should be noted that, in the ψ_{Alg}-range explored, the higher is the fraction of chitlac in the binary polymer solution (and consequently the lower the fraction of alginate present), the higher is the deviation in the zero-shear viscosity from the value expected assuming no interactions between polymers (according to eq 5). This result provided additional confidence on the interpretation of the synergistic effect on the viscosity of these binary polymer solutions as due to electrostatic interactions between the positive charges on the polycation and the negative charges on the polyanion. Moreover, the monotonic increasing curve reported in Figure 5 suggests that in the soluble complexes formed

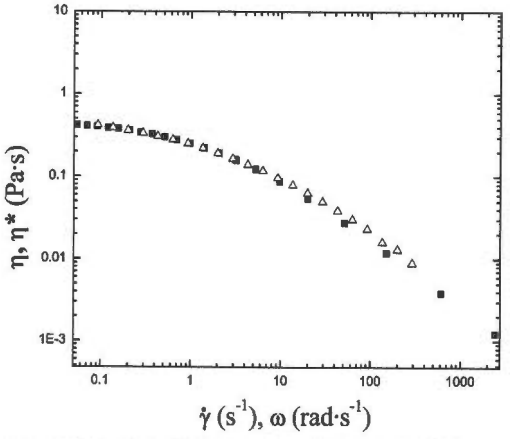

Figure 6. Verification of the Cox–Merz rule for the binary polymer solution containing alginate (1.5%) and chitlac (0.5%) in NaCl 0.15 M, HEPES 10 mM, pH 7.4: (■) dependence of the viscosity (η) on the shear rate ($\dot{\gamma}$) and (△) dependence of the complex viscosity (η^*) on the angular velocity (ω).

between alginate and chitlac, the polycation is likely to induce the aggregation of several alginate chains, thus forming a highly dynamic micronetwork in solution, with an overall negative charge.[37] It is important to note, however, that the formation of the electrostatic interactions between the macromolecules and the relevant increase in viscosity did not lead to the formation of aggregates or microgels on a large scale. In fact, it was found that the Cox–Merz rule[38] held in the case of the binary polymer solution, as reported in Figure 6.

The synergistic effect arising from the interaction between the polycation and the polyanion was quantified in the case of a binary polymer solution containing 1.5% (w/V) alginate and 0.5% (w/V) chitlac in NaCl 0.15 M. Under the assumption that no interaction between the two polymeric components of the mixture occurs, the zero-shear viscosity predicted by eq 5 is approximately 198 mPa·s (equal to that of a 1.95% (w/V) alginate solution). Conversely, the zero-shear viscosity extrapolated for the actual binary mixture ($\psi_{Alg} = 0.75$) was found to be approximately 407mPa·s, corresponding to the zero-shear viscosity of a ~2.45% alginate solution (Figure 3 inset). It therefore seems likely to conclude that the electrostatic contacts between the oppositely charged polyelectrolytes formed (large) soluble complexes which span over a relevant portion of the volume of the solution. The ensuing increase of viscosity corresponds to a "virtual" increase of the total polymer concentration of approximately ≈0.5% (w/V).

A further insight into the main features of the binary polymer mixture of alginate and chitlac was achieved from the analysis of the viscosity (η)-shear rate $\dot{\gamma}$ dependence (Figure 4). In particular, in the case of the binary polymer mixture, a more pronounced shear-thinning behavior was detected, with respect to the pure alginate solutions, directly implying a notable effect on its relaxation time. In fact, Figure 7 shows that by reducing the fraction of alginate (and its concentration) in the binary polymer solution (and consequently increasing the fraction of chitlac), a nonlinear *increase* of the relaxation time of the system was measured. Conversely, a linear *decrease* of τ_{relax} was found for pure alginate solutions with (decreasing) concentration equaling the total polymer concentration present in the binary polymer mixtures (and then with constant $\psi_{Alg} = 1$). Numerically, the relaxation time of the binary polymer solution (τ_{relax} ~ 83 ms) containing the two oppositely charged polysaccharides ($\psi_{Alg} = 0.75$, alginate concentration 1.5% (w/V), chitlac concentration 0.5% (w/V)) resulted to be approximately 1 order

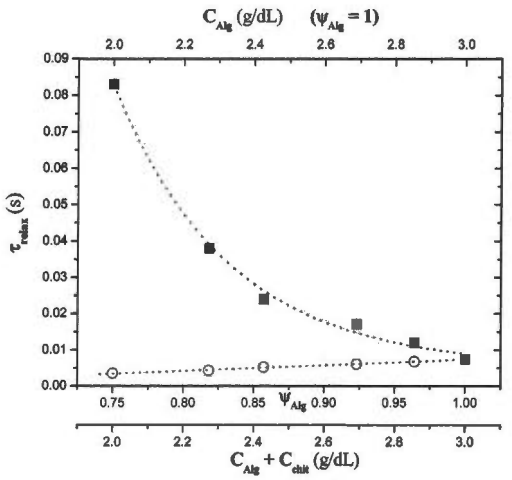

Figure 7. (■, bottom *x*-scale) Dependence of the relaxation time τ_{relax} ($\tau_{relax} = 1/\dot{\gamma}_{cr}$ where $\dot{\gamma}_{cr} = 0.8\dot{\gamma}_0$[34]) on the alginate weight fraction ψ_{Alg} in the case of binary polymer solutions of alginate and chitlac (having the total polymer concentration reported as $C_{Alg} + C_{chit}$). (○, top *x*-scale) Dependence of the relaxation time, τ_{relax}, of alginate solutions on the concentration, with alginate concentrations equaling the total polymer concentrations of the binary systems. The lines have been drawn to guide the eyes.

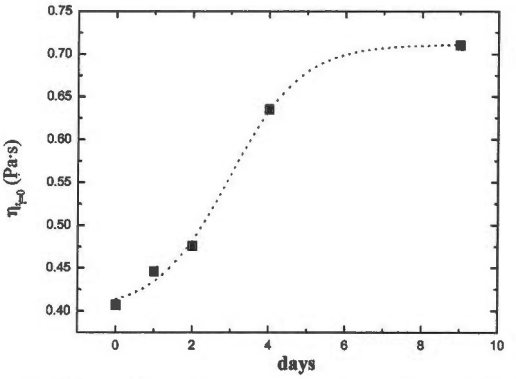

Figure 8. (●) Zero-shear viscosity ($\eta_{\dot{\gamma}=0}$)–storage time relationship for the binary polymer mixture (final concentration: alginate 1.5% and chitlac 0.5%; $\psi_{Alg} = 0.75$; $T = 25$ °C). Solvent: NaCl 0.15 M, HEPES 10 mM, pH 7.4. The line has been drawn to guide the eye.

of magnitude higher than that of an alginate solution (~3.6 ms) of equal total polymer concentration (i.e., 2%). Once more, this behavior is explained by considering the presence, in the semidilute binary polymer solution, of soluble polyanion–polycation complexes which, by fastening polymer chains through electrostatic interactions, likely hamper (to some extent) their molecular motions hence accounting for the higher time required by the system to relax after the application of the stress.

The characterization so far reported has been performed on freshly prepared solutions. However, a preliminary screening of the effect of time on the properties of the mixtures has also been carried out. In particular, it was noted that the zero-shear viscosity increased upon storing the binary polymer mixture ($\psi_{Alg} = 0.75$) at room temperature (Figure 8). Far from being conclusive, this observation seemed to point at the possibility of rearrangements on the soluble complexes which occur on long time-scales and induce further aggregation of polysaccharide chains. In all the cases, however, the formation of networks on a large scale was not detected, since the Cox–Merz rule still applied to the 9-day-stored binary mixture (data not

reported). However, further analyses are needed to confirm the latter observation.

Conclusions

The use of polysaccharide mixtures to develop Extracellular Matrix (ECM)-like systems enabling cell embedding and tissue engineering is a current challenge for biomaterial engineering. In this sense, key aspects such as the polymer miscibility and the presence of synergistic effects on the overall physical properties of the mixture have to be properly addressed and explored for a successful final outcome. Two main contributions deserve to be stressed based on the results of this study:

(i) The miscibility between the polyanion alginate and the polycation chitosan is achieved, under the proper experimental conditions, when a bulky and highly hydrophilic side-chain moiety, namely lactitol, is introduced onto the latter.

(ii) The presence of negative charges on one polysaccharide and of positive charges on the other one leads to interpolymer electrostatic interactions which induce the formation of soluble complexes and account for a notable increase of both viscosity and relaxation time of the binary polymer solution (i.e., synergistic effect). However, the (likely) transient and dynamic nature of the electrostatically based network did not induce large scale aggregations nor microgel formation.

It is worth mentioning that in the present case the choice of polymeric materials for the binary mixture overcomes stringent physicochemical considerations (i.e., solubility and net charge). In fact, by containing at the same time a bioactive (chitlac) and a ionotropic (alginate) polysaccharide, the binary polymer solution presented here might be a very appealing candidate for the development of an engineered semi-IPN hydrogel obtained under cell-friendly conditions. Along this line, the semidilute mixture of alginate and chitlac described in this paper is currently under study for the development of a bioactive scaffold for articular chondrocytes encapsulation with potential applications in cartilage reconstructive surgery.[28] In addition, from the standpoint of a polymeric solution, a synergistic effect on the viscosity could be of interest in all those applications in which one single polysaccharidic component does not provide for adequate viscoelastic properties.

References and Notes

(1) Kirchner, M.; Marshall, D. *Osteoarthr. Cartil.* **2006**, *14*, 154.
(2) Williams, J. M.; Rayan, V.; Sumner, D. R.; Thonar, E. J. M. A. *J. Orthop. Res.* **2003**, *21*, 305.
(3) Balazs, E. A. *Cells Tissues Organs* **2003**, *174*, 49.
(4) Balazs, E. A.; Denlinger, J. L. *J. Rheumatol.* **1993**, *20*, 3.
(5) Soon-Shiong, P.; Feldman, E.; Nelson, R.; Komtebedde, J.; Smidsrød, O.; Skjåk-Bræk, G.; Espevik, T.; Heintz, R.; Lee, M. *Transplantation* **1992**, *54*, 769.
(6) Orive, G.; Hernandez, R. M.; Gascon, A. R.; Calafiore, R.; Chang, T. M. S.; de Vos, P.; Hortelano, G.; Hunkeler, D.; Lacik, I.; Pedraz, J. L. *Trends Biotechnol.* **2004**, *2*, 87.
(7) Rokstad, A. M.; Strand, B.; Rian, K.; Steinkjer, B.; Kulseng, B.; Skjak-Braek, G.; Espevik, T. *Cell Transplant.* **2003**, *12*, 351.
(8) Capoccia, D.; Doherty, P.; Radice, M.; Brun, P.; Abatangelo, G.; Williams, D. F. *Biomaterials* **1998**, *19*, 2101.
(9) Suh, J. K. F.; Matthew, H. W. T. *Biomaterials* **2000**, *21*, 2589.
(10) Dea, I. C. M. In *Polysaccharides on Food*; Blanshard, J. M. V., Mitchell, J. R., eds.; Butterworths: London, 1979; p 229.
(11) Morris, E. R. In *Food Gels*; Harris, P., Ed.; Elsevier Applied Science: London, 1990; p 291.
(12) Viebke, C. *Carbohydr. Polym.* **1995**, *28*, 101.
(13) Parker, A.; Lelimousin, D.; Miniou, C.; Boulenguer, P. *Carbohydr. Res.* **1995**, *272*, 91.
(14) Haug, I. J.; Draget, K. I.; Smidsrød, O. *Carbohydr. Polym.* **2004**, *56*, 11.
(15) Mo, Y.; Kubota, K.; Nishinari, K. *Biorheology* **2000**, *37*, 401.
(16) Secouard, S.; Malhiac, C.; Grisel, M.: Decroix, B. *Food Chem.* **2003**, *82*, 227.
(17) Craig, D. Q. M.; Kee, A.; Tamburic, S.: Barnes, D. *J. Biomater. Sci. Polym. Ed.* **1997**, *8*, 377.
(18) Vårum, K. M.; Ottøy, M. H.; Smidsrød, O. *Carbohydr. Polym.* **1994**, *25*, 65.
(19) Piculell, L.; Bergfeldt, K.; Nilsson, S. In *Biopolymer Mixtures*; Harding, S. E., Hill, S. E., Mitchell, J. R., Eds.; Nottingham University Press: Nottingham, 1995; p 13.
(20) Gåserød, O.; Smidsrød, O.; Skjåk-Bræk, G. *Biomaterials* **1998**, *19*, 1815.
(21) Gåserød, O.; Sannes, A.; Skjåk-Bræk, G. *Biomaterials* **1999**, *20*, 773.
(22) deVos, P.; Faas, M. M.; Strand, B.; Calafiore, R. *Biomaterials* **2006**; *27*; 5603.
(23) Rokstad, A. M.; Holtan, S.; Strand, B.; Steinkjer, B.; Ryan, L.; Kulseng, B.; Skjåk-Bræk, G.; Espevik, T. *Cell Transplant.* **2002**, *11*, 313.
(24) Sandford, P. A. *Front. Carbohydr. Res.* **1992**, *2*, 250.
(25) Donati, I.; Stredanska, S.; Silvestrini, G.; Vetere, A.; Marcon, P.; Marsich, E.; Mozetic, P.; Gamini, A.; Paletti, S.; Vittur, F. *Biomaterials* **2005**, *26*, 987.
(26) Marcon, P.; Marsich, E.; Vetere, A.; Mozetic, P.; Campa, C.; Donati, I.; Vittur, F.; Gamini, A.; Paoletti, S. *Biomaterials* **2005**, *26*, 4975.
(27) Grasdalen, H.; Larsen, B.; Smidsrød, O. *Carbohydr. Res.* **1979**, *68*, 23.
(28) Marsich, E.; Borgogna, M; Donati, I.; Mozetic, P.; Strand, B. L.; Gomez Salvador, S.; Vittur, F.; Paoletti, S. *J. Biomed. Met. Res. A* **2007**, in press.
(29) Lim, F.; Sun, A. M. *Science* **1980**, *210*, 908.
(30) Thu, B.; Bruheim, P.; Espevik, T.; Smidsrød, O.; Soon-Shiong, P.; Skjåk-Bræk, G. *Biomaterials* **1996**, *17*, 1031.
(31) Cross, M. M. *J. Colloid Sci.* **1965**, *20*, 417.
(32) Lapasin, R.; De Lorenzi, L.; Pricl, S.; Torriano, G. *Carbohydr. Polym.* **1995**, *28*, 195.
(33) Morris, E. R.; Cutler, A. N.; Ross-Murphy, S. B.; Rees, D. A. *Carbohydr. Polym.* **1981**, *1*, 5.
(34) Graessley, W. W. *Adv. Polym. Sci.* **1974**, *16*, Chapter 8.
(35) Krause, W. E.; Bellomo, E. G.; Colby, R. H. *Biomacromolecules* **2001**, *2*, 65.
(36) Cowman, M. K.; Matsuoka, S. *Carbohydr. Res.* **2005**, *340*, 791.
(37) Donati, I.; Borgogna, M.; Turello, E.; Cesàro, A.; Paoletti, S. *Biomacromolecules*, submitted.
(38) Cox, W. P.; Merz, E. H. *J. Polym. Sci.* **1958**, *28*, 619.

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