Chitosan Based Systems for Tissue Engineering Part II: Soft Tissues

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Summary

A general introduction to tissue engineering and chitosan as well as its applications in hard tissues has been given in the first part of this review which is previously published in this journal. In this second part, applications of chitosan based systems for the soft tissue engineering will be reviewed. Due to the its properties such as biocompatibility, biodegradability, bioadhesivity as well as its bioactive properties wound healing effect, homeostasis, and antimicrobial activity, chitosan it is a promising scaffold material for tissue engineering. After a brief introduction to tissue engineering in soft tissues such as skin, adipose, cornea, liver, nerve and blood vessel, the application of chitosan for regeneration of these tissues will be discussed in regard to formulation of scaffolds. The strategies to improve their efficacy will also be mentioned.

Key Words: Soft tissue, chitosan, scaffold, nerve, liver, cornea, skin, blood vessel

Doku Mühendisliği için Kitosan İçeren Sistemler Bölüm 2: Yumuşak Dokular

Özet

Doku mühendisliği ve kitosanın sert dokudaki uygulamaları ile ilgili genel bilgiler bu derginin önceki sayılarında yayınlamış olan bu konudaki derlemenin birinci bölümünde verilmiştir. Derlemenin bu kısmında ise, yumuşak doku mühendisliği için kitosan içeren sistemlerin uygulamaları verilecektir. Biyouyumlu, biyoparçalanabilir ve biyoadezif özelliklerinin yanı sıra kendisinin antimikrobiyal, yara iyileştirici, hemostatik gibi birçok biyoaktif özelliğe sahip olması, kitosanı doku iskelesi için ümit verici bir polimer yapmaktadır. Deri, adipoz, kornea, karaciğer, sinir ve kan damarı gibi yumuşak dokularda uygulanan doku mühendisliği yaklaşımları ile ilgili olarak kısa bir girişten sonra, bu dokuların rejenerasyonunda kitosanın uygulamaları doku iskelelerinin formülasyonu yönüyle tartışılacaktır. Ayrıca etkinliği artırmak için yapılanlardan bahsedilecektir.

Anahtar kelimeler: Yumuşak doku, kitosan, doku iskelesi, sinir, karaciğer, kornea, deri, kan damarı

INTRODUCTION

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences in order to fabricate living replacement parts for the body (1). The most common approach for tissue engineering is utilization of scaffolds which are artificial structures capable of stimulating cellular growth, proliferation and cellular differentiation. Materials with suitable biochemical and physio-chemical properties are used for tissue engineering to improve or replace portions of or whole tissues. Scaffolds can be applied both to soft and hard tissues (2). The scientific challenge is to prepare the suitable scaffold with the desirable properties such as adequate mass transport, mimicking the

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biological environment with the controlled pore size, and porosity in order to provide three-dimensional templates for cell adhesion, migration, growth and proliferation.

Soft tissues play crucial functional role in body and soft tissue engineering studies are conducted on many organs such as liver (3), lung (4), muscles (5), skin (6), nerves (7), blood vessels (8), cornea (9), vagina (10), heart valves (11), trachea (12) and adipose tissues (13). Various polymeric scaffolds have been investigated for regeneration of the soft tissues. Among the polymers used for preparation of scaffolds for soft tissues, chitosan which is obtained from deacetylation of chitin obtained from the shells of the crustaceans, has attracted more attention in recent years due to its favorable properties such as bioadhesivity, biodegradability and biocompatibility. Furthermore, it exerts bioactive properties such as hemostatis, wound healing, antimicrobial, etc.

Being structurally similar to extracellular matrix components, chitosan provides stimulation of the attachment, proliferation and viability of tissue cells (14). With chitosan, it is possible to develop scaffolds in various forms such as film, sponge, gel, particulate systems etc. Moreover, chitosan can be modified chemically and enzymatically which enables the improvement of the properties of the scaffolds. The degree of deacetylation (DD), indicating the free amine groups along the chitosan backbone, is a key parameter which changes its physicochemical properties such as solubility, chain conformation and electrostatic properties (15,16). Due to its cationic amine groups, chitosan provides a suitable environment for cell adhesion (17). Degradation products of chitosan are saccharides and glucosamines, which are already available in a mammal metabolism. These compounds can activate macrophages since macrophages have receptors for N-acetyl-D-glucosamine and mannose. It was reported that chitosan increases TGF-β1 secretion and stimulate macrophages to produce platelet-derived growth factor (PDGF) and IL-1, which play an important role in cell growth, division and angiogenesis. Consequently, chitosan promotes granulation and organization which makes it

beneficial for open wounds (14,18). It was shown that chitosan does not increase extracellular matrix (ECM) formation directly, but with the help of growth factors like PDGF and TGF-β1 (14,19). It was reported to cause minimal foreign body reactions, and has stimulating effects on the immune system against viral and bacterial infections (17,20,21).

Chitosan as a promising scaffold material has been investigated using different preparation methods (22,23,24). In general, chitosan based scaffolds for soft tissue engineering were developed by lyophilization, drying by heat, electrospinning and gelation methods.

In the following sections, the applications of chitosan as scaffold for tissue engineering will be reviewed for various soft tissues such as skin, adipose, cornea, liver, nerve and blood vessel.

Skin

Every year, millions of people get burn by hot water, flame, and boiling oil, and these accidents result in major disabilities or even death. Especially in adults, dermis regeneration can not occur spontaneously. Since autologous skin has limited availability and associated with additional scarring (25), this traditional approach for substantial loss of dermis cannot meet the requirements, and tissue engineering became inevitable for skin tissue. Tissue engineered dermal equivalents of full-thickness autografts have been developed which can be used alone or in combination with epithelial sheets (26-28). At present, chitosan is clinically used for skin regeneration in form of bandages and wound dressings, such as Chitodine® (14), Chitoflex® (29) , Chitopack C® (30,31), Chitopoly®, Chitosan Skin® (14), Chitoseal® (30), Clo-Sur® (30), Crabyon® (32), HemCon® (30,33), Tegasorb® (30,31), Tegaderm® (30), TraumaStat® (33), Celox™ (34) and Vulnosorb® (14). Numerous studies have been reported using chitosan for the regeneration of skin tissue. Applications of chitosan in skin tissue engineering are summarized in Table 1.

Chitosan is a favorable scaffold material for skin tissue engineering due to its following properties: hemostasis; acceleration in the tissue regeneration by activation of polymorphonuclear cells (46) and

Table 1. Skin and adipose tissue engineering studies on chitosan in the last 5 years

Tissue	Scaffold content / Pore Size	Chitosan type	Preparation method / Form	Mechanical properties	In vitro testing Cell culture studies on scaffold	In vivo testing	Ref
Skin	CS/PVA hydrogel	Medium MW CS (80% DD)	Drying by heat Film	NA	No significant difference in cell numbers, no cytotoxicity Kidney VERO cells, cementoblast cells	NA	35
Skin	Gold colloid, CS	CS (>90% DD)	Drying by heat Film	NA	Significantly increased attachment, high biological activity, no fusiform fibroblast growth, non-toxic Keratinocytes	NA	25
Skin	Collagen/CS Pore size: 255 μm	CS (Not specified)	Lyophilization Sponge	Highly interconnected pores Porosity: 88.6%	Increased cell proliferation and uniform cell distribution due to perfusion seeding system Fibroblasts	NA	26
Skin	Collagen/CS Pore size: >200 μm	CS (1.0-1.7 10⁵Da, 75–85% DD)	Lyophilization Sponge	Low biodegradation	Cell proliferation and adhesion Fibroblasts	Rabbit Sampling: 3, 7, 14, 28 days Bovine type I collagen preservation, partially degradation of scaffolds	36
Skin	CS (with $Na_2P_3O_{10}$), Collagen Pore size: 40-100 μm	CS (Not specified)	Lyophilization Sponge	Highly interconnected pores	Increased cell proliferation, differentiation to a meshed structure Mouse embryonic steam cells	NS Sampling: 1, 2, 3, 4, 12 days No inflammation, wound healing	37
Skin	CS (with DTBP)	CS (80%, 90%,100% DD)	Lyophilization Sponge	Significantly higher tensile strength with higher DD and with higher crosslinking agent, significantly higher elastic modulus with higher DD, higher elongation percent with 90%>80%>100% DD Porosity: 75.1±5.3, 78.0±0.7, 75.8±2.2	No significant difference in the cell number Detroit 551 fibroblasts	NA	6
Skin	CS/Gelatin/Ha Pore size: 10-20 μm, 65-80 μm	CS (2 x 10⁵ Da, >85% DD)	Lyophilization Sponge	Significant increase in elongation at break, no significant difference in tensile strength	Homogeonus fibroblast dispersion, significant proliferation Adhesion, proliferation and differentiation of fibroblasts and keratinocytes, presence of laminin and type IV collagen, Fibroblasts, Coculture (Fibroblasts, keratinocytes)	NA	38

Tissue	Scaffold content / Pore Size	Chitosan type	Preparation method / Form	Mechanical properties	In vitro testing — Cell culture studies on scaffold	In vivo testing	Ref
Skin	CS-gelatin microspheres with bFGF, CS-gelatin Particle size:4.60± 2.3µm Pore size: 95-160 µm	CS (2 x 10^5 Da, >85% DD)	Lyophilization Sponge	No significant effect of microspheres in porosity and pore size	Significantly higher proliferation and GAG synthesis, significantly increased laminin transcript, significantly decreased type I collagen, no significant difference in type III collagen and fibronectin transcript / Fibroblasts	NA	39
Skin	Collagen/CS/silicone	CS (1x 10^5-1.7x 10^5 Da, 75-85% DD)	Lyophilization Sponge	NA	Good biocompatibility, proliferation / Fibroblasts	Bama miniature pig Sampling: 4 week Angiogenesis of the regenerated dermis	40
Skin	PLGA/CS/PVA Fiber diameter: 275 ± 175 nm	CS (1.65 10^5, 90% DD)	Electrospinning Membrane (Nanofiber)	Decreased tensile modulus and tensile strength, increased elongation	Cell attachment and proliferation / Fibroblasts	NA	41
Skin	Hexanoyl CS Fiber diameter: 0.4-3.2µm ; 83 ± 9µm	CS (88% DD)	Electrospinning Membrane (Microfiber)	NA	Decreased toxicity; increased viability, cell attachment and proliferation, characteristic cell morphology / Mouse fibroblasts, human keratinocytes, human foreskin fibroblasts	NA	42
Adipocyte	PDLLGA scaffold encapsulated with alginate/CS hydrogel capsule Capsule size:3-4.4 mm	CS (Not specified)	Electrospinning Membrane (Microfiber)	Robust for handling, implantation and graft preservation at the site, no sign of gross disintegration	Formation of lipid vacuoles in 30% of the cells and adipocyte layer, cell proliferation, differentiation / Human bone marrow stromal cells	Male MF-1 nu/nu immunodeficient mice (Applied with HBMSC cells) Sampling; 4 or 8 weeks Formation of fibre associated fat tissue, preservation of the adipocyte phenotype	43
Adipocyte	Collagen/CS	CS (Not specified)	Lyophilization Sponge	NA	Cell attachment, proliferation / Preadipocytes	Rat Biocompatible, vascularization	13
Adipocyte	Collagen/CS	CS (Not specified)	Lyophilization Sponge	Porosity: >90%	Cell proliferation / Adipose tissue derived stem cells	NA	44
Adipose	CS/Gelatin	CS(1.8 x 10^6 Da, 83.6% DD)	Drying by heat Film	NA	Differentiation to osteogenic and adipogenic linage cells / Adipose tissue derived stem cells	NA	45

CS: chitosan, DD: degree of deacetylation, DTBP: dimethyl 3-3, dithio bis propionimidate, GAG: glycosamino glycans, HBMSC: human bone marrow stromal cells, MW: molecular weight, NA: not applied, PDLLGA: poly(DL-lactide-co-glycolide), PVA: polyvinyl alcohol

macrophages (41); migration of fibrablasts to the wound area (41) and the synthesis of collagen lead to wound healing (36).

For the construction of artificial skin tissue, chitosan with different deacetylation degrees was investigated (6). No significant differences in the proliferation rate of the fibroblasts on these scaffolds were observed whereas mechanical properties were changed significantly. Higher tensile strength and elastic modulus was observed with higher degree of deacetylation and with higher crosslinking agent, dimethyl 3-3, dithio bis propionimidate (DTBP).

Chitosan based systems at micro and nano scales in combination with other polymers have been developed for skin tissue engineering, using electrospinning method and lyophilization (6,26,36-42).

In a study where PLGA, CS and PVA were used, scaffolds in nanofibrous membrane form were prepared (Figure 1). Increased fibroblast attachment and proliferation was observed with poly(lactide-co-glycolide)/chitosan/poly(vinyl alcohol) (PLGA/CS/PVA) nanofibers (275±175 nm). However mechanical properties were found to be lower than that of PLGA membranes and CS/PVA membranes (41). In a different study, hexanoyl chitosan microfibers, with a diameter of 0.4-3.2 µm at the beginning of the formulation preparation and 83±9 µm at the end, were prepared and investigated in three different cell cultures (mouse fibroblasts, human keratinocytes (HaCaT), human foreskin fibroblasts (HFF) in order to show the biocompatibility of the electrospun scaffold. Studies with fibroblasts showed that the material is
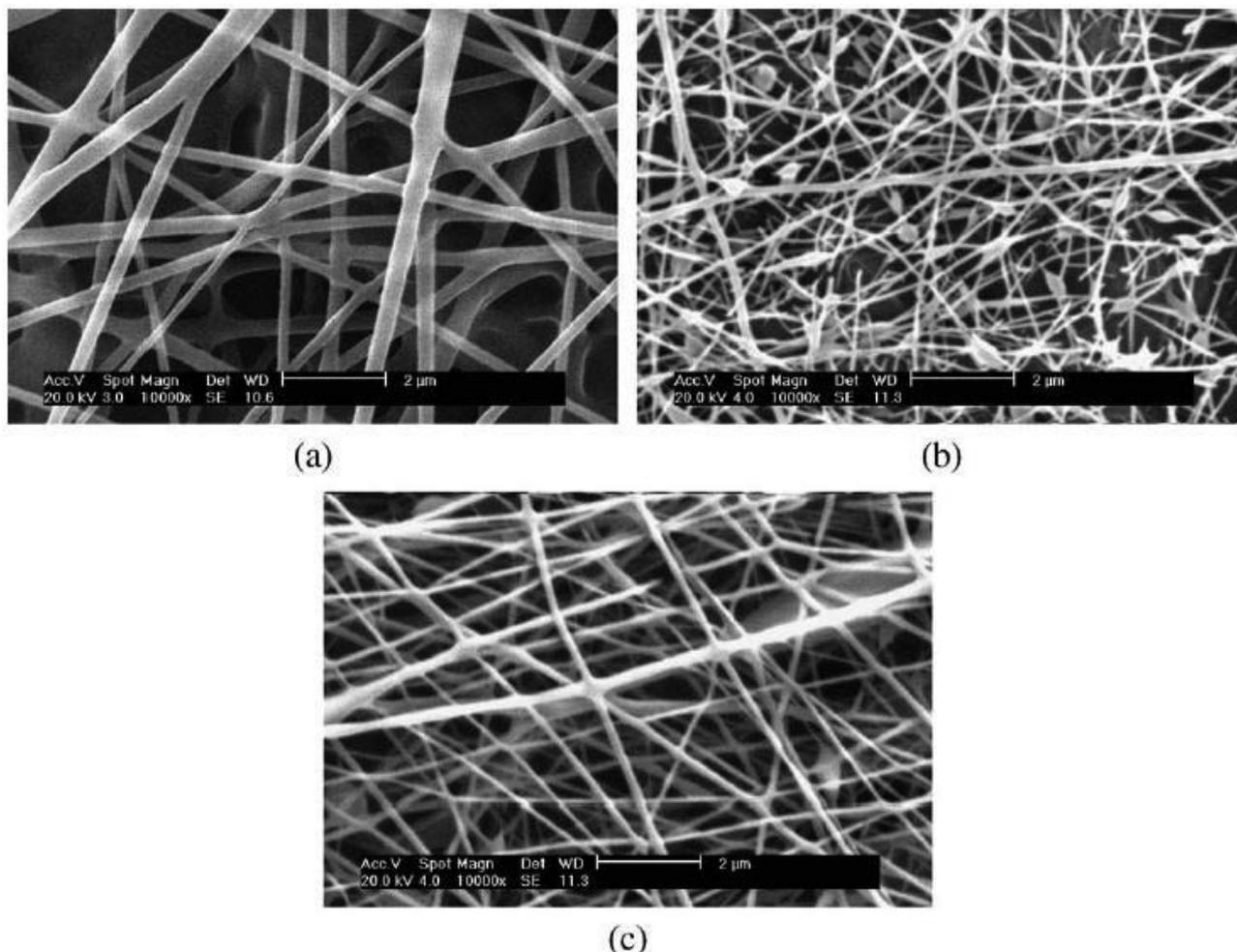

Figure 1. SEM micrographs of electrospun PLGA (a), CS/PVA (b) and PLGA–CS/PVA (c) fibers (41).

non toxic and studies with HaCaT and HFF showed that scaffolds could support the attachment and the proliferation (42).

For skin tissue engineering, pore size for scaffold is suggested to be within the range between 100 to 200 μm, with a porosity of 90% (39). But there are also studies showing that different pore sizes can also increase proliferation of the cells. Collagen–chitosan scaffolds with a 255 μm pore size were prepared using lyophilization method, and seeded by perfusion seeding system. The seeding efficiency and uniformity was shown to increase which resulted in increased cell proliferation (26). On the other hand, significant cell proliferation and differentiation was reported for the scaffolds with lower pore sizes as well. Chitosan/gelatin/hydroxyapatite scaffold with different pore sizes, at the top (65-80 μm) and bottom (10-20 μm), resulted in proliferation and differentiation of fibroblasts and keratinocytes (38). Similarly, lyophilized chitosan scaffold coated with collagen (40-100 μm pore size) was shown to have highly interconnected structure, which increased cell proliferation and wound healing without inflammation (37).

Adipose tissue
Adipose tissue is subcutaneous fat lying directly under the skin layers, and the loss of this tissue often results in a change in the "normal" tissue contour (47). Restoration of this soft tissue is targeted in order to minimize the anxiety, to cushion the skin against trauma, to store energy and to prevent negative psychological feelings associated with disfigurement. Although excess amounts of adipose tissue are found all over the human body, and can be readily obtained through liposuction and transplanted to a target location, the use of autologous fat tissue to repair soft tissue defects is only theoretically applicable and the success in patients of this method is still questionable (43,48). Scaffold designs for adipose tissue engineering, with consideration of surface topology and introduction of surface modifications, adequate pore sizing, and material choice are being investigated. Applications of chitosan in adipose tissue engineering are summarized in Table 1. However, the ideal environment for engineering

adipose tissue has yet to be deciphered.

The combination of PLGA and chitosan/alginate hydrogels to generate a hybrid scaffold has been described for adipogenic tissue engineering and it was suggested that adipocytes derived from the multi-potential population of adult are valuable cell sources for adipose tissue engineering (43). Besides the formation of adiposide layer, preservation of the adipocyte phenotype was also shown on these microfiber based membrane structures.

In another study, collagen and chitosan based sponges were fabricated. Cell attachment and proliferation were observed in preadipocyte cells (PA) and PA seeded scaffolds were subcutaneously applied to rats. This in vivo study showed that scaffolds were biocompatible; on this system, vascularization and adipose tissue formation were observed (13).

Corneal tissue
Cornea can be damaged by various diseases and injury that cause visual impairment and even blindness. The only treatment of irreversible corneal blindness is keratoplasty (49). However, the shortage of corneal donors is a big problem. Additionally, there is some uncertainty and insecurity associated with keratoplasty (50). Acute rejection rates range from 13.3% to 65% within 4 months of keratoplasty, and rejection can occur many years later (51). Therefore, looking for new sources of cornea, and seeking new corneal replacements became an important task. Tissue engineering of cornea is believed to be the right approach to meet this demand.

Unlike other tissues, cornea must be transparent beside the proper chemical and mechanical properties. Biocompatible and transparent chitosan based scaffolds investigated for corneal tissue engineering are summarized in Table 2.

It was reported that chitosan has protective effect against hydrolysis of hyaluronic acid (52), which also stimulates the growth of corneal epithelial cells (53). Moreover chitosan is structurally similar to the glycosaminoglycan, component of the cornea ECM and protects collogen from digestion by collagenase,

Table 2. Corneal tissue engineering studies on chitosan in the last 5 years

| Scaffold content / Pore Size | Chitosan type | Preparation method / Form | In vitro testing | | In vivo testing | Ref |
			Mechanical properties	Cell culture studies on scaffold		
Collagen/CS	CS (400 kDa)	Gelation Gel	Significantly enhanced mechanical strength	Allowed cell attachment, migration, proliferation of corneal epithelial cells; DRG nerve growth, neurite extension in the groups with higher cross-linkers ratio Human corneal epithelial cells (HCECEs), dorsal root ganglia (DRG) from chick embryos	Pig Sampling: 0,2,6,12 months No fibrosis or fibrotic capsule formation, minor immune response the groups with lower cross-linkers ratio Rat Sampling : 1,2,4 months Good transparency, normal epithelium regeneration, sub-epithelial nerve formation	9
Collagen/CS Pore size: NS	CS (5.0 x 10^5 Da -6.0 x 10^5 Da, 85-90% DD)	Drying by heat Membrane transparent with 0.5–0.9% sodium hyaluronate	Relatively strong but elastic, could be placed without torn	Allowed cell attachment, migration, proliferation, confluent monolayer formation in 9 days Rabbit limbal corneal epithelial cells, corneal endothelial cells, keratocytes	New Zealand male albino rabbits Sampling: first week daily then weekly up to 5 months; good biocompatibility, good absorption in cornea,	51
Hydroxypropyl CS/gelatin/ chondroitin sulfate	Hydroxy propyl CS (3.5x 10^4 Da, 75 % DD)	Solvent casting-particulate leaching Membrane	Relatively high elongation at break	Monolayer formation, similar cell adhesion and growth, significantly lower cytosolic enzyme release, more cytocompatible Rabbit corneal epithelial cells	Wistar Rats Sampling:15, 30, 60 days Mild inflammation, no obvious angiogenesis, no vascular growth, totally degradation on day 60	23

CS: chitosan, DD: degree of deacetylation, DRG: dorsal root ganglia, NS: Not specified

and also inhibits fibroblast-mediated contraction of collagen lattices (51,54). Chitosan is known to enhance the permeability of hydrophilic molecules due to the interaction between chitosan and corneal tight junctions (55).

Chitosan based scaffolds for cornea engineering can be in different forms such as membranes or gels. Chen et al. (51) developed collagen-chitosan composite membranes as scaffold for corneal tissue engineering applications with good biocompatibility, transparency, allowing cell attachment, proliferation and migration. Additionally, the prepared scaffold showed desired mechanical stability for in vivo applications.

In another study, an implantable collagen-chitosan scaffold system in gel form was prepared, which was optimally strong, elastic with optical clarity and slightly immunogenic engineered system due to the hybrid polymer networks prepared by either a simple

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/ N-hydroxysuccinimide (NHS) cross-linking system or a hybrid cross-linking system comprised of poly(ethylene glycol) dibutyraldehyde (PEG-DBA) and EDC/NHS. Normal branching nerves and keratocytes were observed with the hybrid cross-linking system in the deeper stroma of the corneas while EDC/NHS system implants showed abnormal vascularization in a twelve-month post-operative study with pigs (9).

Liver tissue

Loss of liver function leads to liver failure which causes over 25,000 deaths/year in United States (56). Despite the advances in medicine, liver transplantation is the only treatment for liver failure. The requirement of immunosuppressive medications, donor organ storage and high cost are the major limitations for liver transplantation (57). To overcome these problems liver tissue engineering aims to

create an artificial liver tissue for the replacement of the liver function in patients. Chitosan is an ideal scaffold material for hepatocyte culture due to its structure, similar to glycosaminoglycans, the components of liver ECM (58,59). Applications of chitosan of liver tissue are summarized in Table 3. Electrospinning and lyophilization are the frequently preferred fabrication methods for chitosan based liver tissue engineering investigations.

Since hepatocytes are anchorage-dependent cells

(63,64) and highly sensitive to the biochemical property of the ECM (64), it is extremely important to mimic their in vivo environment in vitro for their migration, proliferation and differentiation.

Jiankang et al. (58) showed that CS/gelatin scaffolds prepared by lyophilization in a poly-dimethylsilicone (PDMS) mold have porosity larger than 90%. Good biocompatibility, high cell attachment and proliferation, significantly high albumin secretion and urea synthesis were observed with the developed

Table 3. Liver tissue engineering studies on chitosan in the last 5 years

Scaffold content / Pore Size	Chitosan type	Preparation method / Form	In vitro testing		In vivo testing	Ref
			Mechanical properties	Cell culture studies on scaffold		
Galactosylated CS, Poly(ethylene oxide) Nanofiber Diameter: 160nm	Low MW CS (85% DD)	Electrospinling Nanofiber	Similar mechanical properties as an ECM for hepatocytes	Formation of stably immobilized 3D flat aggregates, superior bioactivity with higher levels of albumin secretion, urea synthesis and cytochrome P-450 enzyme Hepatocyte cells	NA	3
CS/gelatin Pore size: 100μm	CS (92% DD)	Lyophilization in a PDMS mold	Well organized structure Porosity: > 90%	Good biocompatibility, allowed cell attachment formation of large colonies in hepatic chambers, completely filled in 7 days, significantly high albumin secretion and urea synthesis Hepatocyte cells	NA	58
Titania/CS Pore size: 180 – 420 μm	CS (Not specified)	Lyophilization Sponge	Significantly increased compression modulus Porosity: > 89%	No significant difference in liver specific functions, long term capability of metabolic activity Cell attachment: 81.2% HL-7702 cells	NA	60
Alginate/ galactosylated CS Pore size: 150 – 200 μm	CS (10 K)	Lyophilization Sponge	Increased tensile strength and elongation at break	Expression of hepatocyte aggregation and cell to cell contact, 1.5 fold decrease in the albumin secretion with a GJIC inhibitor Enhanced increase in albumin secration rates, ammonia elimination rates and ethoxyresorufin-O-deethylase Cell attachment: 72.7% Hepatocyte cells, Coculture (Hepatocyte cells, NIH3T3 cells)	NA	61
Silk fibroin/ chitosan Pore size: 150 – 200 μm	Chitosan (5.3×10^4 Da, 80% DD)	Lyophilization Sponge	NA	Significantly higher cell viability and attachment, spherical shapes, ECM secretion Hepatocyte cells	Rat Sampling: 1,7,14,28 days Acute inflammation, faster degradation	62

CS: chitosan, DD: degree of deacetylation, ECM: extracellular matrix, MW: molecular weight, NA: not appliedq

scaffolds.

The asialoglycoprotein receptors (ASGP-R) are hepatic endocytic recycling receptors, one of the best-characterized systems for receptor-mediated endocytosis via the clathrin-coated pit pathway (65,66). They mediate the endocytosis and degradation of a wide variety of desialylated glycoproteins and neoglycoproteins with terminal galactose (Gal) or N-acetylgalactosamine (Gal- NAc) residues on their N-linked carbohydrate chains (67,68) in order to remove asialoglycoproteins. Fortunately, the ligand of these receptors does not need to be a protein and the interaction is not related with the structure or the properties of the molecule but only depends on the presence of appropriate oligosaccharides (68). In virtue of this property, ASGP-R can interact with galactosylated chitosan (GC) (69) and since ASGP-Rs are specific to hepatocytes, GC is used for hepatocyte-targeting systems.

The relation between the concentration of galactose ligands and hepatocyte interaction has been reported in several studies (3,61,70). Hepatocyte aggregates cultured on GC films have rough and bumpy surface similar to the hepatocyte aggregates formed on GC nanofibers but on nanofibers size distribution is inhomogeneous (Figure 2) (2). It was reported that hepatocytes with high galactose concentration formed larger size and larger number of aggregates,
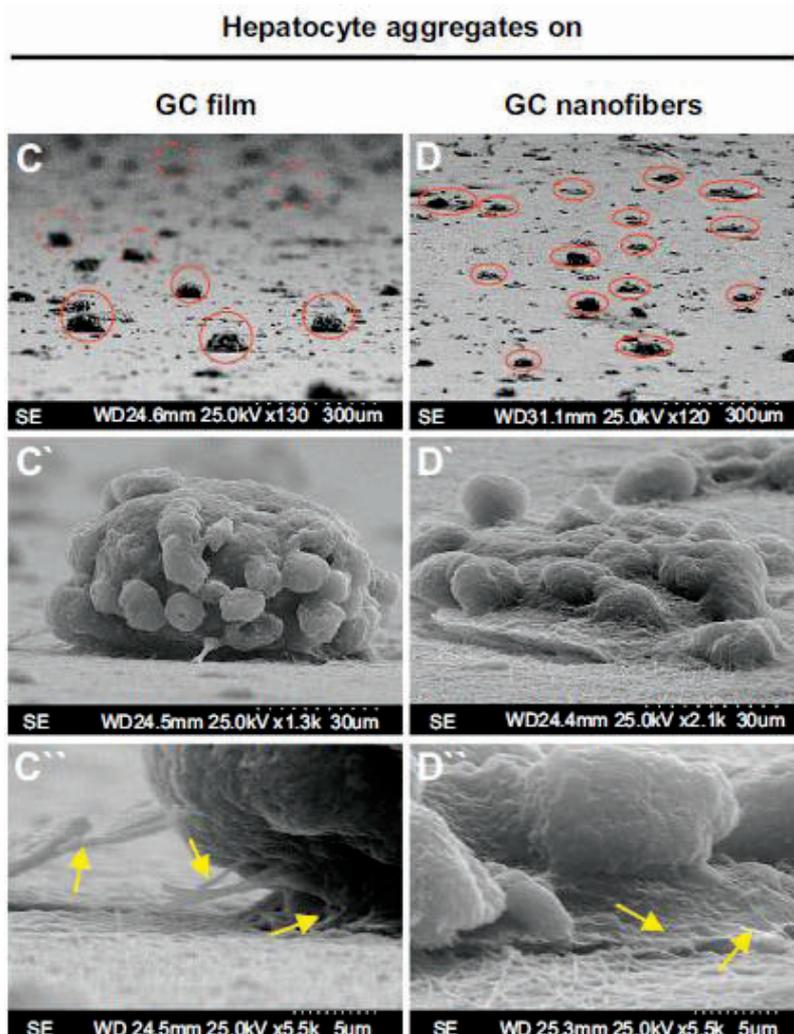

Figure 2. SEM images of hepatocyte aggregates (C–C'',D–D'') after 7-day culture: tightly attached spheroid aggregates of hepatocytes on GC films (C–C''); and perfectly integrated the flat aggregates with GC nanofibers (D–D'') were observed. (Red circles: hepatocyte aggregates; Yellow arrowheads: hepatocyte pseudopods) (3).

which implies higher liver specific function than those at a low galactose concentration. Furthermore, the larger aggregates could maintain higher liver-specific functions for only a short-term culture period. On the other hand, in another study it was suggested that the bigger hepatocyte aggregates the easier to lose their bioactivity in a long-term culture due to the difficulty to provide nutrients to the internal part of the aggregates (3,71). If the nutrients can not be provided to the internal hepatocytes of large aggregates, they will die in a long-term culture period. Besides, large aggregates of hepatocytes have mechanical stability problems against shear stress from culture medium flow (3). Apart from hepatocyte aggregate formation, it was also shown that the ASGP-R and GC ligand interaction leads to increased cell adhesion (61). The study was performed on alginate/galactosylated chitosan (AL/GC) and alginate/chitosan (AL/CS) thin films. Higher cell attachment was observed on AL/GC thin films and optimal GC concentration was determined as 1% (w) since the galactosylation concentration more than 1 % (w) did not change albumin secretion significantly.

Nerve tissue

Nerve defects may lead to movement disorders of the related muscles, bringing physical and psychological problems to the patients. Mature neurons have little capacity for replication and if the nervous system is impaired, it can hardly heal itself (72). Since conventional treatment systems have limitations, nerve tissue engineering application is required. The reader is referred to the articles, which are extensively explained nerve tissue treatment systems and their limitations (59,73,74).

It has been shown that chitosan is a potential candidate material for nerve tissue engineering due to good nerve affinity of the polymer (72), and the affinity was shown for different cell types like neutral stem cells (75), PC-12 cells (76), neuro-2a neuroblastoma cells (77) or N1E-115 cells, derived from mouse neuroblastoma C-1300 (7).

Applications of chitosan based scaffolds in nerve tissue engineering are summarized in Table 4. Wang et al. (75) reported molded chitosan conduit

Table 4. Nerve Tissue engineering studies on Chitosan in the last 5 years

Scaffold content / Pore Size	Chitosan type	Preparation method / Form	In vitro testing		Ref
			Mechanical properties	Cell culture studies on scaffold	
CS/Gelatin, CS tube Pore size: 50-150 μm	CS (1.8 x 10^6 Da, 83.7% DD)	Mantled chitosan tube with chitosan/ gelatin solution Sandwich tabular	Durable pressure:4300-3700 mmHg, native vessel durable pressure:2830 mmHg significantly higher suture-retention strength Porosity: 81.2 %	Improved cell attachment, proliferation, increased cytocompatibility Vascular smooth muscle cells	8
PU, CS coating	CS (Not specified)	Drying by heat then immersing in CS solution Membrane	-	Greatly improved cell attachment and proliferation, acceleration of endothelium regeneration HUVECs	83
Poly(ε-caprolactone), CS coating Pore size: >40 μm	CS (30 kDa, 65% DD)	Particle leaching then lyophilization Sponge	-	Significantly improved cell attachment and proliferation Fibroblasts	84

CS: chitosan, DD: degree of deacetylation, HUVECs: human umbilical vein endothelial cells, PU: polyurethane,

formulation which was applicable for spinal cord injuries or short peripheral nerve defects, and braided CS conduit formulation was be applicable for peripheral nerve regeneration.

Lyophilized chitooligosaccharide has been shown to support cell attachment and proliferation, promote PC-12 cells differentiation to a neuron-like morphology and neurite outgrowth (78). Similarly, with freeze dried chitosan g-glycidoxypropyltrimethoxysilane system, a significant improvement of posttraumatic axagonal regrowth and functional recovery was observed with N1E-115 cells (7).

Feasibility of chitosan tubes for nerve tissue engineering was investigated on two different formulations prepared by braiding of chitosan yams with a textile technique and freeze drying of molded chitosan tubes (75). Mechanical analysis results showed that the suture retention strength of the braided conduit (2.18 ± 0.41 N) was higher than that of fresh nerves and also molded conduits (2.18 ± 0.41) as shown in Figure 3. Both of these scaffolds were shown to have no cytotoxic effect on fibroblasts (L929 cells) or neuroblastoma (Neuro-2a) cells. In another study, 1 - 3% (w/v) chitosan solution was injected into the molded chitosan tube before closing the two ends of the tube and covering it with a thermal insulator, followed by freeze drying. Increased mechanical stability and neuroblastoma cell affinity was observed (77).
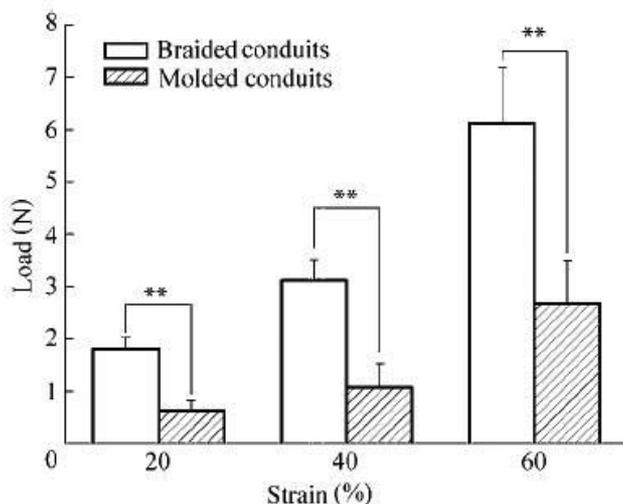

Figure 3. Load-strain relationship during compressive loading (**p<0.01, n=6 in each group) (75).

Apart from sponges and tubes, membranes can also be applicable for nerve tissue engineering. It has been reported that chitosan coated with polylysine (CAP), and a chitosan-polylysine mixture (CPL) membranes promoted nerve cells (gliosarcoma cells and normal cerebral cells) to grow and function normally (72).

Blood vessel tissue

Vascular transplantation is a frequently used method for the treatment of vascular diseases. Limited donor sites and the immune response to allograft and xenograft are limitations of vascular transplantations and blood vessel engineering is regarded as a solution to this problem despite the fact that their degradation products elicit inflammation and immune response, and these units have relatively fast degradation rate (81).

Frequently used biomaterials for blood vessel scaffolds are polyethylene terephthalate (PET) and expanded polytetrafluoroethelene (ePTFE). However these materials perform well only at diameters > 6 mm and these artificial materials are lack of the ability to grow, repair, or remodel. Moreover there is no suitable biomaterial for smaller diameters < 4 mm (82).

Due to the limitations of PET and ePTFET, new approaches are necessary to overcome the problems of long term treatments. Therefore chitosan has been investigated for blood vessel tissue engineering which is summarized in Table 5.

Due to the stress that the structure challenges in vivo, the mechanical properties of blood vessel tissue scaffolds are very important (83). Zhang et al (8) achieved the construction of chitosan-gelatin artificial blood vessel with 50-150 μm pore size as a sandwich tabular structure. It was reported that the artificial structure had higher durable pressure than the natural tissue.

In another study, to provide the desired mechanical properties, polyurethane (PU) scaffold was prepared and surface modification was occurred with chitosan in order to increase cell material interaction (83). It was shown that on the modified scaffold a monolayer of endothelial intima was formed.

Table 5. Blood vessel tissue engineering studies on chitosan in the last 5 years

Scaffold content / Pore Size	Chitosan type	Preparation method / Form	In vitro testing		Ref
			Mechanical properties	Cell culture studies on scaffold	
CS/Gelatin, CS tube Pore size: 50-150 µm	CS (1.8 x 10⁶ Da, 83.7% DD)	Mantled chitosan tube with chitosan/ gelatin solution Sandwich tabular	Durable pressure:4300-3700 mmHg, native vessel durable pressure:2830 mmHg significantly higher suture-retention strength Porosity: 81.2 %	Improved cell attachment, proliferation, increased cytocompatibility Vascular smooth muscle cells	8
PU, CS coating	CS (Not specified)	Drying by heat then immersing in CS solution Membrane	-	Greatly improved cell attachment and proliferation, acceleration of endothelium regeneration HUVECs	83
Poly(ε-caprolactone), CS coating Pore size: >40 µm	CS (30 kDa, 65% DD)	Particle leaching then lyophilization Sponge	-	Significantly improved cell attachment and proliferation Fibroblasts	84

CS: chitosan, DD: degree of deacetylation, HUVECs: human umbilical vein endothelial cells, PU: polyurethane,

Glycosaminoglycans(GAGs)-chitosan membranes were also studied, however these scaffolds were found to inhibit spreading and proliferation of vascular endothelial and smooth muscle cells Heparin-chitosan scaffolds were also investigated in vivo and it was concluded that stimulated cell proliferation and highly vascularized dense granulation tissue was surveyed (84).

Chitosan was also used for surface modification of poly(ε-caprolactone) scaffold and in vitro studies with fibroblast significantly improved cell attachment and proliferation (85).

CONCLUSION

Tissue engineering is a multidisciplinary field aiming to replace damaged or defective tissues and organs, and hereby improve the life quality of millions of patients who can not be treated by conventional treatments. Chitosan seems to be a potential scaffold material for the soft tissues such as skin, adipose, cornea, liver, nerve and blood vessel. It is possible to prepare scaffolds in various forms which is biocompatible, non-toxic, mechanically stable, using different types of chitosan with different molecular weight and deacetylation degree. Combination with other polymers is also possible to enhance the properties of the systems. it is obvious that chitosan is a promising candidate as a supporting material for soft tissue engineering applications owing to its porous structure, gel forming properties, ease of chemical modification, and high affinity to in vivo macromolecules.

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