Chitosan involved Tissue Engineering and Regenerative Medicine

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Abstract

The expanding field of tissue engineering has required the necessity of developing biomaterials that are tissue compatible, biodegradable, and comparable in mechanical properties to that of native tissue.

Chitosan has the desired properties for safe use as a pharmaceutical excipient, having great utility in controlled release and targeting studies of almost all class of bioactive molecules. The novel properties of chitosan make it one of the most promising biopolymers for tissue engineering, gene therapy, and drug delivery vehicles.

Keywords: biocompatible materials, chitosan, drug delivery, tissue engineering**,** scaffolds.

Introduction

Within its natural resources of commercial interest, chitin exists not as a stand-alone biopolymer, but rather in conglomeration with other biomaterials, mainly proteins, lipids, and inorganic salts. The isolation process of chitin starts at the sea-food industry [1].

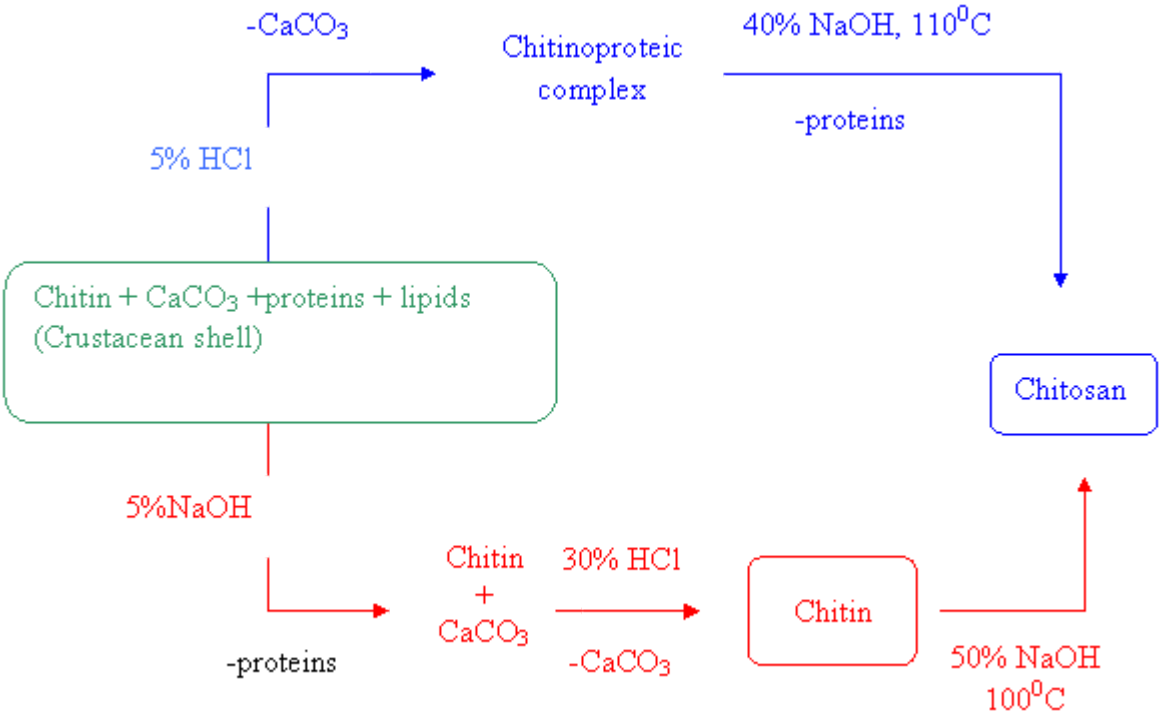
One of the by-products of this industry, *viz.* shells from crab, shrimp, etc. are first crushed into a pulverous powder to help make a greater surface area available for the heterogeneous processes to follow. An initial treatment of the shell with 5% sodium hydroxide dissolves various proteins, leaving behind chitin, lipids and calcium salts (mainly as $CaCO_3$). Treatment with 30% hydrochloric acid hydrolyzes lipids; dissolves calcium salts (demineralization) and other minor inorganic constituents. Chitin thus obtained can be hydrolyzed using 50% sodium hydroxide at high temperature to provide chitosan. Alternatively, if isolation of chitin is not desired, the acid-base sequence may be reversed to directly produce chitosan [1, 2].

In this method, crushed shells are first treated with 5% hydrochloric acid to remove calcium salts. This is then followed by protein and lipids removal by the treatment with 40% sodium hydroxide at higher temperature. During the base treatment a concomitant hydrolysis of acetamido groups in chitin takes place, resulting in the formation of chitosan. Chitosan is a partially deacetylated polymer of N-acetylglucosamine obtained after alkaline deacetylation of the chitin **(Scheme.1).** The average molecular weight of chitin is 1.03 x 10^6 to 2.5 x10^6, but the N-deacetylation reaction reduces this to 1 x 10^5 to 5 x10^5 [1-3]. Depending on the source and preparation procedure, its molecular weight may range from 300 to over 1000 kDa with a degree of deacetylation from 30 to 95% [4, 5].

In general, when the number of N-acetyl-glucosamine units is higher than 50%, the biopolymer is termed chitin. Conversely, when the number of N-glucosamine units is higher, the term chitosan is used. Insoluble in water, chitosan readily dissolves in acidic solutions, which is due to the presence of amino groups in its molecules, the degree of deacetylation necessary to obtain a soluble product being 80–85% or higher [6, 7].

However, some unsatisfactory mechanical properties, such as severe shrinkage, deformation after drying, low solubility in acidic media, pH ≈ 4.5, and compressibility at high operating pressure, limit its application and processing convenience [8]. Therefore, special attention has been paid to the chemical modification of chitosan.

Chitosan has three types of reactive functional groups, an amino group at C-2 position as well as both primary and secondary hydroxyl groups at C-6, and C-3 positions, respectively which allow further chemical modification of chitosan.



Scheme 1. Isolation of chitin and synthesis of chitosan– process schematic

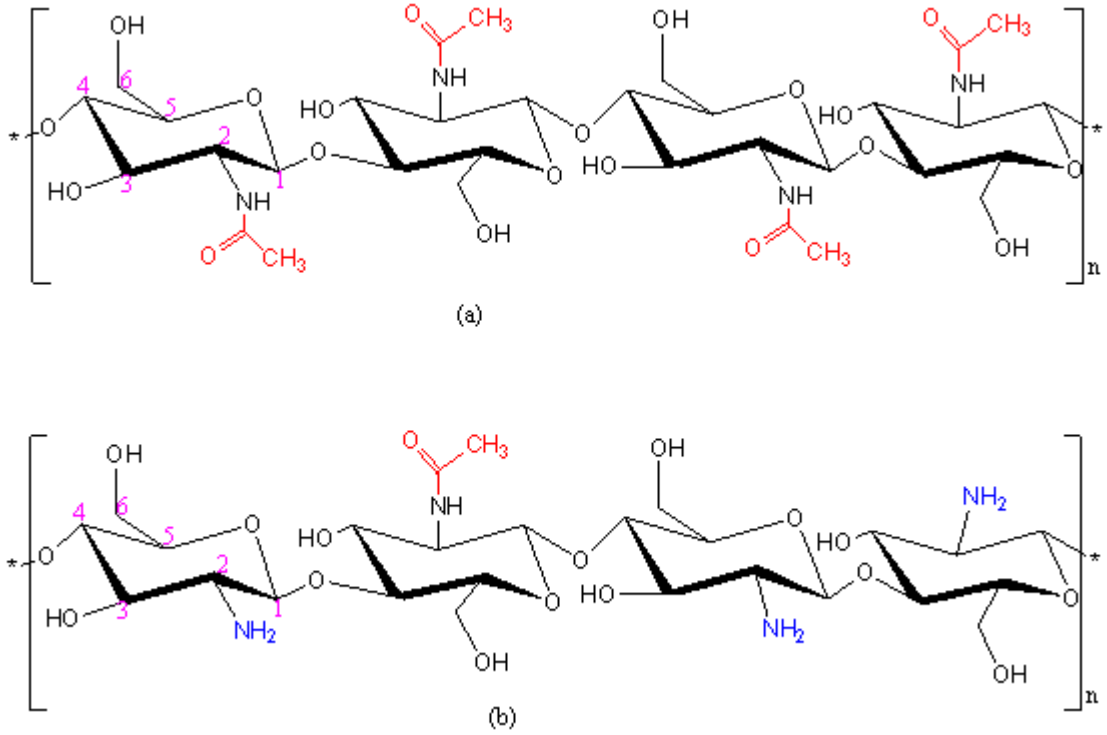


Figure 1. Chemical structures of chitin (a) and chitosan (b)

Cross linking or graft co-polymerizations are well known methods for the modification of chitosan representing convenient and effective ways for improving the physical and mechanical properties for practical uses. The physical properties of chitosan depend on the degree of N-acetylation and the distribution of N-acetyl groups. Relevant physical, chemical and biological properties of chitosan are presented in **Table 1** [9].

Table 1. Physic-chemical and biological proprieties of chitosan

Physic-Chemical properties of chitosan	Biological properties of chitosan
Cationic polyamine	Biocompatibility
When protonated, adheres to negatively charged	Natural polymer

surfaces (bio/mucoadhesive) and forms gels with polyanions	
Forms salts with organic and inorganic acids	Biodegradable to normal constituents
High molecular weight linear polyelectrolyte	Safe an non-toxic
Viscosity, high to low	Haemostatic, bacteriostatic, and fungistatic
Chelates certain transitional metals	Spermicidal
Amiable to chemical modification	Anticancerigen
Reactive amino/hydroxyl groups	Anticholesteremic
High charge density of pH <6,5	Versatile, reasonable cost

1. Chitosan used as Biomaterials

Biomaterials play a central role in tissue engineering. New materials are required to serve as temporary implantable devices, such as hollow molding chambers, porous tissue scaffolds, and bioactive material delivery devices. Manufacturing methods must also be devised to make essential materials broadly available at acceptable costs.

Implantable molds will likely play a role in vivo fabrication of structural tissues, such as bone, that must assume a specific 3D shape. A mold helps to direct the source of blood supply from a predetermined direction to facilitate later surgical transfer. It also helps to concentrate and localize bioactive materials delivered to the anatomic site used for tissue fabrication. This is particularly important in cancer patients in whom the tissues potentially prone to neoplasia should not be exposed to growth inducing factors. Temporary implantable molding chambers for bone fabrication must be fashioned from nontoxic, non-deformable materials that can be made in any shape and do not become incorporated by surrounding tissues after implantation. Silicone rubber, poly (methylmethacrylate), and titanium have been applied experimentally and clinically for these purposes [10, 11].

The second role for biomaterials in tissue engineering is as a scaffold material. The basic requirements are biocompatibility, degradability, mechanical integrity, and tissue conductivity. There are naturally occurring compounds that ordinarily function as structural frameworks which can be used as scaffolds for tissue engineering. Examples of these are hyaluronan[12], glycosaminoglycans [13], collagen [14], chitosan [15], and fibrin [16]. These may be rendered as isolated materials or processed as acellular preparations from sources such as small intestinal submucosal [17] and dermis [18]. Acellular preparations may offer advantages in some applications because more of the native structure and supporting matrix appears to be preserved.

Other types of biomaterials potentially useful as tissue scaffolds are semi-synthetics (i.e., chemical alterations of biologically occurring compounds) and synthetics (i.e., composed completely of unnatural substances). Synthetic polymers are useful in tissue engineering for several reasons. They afford great design flexibility because composition and structure may be tailored to meet specific needs.

Certain polymers degrade under biological conditions. These have chemical bonds that undergo hydrolysis upon exposure to aqueous body fluids, cellular digestion, or enzymatic degradation. Modifying properties such as porosity, hydrophobicity, copolymer ratio, and crystallinity affect the rate of degradation. A variety of porous materials have been useful as scaffolds, including biodegradable polymers [19], calcium ceramics [20], and composites of these materials [21].

Materials can be made with bioactive properties by altering surface characteristics and composition. They may be fabricated to incorporate bioactive molecules that are released as the material degrades. Fashioning the material into microparticles with diameters on the order of 100 μm is a particularly useful approach in this regard. Particles can be created containing bioactive compounds that are released in predictable ways [22].

Biomaterials interact with cells and the extra-cellular matrix at the surface. The surface properties of synthetic and naturally occurring materials may be modified by electrical, topological, and chemical methods to change these interactions in useful ways [23]. Cell adherence [24] and motility [25] can be affected by placing specific adhesion molecules on the material surface. Calcium mineral coating might render degradable polymers more compatible with bone tissue [26, 27]. Naturally occurring materials, such as collagen fibers, may be modified to mimic contours that enhance specific cell adhesion [28]. Cell function can be controlled by

engineering the surface to express specific growth factors [38]. Patterning technology involving micro-contact printing [29], laser photolithography [30], and micro-fluidic channels [31] may be used to modify surfaces to control topology of cell seeding on synthetic materials.

2. Tissue Engineering

2.1. Requirements for the production of tissue engineering scaffolds

Tissue engineering is an interdisciplinary field that applies the principles and methods of engineering and the life sciences toward the fundamental understanding of structural and functional relationships in normal and pathological tissue and the development of biological substitutes to restore, maintain, or improve function.

It is a new field that was formally identified in 1988 during a special workshop of the National Science Foundation. The Tissue Engineering Society was formed in 1996, and the National Institutes of Health offered the first requests for application for funding of tissue engineering research in 1997. Tissue engineering is a specific type of translational research that integrates knowledge from traditional biomedical investigation and applies it to clinical tissue replacement. From this viewpoint, it may be considered tissue modification at the cellular and molecular level for reconstructive surgery.

This fascinating new field of research thus provides an alternative to organ transplantation [31]. Basically, tissue engineering makes use of polymer scaffolds whose specific assignment is to promote adherence, proliferation and differentiation of cells. The scaffolds serve as temporary three-dimensional frameworks on which seeded cells, derived both from biopsies or stem cells, grow and are guide d to form the designed tissues [32].

During the culture, most commonly the scaffolds are degraded or integrated with the tissue, ideally at the rate corresponding to the rate of new tissue formation. Tissues can be grown in vitro on preformed scaffolds (alternatively in membrane reactors) prior to implantation to the body or in situ after injecting gels to the defect site. It involves the in vitro seeding and growing of relevant cells onto a scaffold [33].The scaffold therefore is a very important component for tissue engineering. Several requirements have been identified as crucial for the production of tissue engineering scaffolds [34]:

- the scaffold should possess interconnecting pores to favor tissue integration and vascularization;
- be made from material with controlled biodegradability or bioresorbability so that tissue will eventually replace the scaffold;
- have appropriate surface chemistry to favor cellular attachment, differentiation and proliferation;
- possess adequate mechanical properties to match the intended site of implantation and handling;
- should not induce any adverse response;
- be easily fabricated into a variety of shapes and sizes.

Other functions of the scaffolds are: space filling and controlled release of bioactive molecules (growth factors, nutrients), all of them ideally performed concertedly [35].

Performance of these varied functions, specific of each tissue or organ, sets demanding requirements of the material to be applied: biocompatibility, nontoxicity, biodegradability to nontoxic wastes, nonimmunogenicity, defined structure (porosity and morphology) with the optimal mechanical strength, and adequate mass transport properties that ensure both sustained release of the active substances applied and appropriate transport of gases, nutrients, proteins, cells and metabolites both within the scaffold and between the scaffold and the local environment [36, 37].

Of great importance are specific biological scaffold material–tissue interactions that together with growth factors applied could induce the growth of cells [38]. Currently, the range of potential tissue engineered systems encompasses every tissue or organ, with skin and cartilage constructs for repair of joints and urethral sphincters [39].

A number of synthetic and natural polymers, hydrogels, open-pore structures and fibrils are being investigated for the use in tissue engineering. Among them naturally derived polymers are of special interest [40]. This is due to the fact that as natural components of living structures, they bear biological and chemical similarities to natural tissues, of which that to the native extracellular matrix is crucial. Hence, unlike synthetic polymers, they can be more easily tailored to meet requirements of multifarious tissue systems [41].

Chitosan has been found an appealing candidate for tissue engineering applications, and now along with alginate, collagen and hyaluronic acid belongs to the class of most frequently studied biopolymers in this area

[42]. The choice of chitosan as a tissue support material is governed by multiple ways by which its biological, physical and chemical properties can be controlled and engineered under mild conditions [43]. Also, it has been shown to degrade in vivo, which is mainly by lysozyme-mediated hydrolysis [44]. Chitosan-based materials have been tested for tissue engineering in a number of shapes and physical forms, including porous scaffolds and gels [45].

Excellent porous structures, membranes, blocks, tubes and beads, have been obtained by freezing and lyophilization of chitosan solutions and gels [46] their mean pore size being controlled by varying the freezing temperature/rate, and pore orientation by thermal gradients. Chitosan structures showed promising properties for hepatocyte culture/transplantation, for articular cartilage tissue regeneration and as chitosan–inorganic composites for bone reconstruction [47].

Chitosan gels can be utilized as injectable, in situ gel-forming. Injectable systems, a more recent concept of tissue engineering, offer the following advantages over the use of preformed scaffolds: liquid gels are able to fill any space or shape of a defect site, living cells and therapeutic agents are incorporated prior to the injection within the solution, and more importantly, the systems can be implanted in the site without surgery [48]. The success of these systems strongly depends on the polymer gelation kinetics in the microenvironment involved [49]. In this context, chitosan–calcium phosphate composites were preliminarily evaluated as injectable, resorbable, in situ gelling systems for bone tissue regeneration. Also, temperature sensitive neutral gel forming chitosan/polyol salt solutions were proposed as injectable gels, this being interesting in that, in contrast to pH dependent gelation of most chitosan-based solutions, these solutions gel if heated at body temperature [40].

Additionally, chitosan gels are being proposed for the use in surgery and dentistry as biological adhesives to seal small wounds and to improve wound healing [41]. The present generation of tissue engineering research is based on the seeding of cells onto porous biodegradable polymer matrixes [42]. A primary factor is the availability of good biomaterials to serve as the temporary matrix.

These biomaterials must be capable of being prepared in porous forms to offer a channel for the migration of host cells into the matrix permitting growth into complete tissue analogs and be biodegradable into non-toxic products once they have served their function in vivo [43].

The scaffold material has an essential function concerning cell anchorage, proliferation and tissue formation in three dimensions. Performance of these properties demands usually a porous scaffold structure, with the porosity characteristics being application specific [44]. Chitosan and its derivatives have been reported as attractive candidates for scaffolding materials because they degrade as the new tissues are formed, without inflammatory reactions or toxic degradation [45].

2.2. Functionalized scaffold with chitosan in tissue engineering

To improve the adherent ability for seeding cells, the chitosan allow for a wide range of molecules to be modified. The incorporation of collagen to chitosan as a chitosan-collagen scaffold will enhance its cell attachment ability. Conjugation of chitosan with biologically active containing protein peptides is expected to become a potential technology to develop desirable scaffold materials for the tissue regenerations [46].

An illustration of selected examples of chitosan processing for use in tissue engineering is presented in **Figure 2.** Thus, cells may be encapsulated in gels or seeded in porous matrices including sponge-like or fibrous structures [47].Combinations of chitosan with other biocompatible materials such as calcium phosphate or gelatin are applied to modify biomechanical and cell-matrix-interaction properties. Different adaptations of chitosan may help to optimize cell and tissue differentiation and tailor the transplant to different clinical cell delivery situations.

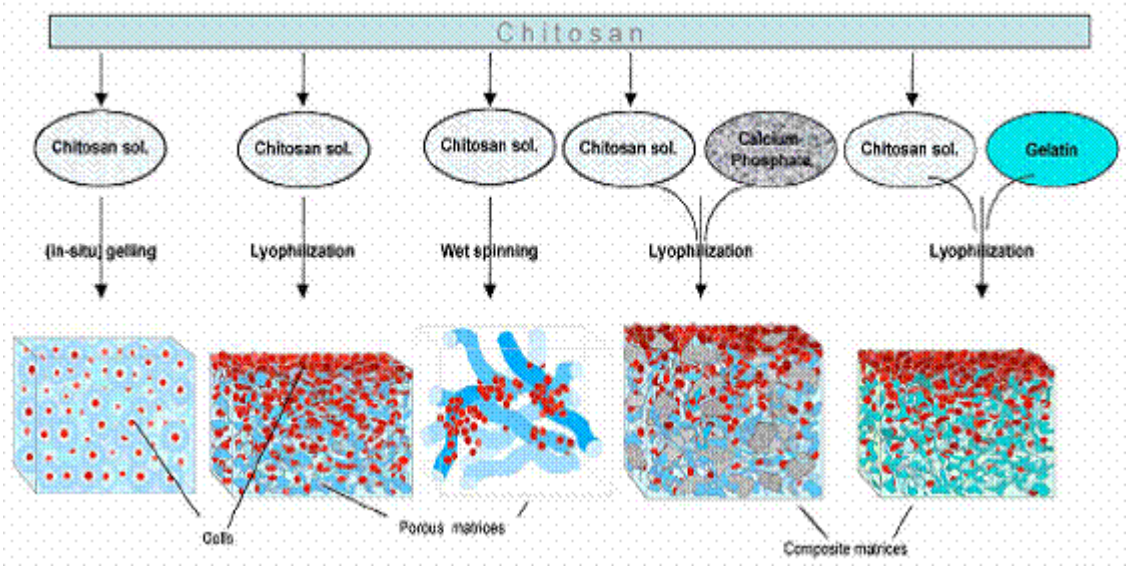


Figure 2. Selected examples of chitosan processing for use in tissue engineering

The poly (glutamic acid) (PGA), a hydrophilic and biodegradable polymer, was also used to modify chitosan matrices and the PGA/chitosan composite biomaterial is shown to be promising biomaterials for tissue engineering applications. Stem cells with self renewal potential and multilineage differentiation capacity have been considered as a best choice for the seeding cells in tissue engineering. Bone marrow derived mesenchymal stem cells have shown promising applications chitosan-grafted - poly-L-lactic acid (PLLA) has the potential application in tissue engineering [48].

The crystallinity of chitosan gradually decreased after grafting, since the side chains substitute the $-NH_2$ groups of chitosan randomly along the chain and destroy the regularity of backing between chitosan chains. In aqueous solutions, the chitosan-grafted-PLLA copolymer was found to be a pH-sensitive hydrogel due to the aggregation of the hydrophobic side chains. The in vitro fibroblast static cultivation on the chitosan-graft - PLLA films showed that the cell growth rate on the copolymers films was faster than chitosan and decreased when the feed ratio of PLLA to chitosan increases [49]. A surface functionalization of biodegradable PLLA was achieved by plasma coupling reaction of chitosan with PLLA. Contact angle and X-ray photoelectron spectroscopy studies demonstrated that the thickness of the grafted chitosan layer was in the order of several manometers [40]. The proliferation and morphology studies of two cell lines, L-929 (mouse fibroblasts) and L-02 (human hepatocytes), cultured on this surface showed that cells hardly spread and tended to become round, but could proliferate at almost the same speed as cells cultured on glass surface. This insight will help to clarify the mechanism of the switch between cell growth and differentiation. This grafted polymer can be used to control the morphology and function of cells, and has applications in tissue engineering [50].

The porous polyglycolide (PGA)–chitosan hybrid matrices are also reported as scaffolds for tissue regeneration. The pore structure, mechanical properties and in vitro degradability of these hybrid matrices were altered by varying the weight ratio of PGA. The 75% PGA hybrid matrix exhibits a high porosity, high strength, good biocompatibility and degradability and is thus a promising biomaterial for tissue engineering applications [51].

Several injectable materials basing on chitosan and its derivatives have been used as osteogenic bone substitutes. Chitosan-calcium phosphate (CP) composites appear to have a promising clinical application [28]. Phosphorylated chitosan to CP composites have been used to fill bony defects in the radius and tibia in vivo. Chemically modified HA (hyaluronic acid)-chitin and chitosan-HA material were reported to be osteoinductive and exhibited rapid degradation and revascularization in vivo [13].The combination of chitosan with antibiotics or growth factors is proved to be suitable for bone tissue engineering [52]. The imidazole-linked chitosan material promoted mineralization, induced bone formation and filled critical size bone defects with the apposition of trabecular bone [53].

It is showed that chitosan has the ability to promote osteogeneic progenitor cell recruitment and attachment, facilitating bone formation [54]. When cultured mesenchymal stem cells are treated in vitro with chitosan, the treated cells show higher averages of colonies per well than the untreated control suggesting that chitosan may promote differentiation of osteoprogenitor cells and bone formation [55].

The calcium phosphate/chitosan coating showed an improved bone marrow stromal cell attachment [56]. The chitosan-alginate gel mesenchymal stem cells/bone morphogenetic protein-2 composites was found to stimulate new bone formation and showed that it could become clinically useful injectable materials to generate new bone [55].Tissue engineering concepts have been introduced to develop cell-based repair approaches for articular cartilage [56]. Tissue engineering of articular cartilage involves the isolation of articular chondrocytes or their precursor cells that may be expanded in vitro and then seeded into a biocompatible matrix, or scaffold, for cultivation and subsequent implantation into the joint. Different cell populations that have been investigated in the experimental studies include matured articular chondrocytes, epiphyseal chondrocytes, mesenchymal stem cells, bone marrow stromal cells, and perichondrocytes [57].

The choice of biomaterial is also critical to the success of such tissue engineering approaches in cartilage repair. A variety of biomaterials, naturally occurring and synthetic, biodegradable and non-biodegradable, have been introduced as potential cell-carrier substances for cartilage repair [58]. The naturally occurring biomaterials include various forms of types I and II collagen-based biomaterial, under the form scaffold matrices, gels, or collagen-alginate composite gels. The synthetic polymer-based biomaterials include polyglycolic acid (PGA) and poly-L-lactic acid (PLLA), or their composite mixture. In cartilage tissue engineering, PGA [15] and PGA-PLLA copolymers have been studied for their efficacy as chondrocyte-delivering scaffolds in vitro and in vivo. Several investigators have also found that some non-biodegradable polymer substances, such as polytetrafluoroethylene, polyethylmethacrylate, and hydroxyapatite/Dacron composites, also facilitate the restoration of an articular surface [57].

The ideal cell-carrier substance should be the one which most closely mimics the naturally occurring environment in the articular cartilage matrix. It has been shown that cartilage-specific extracellular matrix (ECM) components such as type II collagen and glycosaminoglycan (GAG) play a critical role in regulating expression of the chondrocytic phenotype and in supporting chondrogenesis both in vitro and in vivo. Otherwise, chondrocytes may undergo de-differentiation and produce an inferior brocartilaginous matrix rich in type I collagen [58].

This inferior matrix then leads to a failure to form hyaline cartilage. It can assume that the criteria for the choice of biomaterial in cartilage tissue engineering include biological friendliness and biomechanical strength [59].These features may provide a bio-chemically and bio-mechanically appropriate environment necessary for engineered cells to regenerate a long-lasting hyaline cartilage in the defect site. For cartilage tissue engineering, a round cellular morphology is known to be indicative of a normal phenotypic characteristic of non-dedifferentiated chondrocytes. Chitosan has the ability to maintain round morphology and preserve cell-specific ECM molecule synthesis of chondrocytes. Chitosan could improve chondrocyte attachment to poly (L-lactic acid) (PLLA) and alginate, increase cell adhesion, proliferation and biosynthetic activity [20]. The intra-articular injection of chitosan has showed an increase in epiphyseal cartilage in the tibias and femoral joints with an activation of chondrocyte proliferation [58].

When chondrocyte seeded scaffolds were implanted into rabbit knee cartilage defects, partial repair was observed [60]. Chitiosan-based scaffolds were also used to deliver growth factors in a controlled fashion to promote the ingrowths and biosynthetic ability of chondrocytes [61].

The porous collagen/chitosan/GAG scaffolds loaded with transforming growth factor 1 (TGF-1) was reported to promote cartilage regeneration for cartilage defects [51].

A newly injectable material, a thermosensitive water-soluble chitosan-poly(N-isopropylacrylamide) (PNIPAAm) gel was developed and proved to have the ability to differentiate from mesenchymal stem cells (MSCs) to chondrocytes and mass formation. The cell-polymer could be injected easily below the lower critical solution temperature (LCST) around 32°C in the sol state, then they could be transited to the gel at body temperature. Therefore, the combination of chondrogenic differentiated cells from MSCs with a thermo sensitive polymer could be used as an injectable cell-polymer complex [14, 62].With these promising features, they are considered as a very interesting biomaterial for use in cell transplantation and tissue regeneration. This technology has been used to create various tissue analogs including skin, cartilage, bone, liver, and nerve in the past decades [63]. A transparent chitin hydrogel tubes were synthesized from chitosan solutions using acylation and mold casting techniques.

The chitosan tubes were mechanically stronger than their chitin origins and showed significantly enhanced neuritis outgrowth relative to chitin films [41].The alginate-chitosan (AC) microcapsules could support the survival, proliferation, protein secretion by entrapped hepatocytes and the chitosan and PLGA scaffolds were also used for the pancreatic islet culture and transplantation [64].The chitosan based membrane

could provide cell immune-isolation and has the potential for cell cryopreservation. The chitosan hydrogel fibers with micropores could be used to carry recombinant human vascular endothelial growth factor (rhVEGF) for the induction of new vessels. Basing on these properties, chitosan may have prospects in the cell therapy or tissue engineering for nerve guidance, liver cells, and pancreatic islet transplantation [65].

2.3. Chitosan: A versatile biopolymer for orthopedic tissue-engineering

Three-dimensional (3D) scaffolds are essential for the development of engineered articular cartilage [66]. Ideal scaffolds are designed to be biocompatible, bio-absorbable and exhibit predictable porosity and degradation rate. They provide a framework that facilitates new tissue in growth; moreover, mechanical characteristics are matched to those of the native tissue increasing the chances that the reparative process will be compatible with the host's tissue physiology. Chitosan has been used as a scaffolding material in articular cartilage engineering due to its structural similarity with various GAGs found in articular cartilage, involved in modulating chondrocyte morphology, differentiation, and function [67].

Alginate is a suitable biomaterial for cartilage engineering but exhibits weak cell adherence [17]. It was reported an alginate-based chitosan hybrid polymer fibers which showed increased cell attachment and proliferation in vitro compared to alginate [18]. These hybrid polymer fibers showed increased tensile strength, implying a possible use in developing a 3D load bearing scaffold for cartilage regeneration [15].

Chondrocytes cultured on chitosan substrates in vitro maintained round morphology and preserved synthesis of cell specific ECM molecules [68].Chitosan was used to improve chondrocyte attachment to PLLA films the modified substrate showed increased cell adhesion, proliferation and biosynthetic activity. Chitosan was also conjugated with hyaluronan to obtain a biomimetic matrix for chondrocytes. Chondrocyte adhesion, proliferation, and the synthesis of type II collagen were significantly higher on the hybrid fiber than on chitosan [48]. To increase the cellular adhesiveness of chitosan were developed chitosan–alginate–hyaluronan complexes. Cell-seeded scaffolds showed neocartilage formation in vitro [69]. Chitosan based scaffolds can deliver growth factors in a controlled fashion to promote the ingrowth and biosynthetic ability of chondrocytes. The porous collagen/ chitosan /GAG scaffold exhibited controlled release of TG F-b1 and promoted cartilage regeneration improved mechanical properties [44] and stability of the collagen network by inhibiting the action of collagenases [70].

Chitosan and some of its degraded products could be involved in the synthesis of the articular matrix components such as chondroitin, chondroitin-sulfate, dermatane-sulfate, keratin-sulfate and hyaluronic acid. It was studied the effect of intra-articular injection of chitosan on regeneration of articular cartilage. An increase in epiphyseal cartilage in the tibia and femoral joints was seen with an activation of chondrocyte proliferation [41, 54].

2.4. Chitosan in inter-vertebral disc tissue engineering

Possible applications of chitosan in spine tissue engineering encompass three different fields, namely spine fusion, gene therapy and nucleus pulpous regeneration. When a bone graft alternative is applied during spinal fusion procedures, several local biomechanical factors are considered, depending on the type and position of the chosen graft [71]. Anterior interbody grafts are exposed to high compressive forces and need to possess load bearing ability. On the contrary, a posterior applied bone graft is placed along the tension side of the spinal column in absence of local compressive stimuli, and thus bone graft incorporation is less likely to be affected by local biomechanical factors [72].

Materials such as PLA or PLA–PEG have been tested for spinal fusion, and are considered good candidates due to their plasticity, stiffness, biodegradability and ability to support cells and growth factor. A possible application of chitosan could be a composite graft material with a predictable degradation rate and macroporous structure that would allow linking of growth factors and support osteogenic cells for spinal fusion [73, 74]. Gene therapy represents a recent approach to facilitate vertebral body fusion, and is performed via the transfer of the cDNA of osteoinductive genes to the desired cells [12].

Nonviral vectors utilize physico-chemical substrates to facilitate entry of the genetic material into the cell. This method of delivery is advantageous because the size of the genetic material that can be introduced into the cell is not limited and therefore large genes could be introduced with these vectors. The efficiency of

transfection with non viral vectors is low, and the duration of express ion of the protein product tends to be short partly due to the episomal nature of the transgenic itself. The use of plasmids for gene delivery is restricted because only a few cell types will take up this naked form of DNA [42]. This problem can be partially overcome by combining the genetic material with bioabsorbable scaffolds to form what has been termed the gene-activated matrix (GAM). The scaffolds serve as a substrate for cell adhesion and facilitate the contact of naked DNA with the target cells. GAMs have been successfully used to promote fracture healing in rodents and canines. Through its ability to complex DNA and its good bio-absorbability, chitosan can be considered as an excellent candidate matrix for non-viral gene therapy for spinal fusion applications [75].

Inter-vertebral discs possess different functional and anatomic regions: the inner nucleus pulpous, a hydrated gelatinous tissue rich in proteoglycans, and the outer annulus fibrosis made of concentric collagen lamellae. Loss of proteoglycans and water content in the inner nucleus pulpous initiates degenerative spinal disease. Biologic disc regeneration is considered as a promising approach to restore biological integrity and function of a pathologically altered disc [66]. Several strategies can be employed for different stages of disc degeneration that utilize direct drug/growth factor delivery to the disc, as well as gene transfer to disc cells cultured in vitro An human-skin (HS), heparin sulphate [77].

Chitosan gel has been used as a scaffold for nucleus pulpous cells, and growth factors (GFs) were used to modulate matrix synthesis in an attempt to produce a tissue with a similar molecular composition to native nucleus pulpous tissue. In vitro formation of nucleus pulpous tissue did not appear to be facilitated by using of a artificial 3D scaffold, although nucleus pulpous cells implanted in gel synthesized aggrecan, small proteoglycans as well as Type I and II collagen, retention of matrix molecules within the scaffolds was low and synthetic levels did not exceed 35% of the native nucleus pulpous tissue. Chitosan gels were superior to other scaffolds, such as collagen and hyaluronan, with increased matrix synthesis and stable cell phenotype [78].

Another idea is to complex cationic chitosan to DNA forming chitosan nano-particles that could be transfected into nucleus pulpous cells to promote cell differentiation and matrix synthesis in both in vitro and in vivo studies. Chitosan has been extensively used in bone tissue engineering since it was shown to promote growth and mineral rich matrix deposition by osteoblasts in culture. Several studies have focused on the use of chitosan –calcium phosphate (CP) composites for this purpose [79]. A 3D macroporous CP bioceramic embedded with porous chitosan sponges is developed [53]. In this scaffold, a nested sponge enhanced the mechanical strength of the ceramic phase via matrix reinforcement and preserved the osteoblast phenotype [80]. Similarly, gentamycin-conjugated macroporous chitosan scaffolds reinforced with beta-tricalcium phosphate and calcium phosphate has been developed for bone engineering [35].

Macroporous chitosan scaffolds incorporating hydroxyapatite (HA) or CP glass with an interconnected porosity of approximately 100 nm have been synthesized. Overall composites of chitosan – calcium phosphate appear to have a promising clinical application in the future for osteoinductive effect and neovascularization in vivo [35]. Formation of new bone was observed when a chitosan-hydroxyapatite paste was applied on the surface of the tibia after periosteum removal, indicating suitability of this paste for further clinical studies as a bone filling material [54]. A chitosan-hydroxyapatite multilayer nanocomposite with high strength and bending modulus rendering the material suitable for possible application in internal fixation of long bone fractures. A macroporous chitosan -gel/b- calcium phosphate composite scaffold for bone tissue engineering using freeze-drying process is developed [41].Chitosan is used as an adjuvant with bone cements to increase their injectability while keeping the chemical and physical properties suitable for surgical use.

The rationale of using chitosan for this purpose is based on the property that chitosan solutions gel in response to a pH change from slightly acidic to physiological; in fact, the chitosan – calcium phosphate composites address the need to develop bone fillers that set in response to physiological conditions. Many of these chitosan gel composites are proposed mainly for non load bearing bony defects [37]. A composite material as phosphorylated chitosan to CPC was biocompatible, osteoinductive, bioresorbable and remodeled through a creeping substitution process. This injectable, bioabsorbable composite material possessed interconnected macropores and provided strength to the implant during tissue regeneration [29].

The intramolecular hydrogen bonds of chitosan provide interacting macromolecules with a good resistance to heat. To exploit this property, composites of chitosan with poly methyl methacrylate (PMMA) were developed that exhibited lower exothermic curing temperatures. This composite material possessed a high interconnected porosity with a pore size suitable for osteo conduction and better anchorage to the surrounding bone. It was observed that the pore size of this composite material increased with time due to biodegradation of the chitosan. The ability of chitosan to bind growth factors is suitable for bone tissue engineering [68].

Due to its cationic nature and predictable degradation rate, chitosan-based materials bind growth factors and release them in a controlled fashion. The cationic nature of chitosan can be further enhanced by the introduction of a covalently linked imidazole group. The biochemical significance of imidazole addition is that this group inhibits thromboxane synthetase, acts as antioxidant and facilitates intracellular buffering for the tissue healing process. This imidazole-linked chitosan material promoted mineralization, induced bone formation and filled critical size bone defects with the position of trabecular bone [25].

Titanium (Ti) surface coated with chitosan via silane–glutaraldehyde chemistry exhibited increased osteoblast attachment and proliferation. The chitosan coatings could promote osteointegration of Ti implant devices commonly used for orthopedic and craniofacial implants, although chitosan bond strength was found to be less compared to CP coatings [29].

Chitosan were used to increase the biocompatibility of electrolytically deposited apatite coatings on Ti alloys. A hybrid calcium phosphate/chitosan coating, developed through electrodeposition, exhibited an increased dissolution rate in both acidic and neutral simulated physiologic solution. Moreover, the calcium phosphate/ chitosan coating showed an improved bone marrow stromal cell attachment. The ability to reconstitute tissue structure and function using chitosan has shown tremendous clinical implications and is likely to play a key role in cell and gene therapies in the future [29].

2.5. Wound Healing

Grafted chitosan presents interesting properties for wound-healing applications, because chitosan derivatives can exhibit enhanced bacteriostatic activity with respect to pure chitosan. Ethylene diamine tetra-acetic acid (EDTA) grafted onto chitosan increases the antibacterial activity of chitosan by chelating magnesium that under normal circumstances stabilizes the outer membrane of gram-negative bacteria.

The increase in chitosan antimicrobial activity is also observed with carboxymethyl-chitosan, which makes essential transition metal ions unavailable for bacteria or binds to the negatively charged bacterial surface to disturb the cell membrane [11].The grafted chitosans are used in wound healing systems, such as carboxymethyl-chitosan for the reduction of periodontal pockets in dentistry and chitosan grafted with EDTA as a constituent of hydro-alcoholic gels for topical use [12].

It was carried out the grafting of acrylic acid onto ozone-treated poly (3-hydroxybutyric acid) (PHB) and poly (3-hydroxybutyric acid-coK3-hydroxyvaleric acid) (PHBV) membranes. The resulting membranes were further grafted with chitosan via esterification. These chitosan grafted membranes showed antibacterial activity against Escherichia coli, Pseudomonas aeruginosa, methicilin-resistant Staphylococcus aureus (MRSA), and S. aureus [25, 46]. Acrylic acid grafting increased the biodegradability with Alcaligens faecalis, where as chitosan and chitooligosaccharide grafting reduced the biodegradability [34]. In addition, chitosan-grafted- poly (3-hydroxybutyricb-acid-coK3-hydroxyvaleric acid) membrane showed higher antibacterial activity and lower biodegradability than chitooligosaccharide grafted membrane. Two anionic soluble monomers, mono (2-methacryloyloxyethyl) acid phosphate and vinyl sulfonic acid sodium salt, were grafted onto chitosan to obtain copolymers with zwitterionic property. The grafting reaction improved the antimicrobial activities of chitosan against Candida albicans, Trichophyton rubrum, and Trichophyton violaceum, which depends on the amount and type of grafted chains, as well as on the changes of pH. The antibacterial activity of the polypropylene was enhanced by the modification of γ-ray radiation induced grafting of acrylic acid and the immobilization of chitosan onto the polypropylene-graft-acrylic acid modified polymer [15, 81].

Chitosan possess the characteristics favorable for promoting rapid dermal regeneration and accelerated wound healing. It is observed that chitosan oligosaccharides have a stimulatory effect on macrophages, and both chitosan and chitin are chemo attractants for neutrophils both in vitro and in vivo, an early event essential in accelerated wound healing. Chitin and chitosan may further facilitate wound healing by stimulating granulation tissue formation or re-epithelization [82].

Chitosan membranes did not restrict normal human skin fibroblasts, but impeded keloid fibroblast by inhibiting type I collagen secretion and suggested a role for wound healing in keloid control [23]. The argon-plasma-treated chitosan membranes exhibited excellent attachment, migration, and proliferation of the human-skin-derived fibroblasts compared to the untreated ones. Various forms of chitosan have been tested in wound healing. In a comparative study of chitin, chitosan, water-soluble chitin (WSC) powders and WSC solution, WSC solution was found to have the highest tensile strength with the healing rate fastest for WSC solution, followed by WSC powder, chitin powder, and chitosan powder. The superior biodegradability and hydrophilicity

of WSC can enhance its compatibility with wounded tissues and increase its activity as a wound-healing accelerator [83]. To improve the healing process, chitosan has been combined with a variety of modified materials such as growth factors, extracellular matrix components and antibacterial agents. The incorporation of basic fibroblast growth factor (bFGF) with chitosan accelerated the rate of healing [84].

The inclusion of antimicrobial agents into wound dressings is another important strategy. Silver sulfadiazine was introduced to the chitosan gel and membrane. The controlled release of sulfadiazine was found to be effective in controlling infection in wound healing [11]. A chlorhexidine containing chitosan-based wound dressing also showed antibacterial efficacy [85].

A surgical dressing made of a chitosan-gelatin complex was further developed and this experimental dressing displayed excellent adhesion to subcutaneous fat. The chitosan gelatin scaffolds were wet table and adsorbed more water than did chitosan alone [86]. Another chitosan derivative, 5-methylpyrrolidinone chitosan, can be compatible with other polymer solutions, including gelatin, poly (vinyl alcohol), poly (vinyl pyrrolidone), and hyaluronic acid [40].

The biomaterial could be fabricated into many different forms, such as filaments, non-woven fabrics, and so forth. The advantages include healing of wounded tissues, and of decubitus ulcers, depression of capsule formation around prostheses, limitation of scar formation and retraction during healing. Some wound-covering materials have been developed from chitin non-woven fabric or polyelectrolyte complexes of chitosan with sulphonated chitosan or N-carboxybutyl chitosan [87]. These materials were found to be effective in regenerating the wounded skin tissue. Chitosan in combination with alginate as polyelectrolyte complex (PEC) films have also been prepared and display greater stability to pH changes and are more effective as controlled release membranes than either the chitosan or alginate separately. The PEC membranes were found to promote accelerated healing of incisional wounds in a rat model [51, 88].

A collagen-chitosan tissue-engineered skin sponge was developed and serves as a scaffold for the reconstruction of a tissue-engineered skin in vitro [14]. This reconstructed skin promoted the remodeling of an extracellular matrix nearly similar to normal dermis, and provides new perspectives to increase nerve regeneration within the tissue-engineered skin by linkage of neurotrophic factors in the sponge before transplantation [89].

It was created a skin equivalent with characteristic dermal and epidermal architecture by combining dermal stem cells and hair follicle epidermal stem cells on a chitosan/collagen-based scaffold. The results showed that the chitosan/collagen matrix provide a suitable substrate for the tridimensional growth of skin stem cells [90].

Conclusions

Numerous papers published during the last 3 years on the application of polymers in the fields of tissue engineering, regenerative medicine, and medical devices, attest to the great and still growing interest of the scientific community in the biomedical applications of macromolecules.

Chitosan, the deacetylated derivative of chitin, has a number of properties such as biocompatibility, biodegradability, non-toxicity, and antimicrobial activity, which have attracted much scientific and industrial interest in such fields as biotechnology, pharmaceutics, waste water treatment, cosmetics and food science. Chitosan has been shown to be a promising scaffold for various applications in tissue engineering.

Recent studies of the chemical modification of chitosan are discussed from the viewpoint of biomedical applications. Chitosan has been shown to be useful as a wound dressing material, drug delivery vehicle and increasingly a candidate for tissue engineering. Chitosan is almost the only cationic polysaccharide in nature, and it is nontoxic and biodegradable in human body. Chitosan is a suitable material as a bone graft alternative in orthopedic procedures. Chitosan a promising candidate scaffold material for cartilage, inter-vertebral disc and bone tissue engineering in clinical practice.

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