Carbohydrate Polymers 76 (2009) 167–182

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Review

Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone

Riccardo A.A. Muzzarelli *

Institute of Biochemistry, University of Ancona, Via Ranieri 67, IT-60100 Ancona, Italy

ARTICLE INFO

Article history:
Received 14 October 2008
Received in revised form 4 November 2008
Accepted 5 November 2008
Available online 13 November 2008

Keywords:
Wound healing
Hemostasis
Neoangiogenesis
Metalloproteinase
Macrophage
Skin
Nerve
Cartilage
Bone
Chitin
Chitosan

ABSTRACT

This review provides a balanced integration of the most recent chemical, biochemical and medical information on the unique characteristics of chitins and chitosans in the area of animal/human tissue regeneration. Hemostasis is immediately obtained after application of most of the commercial chitin-based dressings to traumatic and surgical wounds: platelets are activated by chitin with redundant effects and superior performances compared with known hemostatic materials. To promote angiogenesis, necessary to support physiologically ordered tissue formation, the production of the vascular endothelial growth factor is strongly up-regulated in wound healing when macrophages are activated by chitin/chitosan. The inhibition of activation and expression of matrix metalloproteinases in primary human dermal fibroblasts by low MW chitosans prevents or solves problems caused by metalloproteinase-2 such as the hydrolysis of the basement membrane collagen IV. Experimental biocompatible wound dressings derived from chitin are today available in the form of hydrogels, xerogels, powders, composites, films and scaffolds: the latter are easily colonized by human cells in view of the restoration of tissue defects, with the advantage of avoiding retractive scar formation. The growth of nerve tissue has been guided with chitin tubes covalently coated with oligopeptides derived from laminin. The regeneration of cartilage is also feasible because chitosan maintains the correct morphology of chondrocytes and preserves their capacity to synthesize cell-specific extracellular matrix: chitosan scaffolds incorporating growth factors and morphogenetic proteins have been developed. Impressive advances have been made with osteogenic chitosan composites in treating bone defects, particularly with osteoblasts from mesenchymal stem cells in porous hydroxyapatite-chitin matrices. The introduction of azido functions in chitosan has provided photo-sensitive hydrogels that crosslink in a matter of seconds, thus paving the way to cytocompatible hydrogels for surgical use as coatings, scaffolds, drug carriers and implants capable to deliver cells and growth factors. The peculiar biochemical properties of chitins and chitosans remain unmatched by other polysaccharides.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

The wound healing progression involves the orchestration of complex interactions among cells, extracellular matrix components and signaling compounds (Clark, 1996; Ferguson & O'Kane, 2004; Miller & Nanchahal, 2005; Roberts & Sporn, 1996; Turner, Schmidt, & Harding, 1986; Werner & Grose, 2003). After hemostasis and clot formation, the healing process can be divided into three overlapping phases: inflammation, proliferation and scar maturation. Inflammation sets in within minutes of a skin injury; the first inflammatory responders are leukocytes, namely neutrophils that transmigrate across endothelia from local blood vessels, and monocytes that migrate from blood into tissues and differentiate into macrophages. Being part of the first line of defense, the latter initiate inflammatory responses by secreting cytokines, and they recruit further immune cells to the site of infection. Cytokines are small soluble compounds, mainly peptides, that act as messengers essential in integrating and coordinating the immune responses: they include tumor necrosis factor alpha (TNF-α), interferon-gamma (INF-γ), interleukins and others. The proliferation phase proceeds over the next 5–14 days and involves the initial repair processes for both the epidermal and dermal layers. Fibroblasts, macrophages and vascular tissues coordinately enter the wound to begin formation of a new dermal composite, the granulation tissue. Fibroblasts and myofibroblasts lay down collagen-rich connective tissues comprising this composite and myofibroblasts also contribute to wound contraction. Simultaneously, in a process termed re-epithelialization, keratinocytes at the wound edge migrate over the granulation tissue to differentiate the new outer layer of epidermis.

The healed wound finally enters the maturation phase, and granulation tissue continues to be remodeled by its constituent cells. Synthesis of structural proteins, such as collagen, remains elevated for 6–12 months, although the scar reaches, at best, 70% of the tensile strength of intact skin. Hypertrophic or keloid scars

* Tel.: +39 071 36206.
E-mail addresses: Muzzarelli@univpm.it, Riccardoposta@excite.it

0144-8617/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2008.11.002

are associated with excess of granulation. The appearance of a scar is influenced by several cell- and tissue-specific factors: for example, the local changes of repaired skin coloration result from the disruption of collagen organization (Rhett et al., 2008).

Investigations into the ability of embryonic wounds to heal without apparent scars have been influential in the development of interest in hyaluronan: there are examples based on the manipulation of hyaluronan and fibromodulin, extracellular matrix components present at elevated levels in embryonic wounds. Thanks to the high concentration of hyaluronan, the wounds in the fetus heal with the correct tissue reconstitution (West, Shaw, Lorenz, Adzick, & Longaneker, 1997), where the scar imprint typical of the adult tissue is absent.

Recent evidence points to the DG42 protein, a chitooligomer synthase, that during embryogenesis, produces chitooligomers acting as primers in the synthesis of hyaluronan (Bakkers et al., 1997). Most preparations of hyaluronan have chitooligomers at their reducing end, that act as templates for hyaluronan synthesis (Varki, 1996). The propensity of embryos for scar-less healing correlates inversely with the maturation of the cellular immune response during development. Cyclooxygenase-2 (COX-2) is one of the enzymes responsible for production of prostaglandin, a well characterized mediator of the inflammatory response. COX-2 inhibition might decrease scar collagen deposition after cutaneous injury (Willoughby & Tomlinson, 1999).

Transforming growth factors-β are secreted by platelets, fibroblasts and macrophages within the injury and are thought to act in various capacities as attractants or inhibitors of keratinocyte, fibroblast and inflammatory cell migration, in up-regulation of collagen synthesis and modulation of matrix turnover via effects on matrix metalloproteinases (MMPs) and their inhibitors (Bottomley, Bradshaw, & Nixon, 1999; Witte, 1998). Additionally, TGF-β1 induces differentiation of myofibroblasts, a cell type critical to wound contraction and marked by active synthesis of granulation tissue constituents, including collagen and fibronectin.

During the last few years, chitins and chitosans have become protagonists in the scenario concisely recalled above, thanks to their outstanding properties summarized in Table 1. Basic information on these polysaccharides, relevant to this topic, can be found in books and review articles (Chen & Chen, 1998; Chopra et al., 2006; Dahiya, Tewari, & Hoondal, 2006; Degim, 2008; Jiang 2001; Jollès & Muzzarelli, 1999; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Kurita, 2006; Mourya & Inamdar, 2008; Muzzarelli, 1977, 2008a, 2008b, 2008c; Muzzarelli & Muzzarelli, 2006; Rinaudo, 2006a, 2006b; Ruel-Gariepy & Leroux, 2006; Sogias, Williams, & Khutoryanskiy, 2008; Terbojevich & Muzzarelli, 2000; Varlamov, Bykova, Vikhoreva, Lopatin, & Nemtsev, 2003; Varma, Deshpande, & Kennedy, 2004; Yamada & Kawasaki, 2005; Yuan, Chestnutt, Haggard, Bumgardner, & Muzzarelli, 2008a).

Table 1
Characteristic properties of chitosan in regenerative medicine.

1 = Wound healing
Chemoattraction and activation of macrophages and neutrophils to initiate the healing process; promotion of granulation tissue and re-epithelization; entrapment of growth factors to accelerate the healing; limitation of scar formation and retraction; stimulation of integrin-mediated cell motility and increased *in vitro* angiogenesis; integrin-dependent regulation of the pro-angiogenic transcription factor Ets1; release of glucosamine and *N*-acetylglucosamine monomers and oligomers, and stimulation of cellular activities; intrinsic antimicrobial activity and controlled release of exogenous antimicrobial agents to prevent infection
2 = Tissue engineering
Non-toxic and rapidly biodegradable; easy to develop in various forms; chemically and enzymatically modifiable; mucoadhesive; suitable for controlled release of cytokines, extracellular matrix components and antibiotics and for retention of the normal cell morphology, promotion of the attachment, proliferation and viability of tissue cells including stem cells

2. Chitins and chitosans in wound healing and scar differentiation

In the context of veterinary medicine, chitosan was found to enhance the functions of polymorphonuclear leukocytes (PMN) (phagocytosis, and production of osteopontin and leukotriene B4), macrophages (phagocytosis, and production of interleukin-1, transforming growth factor β1 and platelet-derived growth factor), and fibroblasts (production of interleukin-8). As a result, chitosan promotes granulation and organization, and therefore it is beneficial for open wounds; certain PMN functions are enhanced, such as phagocytosis and the production of chemical mediators (Ueno, Mori, & Fujinaga, 2001a).

Mouse fibroblasts L929 were cultured with chitosan and the production of extracellular matrix was evaluated *in vitro*. Type I and III collagens and fibronectin were secreted by L929 with or without chitosan; however, there was no significant difference in the amount of extracellular matrix between the control and the chitosan groups. Secondly, macrophages were stimulated with chitosan, and then TGF-β1 and platelet-derived growth factor (PDGF), messenger ribonucleic acid (mRNA) expressions and production of their proteins were assayed *in vitro*. The result that chitosan promoted the production of TGF-β1 and PDGF indicates that chitosan does not directly accelerate the extracellular matrix production by fibroblasts, but, rather, by the growth factors (Ueno et al., 2001b).

A peculiarity of chitosan is the ability to foster adequate granulation tissue formation accompanied by angiogenesis and regular deposition of thin collagen fibers, a property that further enhances correct repair of dermo-epidermal lesions (Shi et al., 2006). In fact, the main biochemical effects of chitins and chitosans are fibroblast activation, cytokine production, giant cell migration and stimulation of type IV collagen synthesis.

Chitin too has quite a relevant biochemical significance, in particular it accelerates macrophage migration and fibroblast proliferation, and promotes granulation and vascularization. While some chitin and chitosan derivatives also have biochemical significance, some other are rather inert, as it is the case for dibutyryl chitin; in general, however, they are biocompatible. The high biocompatibility of dibutyryl chitin in the form of films and non-wovens has been demonstrated for human, chick and mouse fibroblasts by various methods: this water-insoluble modified chitin was also tested in full-thickness wounds in rats with good results (Muzzarelli et al., 2005). Traumatic wounds in a large number of patients were treated with chitosan glycolate dressings; in all cases they healed with satisfactory results (Muzzarelli et al., 2007). Biochemical efficacy of chitin on matrix metalloproteinases has been documented.

3. Inhibition of matrix metalloproteinases by chitosans

The effect of chitin, chitosan and their derivatives on matrix metalloproteinases has been the object of a limited number of scientific articles so far. These enzymes are a family of secreted or transmembrane endopeptidases that share structural domains and degrade extracellular matrix components. They are classified into five major groups, in part based on substrate specificity: the most important groups are the interstitial collagenases MMP1, 8 and 13, that recognize collagen types I, II and III; the stromelysins MMP3, 10 and 11, with specificity for laminin, fibronectin and proteoglycans; and the gelatinases MMP2 and 9 that cleave collagen types IV and V. Regulation of gene expression of most MMPs is controlled by two major transcription factors. Under normal physio-

logical conditions, MMP transcripts are expressed at low concentrations, that rise promptly, however, when tissues locally undergo remodeling events such as inflammation, wound healing, cancer and arthritis. Therefore, inhibition of MMP is a primary therapeutic target: several drugs have been developed and produced for the treatment of said diseases that involve excessive extracellular matrix degradation (Bottomley et al., 1999).

Partially hydrolysed chitosans are often preferred in pharmaceutical and medicinal applications, thanks to their high solubility and absence of toxicity. They inhibit activation and expression of MMP2 in primary human dermal fibroblasts, the highest inhibitory effect being exerted by hydrolysed chitosans with molecular weights as low as 3–5 kDa. The inhibition is caused by the decrease of the gene expression and transcriptional activity of MMP2. Therefore said chitosans may prevent and treat several health problems mediated by MMP2 (that can hydrolyse the basement membrane collagen IV) such as wound healing and wrinkle formation. It was speculated that the inhibitory effect might be explained by the effective chelating capacity of chitosan for Zn^{2+} that would become unable to exert correctly its cofactor role in MMP2 (Kim & Kim, 2006; Muzzarelli & Sipos, 1971).

Similarly, chitosan (ca. 500 kDa, degree of acetylation 0.30) decreases the invasiveness of human melanoma cells, via specific reduction of MMP2 activity. While the expression level of MMP2 was not affected, the amount of MMP2 in the cell supernatant was reduced, indicating a post-transcriptional effect of chitosan on MMP2. Atomic force microscopy revealed a direct molecular interaction between MMP2 and chitosan forming a complex with a diameter of 349.0 ± 69.06 nm and a height of 26.5 ± 11.50 nm. Affinity chromatography revealed a high binding-specificity of MMP2 to chitosan, and a colorimetric assay suggested a non-competitive inhibition of MMP2 by chitosan (Gorzelanny, Poppelmann, Strozyk, Moerschbacher, & Schneider, 2007). It should be mentioned however, that Okamura, Nomura, Minami, and Okamoto (2005) and Nakade et al. (2000) reported that squid chitin and chitosan powders or sponges stimulate human fibroblasts to release MMPs by physical stimuli, in the same way as latex beads.

Research was carried out on human fibrosarcoma cells HT1080 with glucosamine sulfate ester, *N*-succinyl glucosamine and partially hydrolysed chitosans: all of these compounds were found to inhibit MMP9 that increases in the majority of malignant tumors and plays a major role in the establishment of metastases. The partially hydrolysed chitosans were found to be potent inhibitors of gene and protein expression of MMP9 (Mendis, Kim, Rajapakse, & Kim, 2006; Rajapakse, Mendis, Kim, & Kim, 2007; VanTa, Kim, & Kim, 2006).

Treatment of knee osteoarthritis with glucosamine sulfate for 12 months and up to 3 years may prevent total joint replacement in an average follow-up of 5 years after drug discontinuation (Bruyere et al., 2008; Chu et al., 2006; Muzzarelli & Muzzarelli, 2006). Clinical trials that have demonstrated a good efficacy of glucosamine are those that tested glucosamine sulfate, and are limited almost exclusively to those carried out with glucosamine sulfate produced by Rottapharm (Felson, 2008).

Interleukin-1, a cytokine released by synovial cells and invading macrophages in inflamed joints, induces MMPs and chondrocyte apoptosis that is important in pathogenesis of osteoarthritis, while depressing extracellular matrix synthesis. Primary rabbit chondrocytes were cultured and induced to apoptosis by 10 ng/ml interleukin-1 After treatment with various concentrations of carboxymethyl chitosan (50, 100, 200 μg/ml), the following parameters were measured: apoptotic rate, mitochondrial function, nitric oxide production, levels of inducible nitric oxide synthase mRNA and reactive oxygen species. The results showed that carboxymethyl chitosan could inhibit chondrocyte apoptosis in a dose-dependent manner. Furthermore, it could partly restore the levels of mitochondrial membrane potential and ATP, decrease nitric oxide production by down-regulating nitric oxide synthase mRNA expression, and scavenge reactive oxygen species in chondrocytes. The inhibitory effects of carboxymethyl chitosan on IL-1β-induced chondrocyte apoptosis were possibly due to protection of the mitochondrial function, and decline of nitric oxide and reactive oxygen species (Chen, Liu, Du, Peng, & Sun, 2006).

The signal transduction pathway involved in the glucosamine influence on the gene expression of matrix metalloproteinases was investigated in chondrocytes stimulated with IL-1β. Glucosamine inhibited the expression and the synthesis of MMP3 induced by IL-1β, and that inhibition was mediated at the level of transcription. Inhibition of the p38 pathway in the presence of glucosamine substantially explains the chondroprotective effect of glucosamine on chondrocytes that regulate COX-2 expression, PGE(2) synthesis and NO expression and synthesis (Lin et al., 2008).

4. Hemostasis and angiogenesis

Chitosan, preferably with deacetylation degree ca. 0.7, is degraded by enzymes such as lysozyme, *N*-acetyl-D-glucosaminidase and lipases. It is not excluded that NO also plays a role in a chemoenzymatic degradation process. Histological findings on wounded skin dressed with chitosan, indicated that collagen fibers were fine in the wounds and more mature than in the control (7 days post-operation): their arrangement was similar to that in normal skin. The tensile strength was clearly superior compared to controls. At 7 days, the wounds were completely re-epithelialized, granulation tissues were almost replaced with fibrosis and hair follicles were almost healed. Evidence has been collected that small but significant portions of chitin-based dressings are depolymerized, and that oligomers are further hydrolysed to *N*-acetylglucosamine, a common aminosugar in the body, which enters the innate metabolic pathway to be incorporated into glycoproteins.

Chitooligomers act as building blocks for hyaluronan synthesis. Hyaluronan has been shown to promote cell motility, adhesion and proliferation and to have important roles in morphogenesis, inflammation and wound repair. The *in vitro* biocompatibility of wound dressings in regards to fibroblasts has been assessed and compared with three commercial wound dressing made of collagen, alginate and gelatin; methylpyrrolidinone chitosan and collagen were found to be the most compatible materials. The use of wheat germ agglutinin (WGA), a lectin, for modifying chitosan and enhancing the cell–biomaterial interaction was examined by Wang, Kao, and Hsieh (2003).

The percentage of living fibroblast cells on the surfaces of tissue culture polystyrene control, WGA-modified chitosan, and plain chitosan films increased to 99%, 99% and 85%, respectively, after seeding for 48 h. DNA staining revealed that a portion of fibroblasts cultivated on chitosan films were undergoing apoptosis. In contrast, fibroblasts growing on WGA-modified chitosan film surfaces did not show any indication of apoptosis. The number of fibroblast cells was the highest on the WGA-modified chitosan surfaces, followed by the polystyrene and unmodified chitosan surfaces. Thus, WGA and other lectins enhance the cell–biomaterial interaction via oligosaccharide-mediated cell adhesion and improve cell adhesion and proliferation, the two key issues in tissue engineering (Wang et al., 2003).

Chitosan has been associated with other biopolymers and with synthetic polymers: wound dressings composed of a spongy sheet of chitosan and collagen, laminated with a polyurethane membrane impregnated with gentamycin sulfate, have been produced and clinically tested with good results.

Fischer, Bode, Demcheva, and Vournakis (2007) investigated the relationship between conformation of chitins and activation of

hemostasis, including Syvek-Patch® whose chitin fibers are organized in a parallel tertiary structure that can be chemically modified to an antiparallel one; and hydrogels consisting of either partially or fully deacetylated daughter chitosans. Several studies were performed with said chitosans, including (1) an analysis of the ability of chitosans to activate platelets and turnover of the intrinsic coagulation cascade, (2) an examination of the viscoelastic properties of mixtures of platelet-rich plasma and chitosans via thrombo-elastography and (3) scanning electron microscopy to examine the morphology of the chitosans. The hemostatic responses to the chitosans were highly dependent on their chemical nature and tertiary/quaternary structure, while the microalgal chitin fibers were found to have superior hemostatic activity compared to the other chitosans.

Of course the hemostatic activity is important in the early treatment of an injury, but other aspects are even more important for the restoration of the lost tissues, and for the formation of physiologically and biochemically regular tissues. Angiogenesis is a hallmark of cutaneous wound healing and is necessary to support new tissue formation. The production of the vascular endothelial growth factor (VEGF) is strongly up-regulated in wound healing, because it is secreted by activated macrophages and keratinocytes promoting new capillary formation within the wound bed. Impairment of new vessel formation results in low-quality wound healing due to poor blood circulation (Galiano et al., 2004; Hong et al., 2004; Tonnesen, Feng, & Clark, 2000), thus efforts have been made to increase vascularization for tissue regeneration and repair of chronic, non-healing ischemic wounds. In particular, the Syvek-Patch® was used clinically as a hemostatic agent (Palmer, Gantt, Lawrence, Rajab, & Dehmer, 2004). Recent data show that chitin microfiber-treated platelets are fully activated and the consequence of this activation is a marked increase of the formation of a fibrin matrix (Thatte, Zagarins, Khuri, & Fischer, 2004). Of importance, platelet activation by chitin fibers is mediated by their association with integrin β3 and activation of integrin-mediated signaling. Indeed, chitin fibers have been shown to bind integrins specifically in pull-down assays (Fischer et al., 2005).

Electrophoretic and Western blot analysis of red blood cell surface proteins demonstrated that chitin microfibers were bound to band three of the red blood cells. An important and unique result of the interaction of red blood cells with chitin fibers was the activation of the intrinsic coagulation cascade associated with the presentation of phosphatidylserine on the outer layer of the surface membrane of nanofiber bound red blood cells. The results demonstrate that red blood cells play a direct and important role in achieving surface hemostasis by accelerating the generation of thrombin (Fischer et al., 2008).

The treatment of cutaneous wounds with chitin derived membranes results in faster kinetics of wound healing attributed, in part, to a marked increase of angiogenesis (Pietramaggiori et al., 2008). In the absence of growth factor or serum, the chitin treatment induces endothelial cell motility and increases *in vitro* angiogenesis as measured by cord formation in Matrigel assays. Chitin-induced cell motility is found to be mediated by integrins and results in mitogen-activated protein kinase (MAPK), with increased expression of the pro-angiogenic transcription factor Ets1, the vascular endothelial growth factor VEGF, and the interleukin-1, IL-1. The effect of chitin is not a consequence of its induction of VEGF: the blockade of VEGF receptor did not block the induction of Ets1. Importantly, Ets1 is required for chitin-induced cell motility. Experimental findings by Vournakis, Eldridge, Demcheva, and Muise-Helmericks (2008) further support a role for Ets1 in the induction of cell motility by chitin: in fact a role for Ets1 in the transcriptional regulation of a number of integrin subunits has been indicated (Lu, Heuchel, Barczyk, Zhang, & Gullberg, 2006; Oda, Abe, & Sato, 1999; Rosen, Barks, Iademarco, Fisher, & Dean, 1994; Tajima, Miyamoto, Kadowaki, & Hayashi, 2000).

Pietramaggiori et al. (2008) demonstrated that treatment of full-thickness cutaneous wounds in a diabetic mouse model with chitin-containing membranes results in an increased wound closure rate correlated with impressive rise of angiogenesis. Serum-starved endothelial cells were treated with VEGF or with different concentrations of chitin: as compared with the total number of cells plated (control), at 48 h after serum starvation, there was a twofold reduction of the number of cells, but this reduction was compensated upon addition of VEGF or chitin at either 5 or 10 μg/ml. These results indicate that like VEGF, chitin treatment prevents cell death induced by serum deprivation. However, chitin does not result in a higher metabolic rate (by MTT assays), suggesting that this polymeric material is not causing marked increases in cellular proliferation but is rescuing cells from dying by serum deprivation.

The fact that chitosan can stimulate wound healing and increase angiogenesis, at least in part by integrin engagement and by enhancing the expression of cytokines and growth factors, suggests its potential uses not only in a clinical context but also as a tool to distinguish the molecular mechanisms regarding cell–cell and cell–matrix interactions in the course of wound healing.

5. Chitosans and macrophages

Chitin- and chitosan-based biomaterials are endowed with biochemical significance not encountered in cellulose, starch and other polysaccharides: they can be considered primers on which the normal tissue architecture is organized. Key factors in the rebuilding of physiologically valid tissues exerted by chitosans are an enhanced vascularization and a continuous supply of chitooligomers to the wound that stimulate correct deposition, assembly and orientation of collagen fibrils, and are incorporated into the extracellular matrix components.

It is known that chitosan activates macrophages for tumoricidal activity and for the production of interleukin-1. Oligochitosan had an *in vitro* stimulatory effect on the release of tumor necrosis factor-α and interleukin-1β in macrophages. Moreover, oligochitosan could be collected by macrophages: Scatchard analysis of 2-aminoacridone-oligochitosan in macrophages indicated that its internalization was mediated by a specific receptor on macrophage membrane with K_d 2.1×10^{-5} M. Oligochitosan internalization is mediated by a macrophage lectin receptor like with mannose specificity. In fact chitin/chitosan items administered intravenously to mice become bound to macrophage plasma membrane mannose/glucose receptors that mediate their internalization.

Shibata, Foster, Metzger, and Myrvik (1997a) and Shibata, Metzger, and Myrvik (1997b) showed that mouse spleen cells produced IL-12, TNF-α and IFN-γ when stimulated with phagocytosable chitin particles (1–10 μm). Their results indicate that mannose receptor-mediated phagocytosis is highly associated with the production of IFN-γ-inducing signaling factors such as IL-12 and TNF-α. Thus, chitosan shows immuno-potentiating activity: the mechanism involves, at least in part, the production of interferon-γ. Chitosan has also *in vivo* stimulatory effect on both nitric oxide production and chemiotaxis, and modulates the peroxide production. Chitin/chitosan oligomers generated by chemo-enzymatic degradation in the wound environment, exert significant biochemical effects: the migratory activity of the mouse peritoneal macrophages was enhanced significantly by chitin/chitosan oligomers (Moon et al., 2007; Mori et al., 2005; Okamoto et al., 2003).

β-Chitin grafted with poly(acrylic acid) was prepared with the aim of obtaining a hydrogel suitable as wound dressing. Acrylic acid was first linked to chitin, via ester bonds between the chitin

primary alcohol groups and the carboxyl groups of acrylic acid, as the active grafted moiety that was further polymerized upon addition of an initiator to form a network. The chitin–polyacrylate films were synthesized at various acrylic acid contents: the degree of swelling of the chitin–polyacrylate films was in the range of 30–60 times of their original weights depending upon the monomer feed content. The chitin–polyacrylate film with 1:4 weight ratio of chitin: acrylate, possessed optimal physical properties. The cytocompatibility of the film was tested with L929 mouse fibroblasts that proliferated and adhered well onto the film: thus, it was found to be interesting, considering also the absence of unreacted acrylic acid. The morphology and behavior of the cells on the chitin–polyacrylate film were found to be normal after 14 days of culture (Tanodekaew et al., 2004).

Chitosans possess favourable characteristics for promoting physiologically ordered dermal regeneration during wound healing. Chitosan oligosaccharides have a stimulatory effect on macrophages, and both chitosan and chitin are chemo-attractants for neutrophils *in vitro* and *in vivo*. Chitin and chitosan may further facilitate wound healing by stimulating granulation tissue formation or re-epithelialization. Carboxymethyl chitosan promoted the proliferation of the normal skin fibroblasts significantly but inhibited the proliferation of keloid fibroblasts: it reduced the ratio of collagens I/III in keloid fibroblasts by inhibiting the secretion of collagen type I while being with no effect on the secretion of collagens I and III in the normal skin fibroblasts (Chen, Wang, Liu, & Park, 2002; Janvikul, Uppanan, Thavornyutikarn, Prateepasen, & Swasdison, 2007; Zhu, Chian, Chan-Park, & Lee, 2005). The argon-plasma-treated chitosan membranes exhibited excellent attachment, migration and proliferation of the human skin fibroblasts compared to the untreated ones (Kojima et al., 2004).

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. It was found that the incorporation of basic fibroblast growth factor (bFGF) into chitosan accelerated the rate of healing (Obara et al., 2003).

6. Chitin/chitosan scaffolds and hydrogels

Tissue engineering involves the *in vitro* seeding and growing of cells onto a scaffold: Lee and Mooney (2001) reviewed this topic for a number of hydrogels from natural and synthetic polymers endowed with optimized porosity, pore interconnections, superficial biocompatibility and convenient rigidity. For example, β-chitin scaffolds were prepared by dissolving chitin in the saturated calcium chloride alcoholic solution ($CaCl_2{\cdot}6H_2O$ in C_2H_5OH) followed by dialysis to precipitate the chitin hydrogel, and lyophilization. They were also immersed in saturated aqueous $CaCl_2$ and Na_2HPO_4 solution for 12 h: SEM showed that, after several days, crystalline apatite could grow at the surface as well as inside the β-chitin scaffolds (Maeda, Jayakumar, Nagahama, Furuike, & Tamura, 2008). The versatility of chitosan and its derivatives paves the way to a wide range of applications; besides the inorganic compounds, scaffolds can also provide controlled release of drugs, growth factors and extracellular matrix components.

Scaffolds can be prepared by spinning: for example, by using a "one pot" approach, L-lactide oligomers were grafted to chitosan via ring opening polymerization. Side chains were prevalently attached to hydroxyl groups on carbons 3 and 6 of the glucosamine ring, while the amino groups remained in the primary form. This synthetic route imparts solubility in a broad range of organic solvents, facilitating formation of ultrafine fibers via electrospinning. Cytotoxicity tests using fibroblasts performed on electrospun L-lactide modified chitosan fibers showed that the specimen with the highest molar ratio of L-lactide (1:24) was the most interesting material for tissue engineering purposes. This approach allowed the manipulation of biodegradation rate and hydrophilicity of the scaffold material (Skotak, Leonov, Larsen, Noriega, & Subramanian, 2008; Xiao et al., 2008).

The incorporation of collagen into chitosan as a chitosan-collagen scaffold enhances attachment of seeded cells (Cuy, Beckstead, Brown, Hoffman, & Giachelli, 2003). The γ-poly(glutamic acid), a hydrophilic and biodegradable polymer, was also used to modify chitosan matrices and the resulting composite biomaterial was shown to be suitable for tissue engineering applications (Hsieh, Tsai, Wang, Chang, & Hsieh, 2005). Under other conditions, poly(γ-glutamate) promoted self-assembly of trimethyl chitosan-poly(γ-glutamate) nanoparticles whose TEM micrographs closely resembled chitosan tripolyphosphate nanoparticles. For both types of nanoparticles, trimethyl chitosan with 60 kDa, deacetylation ca. 0.85, quaternization ca. 0.55 was used. By varying the conditions, samples with degrees of quaternization ca. 0.25 and 0.40 were synthesized according to Polnok et al. (2004): they were purified by dialysis and freeze-dried. The nanoparticles were prepared by stirring at room temperature: aqueous poly(γ-glutamate) (MW 60 kDa, 1.0 mg/ml, 2 ml) was added to an aqueous trimethyl chitosan (1.2 mg/ml, 10 ml, pH 6.0). The self-assembled nanoparticles were collected after 50 min centrifugation at 15,000 rpm (Mi et al., 2008; Sieval et al., 1998).

Stem cells with self-renewal potential and multilineage differentiation capacity have been considered as the best choice for seeding cells (Riha, Lin, Lumsden, Yao, & Chen, 2005) and bone marrow derived mesenchymal stem cells have been extensively studied (Short, Brouard, Occhiodoro-Scott, Ramakrishnan, & Simmons, 2003). In fact, chitosan has good characteristics for the attachment, proliferation and viability of mesenchymal stem cells (Cho et al., 2004; Dang et al., 2006). With these promising features, they were considered for use in cell transplantation and tissue regeneration. This technology has been used so far to create various tissue analogs including skin, cartilage, bone, liver and nerve.

7. Repair of dermal tissues

Several chitin-based wound dressings are commercially available (Table 2). In most instances their technological content is as high as their biological significance: in fact the manufacture of these products has requested the competence of chemists, spinning technologists, experimental surgeons, materials scientists and other specialists. The ordered regeneration of wounded tissues requires the use of chitins and chitosans in the form of non-wovens, nanofibrils, composites, films and sponges.

The HemCon® hemostatic dressing listed in Table 2, was studied as a topical antimicrobial dressing having favourable effects on healing of excisional wounds that were or were not infected with *Staphylococcus aureus*, in mice. In order to study the conflicting clamping and stimulating effects of chitosan acetate bandage on normal wounds, the bandages were removed from wounds at times after application ranging from 1 h to 9 days. Three days application gave the earliest wound closure at day 10, and all application times gave a faster healing after the dressing was discontinued compared with control wounds without chitosan. Chitosan acetate bandage, besides being strongly bactericidal, reduced the number of inflammatory cells in the wound at days 2 and 4, and had an overall beneficial effect on wound healing especially during the early period where its antimicrobial effect is most important (Burkatovskaya, Castano, Demidova-Rice, Tegos, & Hamblin, 2008). The bandage TraumaStat® has been recently introduced as an alternative to HemCon®(Englehart et al., 2008).

Membranes made of chitosans in combination with alginates as polyelectrolyte complexes have also been prepared: they display

Table 2
Some commercial chitin- and chitosan-based bandages and wound dressings.

Chitin-based:
Beschitin® Unitika. Non-woven material manufactured from chitin. Available in Japan since 1982. Favours early granulation, no retractive scar formation. For traumatic wounds, surgical tissue defects
Syvek-Patch® Marine Polymer Technologies. Made of chitin microfibrils from the centric diatom *Thalassiosira fluviatilis* grown under aseptic conditions. It is claimed to be 7 times faster in achieving hemostasis than fibrin glue, because it agglutinates red blood cells, activates platelets whose pseudopodia make a robust contact with chitin and promotes fibrin gel formation within the patch, thus acting in a redundant way even on heparinized patients
Chitipack S® Eisai Co. sponge-like chitin from squid. For traumatic wounds and surgical tissue defects. Favours early granulation, no retractive scar formation
Chitipack P® Eisai Co. dispersed and swollen chitin supported on poly(ethylene therephthalate). For the treatment of large skin defects. Favours early granulation. Suitable for defects difficult to suture

Chitosan-based:
Chitodine® IMS. Chitosan powder with adsorbed elementary iodine, for the disinfection and cleaning of wounded skin and for surgical dressing
Chitoflex® HemCon. Antibacterial, biocompatible wound dressing designed to be stuffed into a wound track to control moderate to severe bleeding. It adheres strongly to tissue surfaces forming a flexible barrier that seals and stabilizes the wound surface. Easily removed with saline or water
Chitopack C® Eisai. Cotton-like chitosan obtained by spinning chitosan acetate salt into a coagulating bath of ethylene glycol, ice and NaOH; fibers washed with water and methanol. Complete reconstruction of body tissue, rebuilding of normal subcutaneous tissue and regular regeneration of skin
Chitopoly® Fuji spinning. Chitosan and polynosic Junlon poly(acrylate) for the manufacture of antimicrobial wears, suitable to prevent dermatitis
Chitosan Skin® Hainan Xinlong non-wovens. A chitosan-based skin substitute
Chitoseal® Abbott, based on chitosan, for bleeding wounds
Clo-Sur® Scion. A non-woven
Crabyon® Ohmikenshi. Made of cellulose viscose and chitosan mainly for comfortable sportwear
HemCon® HemCon. Freeze-dried chitosan acetate salt, for emergency use to stop bleeding. Used on battlefields since the time of the Desert Storm expedition in Iraq
Tegasorb® 3M. The dressing contains chitosan particles that swell while absorbing exudate and producing a soft gel. A layer of waterproof *Tegaderm® film dressing covers the hydrocolloid.* For leg ulcers, sacral wounds, chronic wounds. Reportedly superior to Comfeel® and Granuflex®
TraumaStat® Ore-Medix. Freeze-dried chitosan containing highly porous silica
Vulnosorb® Tesla-Pharma. Freeze-dried sponge of collagen and chitosan, on the European market since 1996

greater stability to pH changes and are more effective as controlled release membranes than either the chitosan or alginate separately. Membranes with wet thickness from 106 to 633 μm, tensile strength 6.86–31.14 MPa, elongation at break 3.97–8.42%, and maximum uptake 19 g of water per gram of membrane were obtained in a fairly reproducible way. The membranes were used on highly exuding wounds and prevented the bacterial infections (Rodrigues, Sanchez, daCosta, & Moraes, 2008). A spongy collagen-chitosan skin was developed as a scaffold for the reconstruction of skin *in vitro*: this artificial skin promoted the remodeling of an extracellular matrix similar to normal dermis (Berthod, Sahuc, Hayek, Damour, & Collombel, 1996).

Flexibility, softness, transparency and conformability permit to use chitin films as occlusive, semi-permeable wound dressings. The chitin films are generally non-absorbent, exhibiting a total weight gain of only 120–160% in physiological fluid. Dry chitin films transpire water vapor at a rate of about 600 g/m^2/24 h, (similar to commercial polyurethane-based film dressings), that rises to 2400 g/m^2/24 h when wet (higher than the water vapor transmission rate of intact skin): the chitin films are non-toxic to human skin fibroblasts, maintaining 70–80% cell viability. Wound studies using a rat model showed no signs of allergenicity or inflammatory response. The chitin films displayed accelerated wound healing properties. Wound sites dressed with the chitin films healed faster and appeared stronger than those dressed with Opsite® and gauze (Yusof, Wee, Lim, & Khor, 2003).

Water-soluble chitin was prepared with the desired deacetylation degree of 0.50 and molecular weight of 800 kDa: to this end plain chitin was dissolved in NaOH 40% at 0 °C and stirred at 25 °C for 60 h (so-called homogeneous deacetylation), and then an ultrasonic treatment was applied. The resulting 0.50-chitin was water-soluble and more susceptible to the action of lysozyme than chitosan. Full-thickness skin incisions were made on the backs of rats and then chitin, chitosan, 0.50-chitin powders and the 0.50-chitin hydrogel were embedded in the wounds. The 0.50-chitin was found to be more efficient than chitin or chitosan as a wound healing accelerator: the wounds treated with 0.50-chitin hydrogel were completely re-epithelialized, granulation tissues were nearly replaced by fibrosis and hair follicles were almost healed at 7 days after initial wounding. Also, the 0.50-chitin hydrogel treated skin had the highest tensile strength and the arrangement of collagen fibers in the skin was similar to normal skins. The 0.50-chitin hydrogel was considered to be a suitable wound healing agent due to its easy application and high effectiveness. It is likely that the superior enzymatic degradability and hydrophilicity of water-soluble chitin enhances its activity as a wound healing accelerator (Cho, Cho, Chung, Yoo, & Ko, 1999).

Taurine, 2-aminoethane sulfonic acid, a strongly acidic amino-acid, plays various important physiological roles in almost all mammalian tissues. Taurine dissolves chitosan beads, exhibits an antioxidant effect and influences cell proliferation, inflammation and collagenogenesis. Many antioxidants have been used to eliminate the negative effects of oxygen free radicals on wound healing. In mice, the taurine-chitosan gel releases taurine slowly. A plain chitosan solution (1.5%) and the same with added taurine (50 mM) were applied to full-thickness skin wounds of mice once a day for 7 days and then malondialdehyde and hydroxyproline levels along with the tensile strength of wound tissues were measured. Locally administered chitosan taurine salt increased wound tensile strength by decreasing malondialdehyde and increasing hydroxyproline levels, as supported by histological findings. Thus the taurine gel may be effective in wound healing (Degim et al., 2002; Huxtable, Azuma, Kuriyama, Nakagawa, & Baba, 1996) because it would act synergistically with chitosan.

Some wound-covering materials have been developed from chitin non-woven fabric and polyelectrolyte complexes of chitosan with its own anionic derivatives (sulfated chitosan or *N*-carboxybutyl chitosan): these materials were found to be effective in regenerating the wounded skin tissue.

A remarkable wet spun alginate composite containing 0.15–2.0% chitin nanofibrils was characterized in view of its use as a wound dressing: the essential result is that the overall susceptibility to lysozyme is improved by the tiny amounts of chitin nanofibrils. Moreover, the release of chitin oligomers as a consequence of the enzymatic hydrolysis is a significant contribution to the efficacy of the Ca alginate dressings (Turner et al., 1986; Watthanaphanit, Supaphol, Tamura, Tokura, & Rujiravanit, 2008).

Generally speaking, the efficacy of chitosan oligomers is superior to other forms of chitins and chitosans. In fact, chitins (including *N*-acetyl-D-glucosamine and a mixture of its oligomers up to the pentamer) and chitosans (including D-glucosamine and a mixture of its oligomers up to the hexamer) were compared using a linear incisional wound model in rats. Wound break strength in the chitosan group was higher than in the chitin group. Collagenase activity was also higher in the chitosan group than the chitin group. There was no significant relationship between the concentration of the sample and the break strength or the collagenase activity in general. Collagen fibers run perpendicular against the incisional line in the oligomers groups, and many activated fibroblasts were observed in the wounds in the chitosan group. As for the deacetylation degree, the strength at break of the regenerated tissues was linearly dependent on the deacetylation degree with

typical values of 0.27 ± 0.03 or 0.44 ± 0.08 N/mm^2 for degrees of deacetylation 0.33 or 0.96, respectively (Minagawa, Okamura, Shigemasa, Minami, & Okamoto, 2007).

In fact, chitosan oligomers supplementation enhanced the immune organ development: in 2400 broilers, dietary supplementation with chitosan oligomers increased serum concentrations of IgA, IgG and IgM, with the greatest response for the 100 mg kg^{-1} chitosan oligomer supplementation, compared to controls. Besides that, chitosan oligomers were confirmed to be a type of prebiotic that favourably alters the intestinal microflora balance, inhibits the growth of harmful bacteria, promotes good digestion and boosts immune function (Huang et al., 2007).

A number of articles have addressed the reduction of silver ions in the presence of stabilizing or protecting agents to prevent the agglomeration of the nanoparticles. Examples are silver ions reacting with $NaBH_4$ and thiosalicylic acid, $NaBH_4$ and sodium oleate, ascorbic acid and various surfactants, aniline or sodium citrate, sodium dodecyl sulfate, liquid ammonia, supercritical water and supercritical carbon dioxide, ethylene glycol and poly(vinyl pyrrolidone), formamide and poly (ethylene glycol). Methods are available for the formation of silver nanoparticles, based on phase-transfer processes, UV-activation, microwave irradiation, spray pyrolysis and microemulsion. Preparations of silver nanocomposites have been made with increasing frequency: examples are silver composites with chitosan, polyacrylonitrile, siloxane, epoxy resin and porous silica. Twu, Chen, and Shih (2008) expressed a preference for suspended amorphous chitosan with Ag and subsequent annealing at 600 °C with production of silver nanoparticles. Muzzarelli (unpublished data) used silver carbonate aqueous solution to contact freeze-dried chitosan-based sponges, and reduced the chelated Ag^+ to atomic Ag with the aid of $NaBH_4$ *in situ* while keeping intact the porous structure.

For example, a wound dressing composed of nano-silver and chitosan was fabricated using chitosan films preliminarily sterilized by immersion in 75% alcohol solution overnight, exposed to ultraviolet light for 1 h on each side and rinsed with sterile water. Nano-silver was prepared by adding trisodium citrate solution to boiling $AgNO_3$ aqueous solution (Katrin et al., 1997). Sterile chitosan films were immersed in nano-silver solution at 4 °C for 12 h for self-assembly of the nano-silver chitosan films via Ag–N bonding and to obtain 0.35% w/w Ag in the material dressing was determined by flame atomic absorption spectroscopy. Atomic force microscopy was used to examine the dressing, and scanning electron microscopy at room temperature identified the nano-silver immobilized on the chitosan film. Sterility and pyrogen testing assessed biosafety, and efficacy was evaluated using Sprague–Dawley rats with deep partial-thickness wounds: silver sulfadiazine and chitosan film dressings were used as controls. At day 13, the healing rate of the Ag-chitosan dressing group was higher than the rates of the control groups at 99% compared with 82% for the Ag sulfadiazine group. The healing time was 13.51 ± 4.56 days for the Ag-chitosan group, and 17.45 ± 6.23 days for the Ag sulfadiazine group, respectively: on average, 4 days shorter for the Ag-chitosan group.

Observations made on the histological sections on day 9 post-operatively indicated that in the nano-silver dressing group a continuous epithelial lamina was formed, together with some sebaceous glands. The Ag sulfadiazine group showed no epithelial laminae, whilst the chitosan film group exhibited patchy epithelial laminae with a few sebaceous glands. At 13 days the blood silver content was 5 times normal and on the 45th day post-operatively, the silver content of liver, kidney and brain had increased in both nano-silver and Ag sulfadiazine groups but more so in the latter, where the liver silver content was 100 times greater than normal. Thanks to its distinct antimicrobial action towards a broad range of bacteria, yeast, fungi and viruses, the Sprague–Dawley rats of the nano-silver group showed less post-operative infection than the control groups. Nano-silver is far less affected by the wound environment than is the ionic form of silver: therefore at the same concentration, the bactericidal activity of nano-silver is greater than that of the ionic form (Lu, Gao, & Gu, 2008).

8. Guided regeneration of nerve tissues

The anastomosis of peripheral nerves is a demanding procedure that presents potential complications due to foreign body reactions elicited by sutures. As an alternative to autologous grafts, hollow guides are needed for correct nerve growth. The tendon of the crab leg muscle consists of crystalline chitin, calcium phosphate and proteins; it has an aligned molecular structure that imparts exceptional tensile strength (ca 68 MPa, that reaches 235 MPa upon heating at 120 °C due to hydrogen bonding). The tendon exhibits a flattened tube structure with a diameter in the order of 1–5 mm. It is amenable to the deacetylated form by current protocols: both chitin and chitosan tubes do not produce inflammation nor encapsulation when implanted in rats (Itoh et al., 2003; Yamaguchi et al., 2003). A conduit can be easily manufactured from the crustacean tendon and used to bridge the ends of a dissected nerve, thereby serving as a barrier against scar tissue formation.

While laminin (a 800 kDa glycoprotein from basement membrane) can aid nerve growth, synthetic laminin peptides were preferred in studies intended to facilitate nerve regeneration *in vivo* with the aid of chitosan tubes covalently coated with said peptides (Chavez-Delgado et al., 2003; Matsuda, Kobayashi, Itoh, Kataoka, & Tanaka, 2005; Mochizuki et al., 2003; Suzuki et al., 2003). Semi-synthetic conduits were prepared also from *O*-carboxymethyl chitosan crosslinked with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (Lu et al., 2007).

To improve cell adhesion and neurite outgrowth, poly-D-lysine too was immobilized onto chitosan via azidoaniline photo-coupling. Fetal mouse cortical cells were cultured on films and within thermally responsive chitosan glycerophosphate hydrogels, in order to assess their biocompatibility in terms of cell number and neurites per cell. Osmolarity of the hydrogel was a critical factor in promoting cell survival with isotonic glycerophosphate concentrations providing optimal conditions. Increased poly-D-lysine concentrations did not alter cell survival in cultures but neurite outgrowth was significantly depressed. Neurons exhibited a star-like morphology typical of 2D culture systems. The effects of poly-D-lysine on cell number, cell morphology and neurite outgrowth were more distinct in gel culture where neurons exhibited larger cell bodies and sent out single neurites. Immobilized poly-D-lysine improved cell survival up to the optimum concentration of 0.1%, however, further increases resulted in reduced cell number and neurite outgrowth, due to a higher cell interaction with poly-D-lysine within the hydrogel instead of the film surface. Thus, thermally responsive chitosan glycerophosphate hydrogels provided a suitable 3D scaffolding environment for neural tissue growth (Crompton et al., 2007). This artificial skin sustains nerve growth and provides new perspectives to increase nerve regeneration within the tissue engineered skin by linkage of neurotrophic factors in the sponge before transplantation (Gingras, Paradis, & Berthod, 2003). Transparent chitin hydrogel tubes were synthesized from chitosan solutions using acylation chemistry and mold casting techniques: they showed significantly enhanced neurite outgrowth compared to chitin films (Freier, Koh, Kazazian, & Shoichet, 2005a; Freier, Montenegro, Koh, Shoichet, 2005b).

Nerve guides are currently being fabricated by adding certain proteins that support nerve repair and regeneration, and by optimizing the biological properties of a nerve guide. In particular, glial cell line-derived nerve growth factor and laminin were blended

with chitosan to fabricate factor + laminin + chitosan guides. As said factor is known to provide trophic support to motor neurons, the functional restoration of an injured sciatic nerve treated with said material was studied. Results indicated an increase in the functional recovery of the factor + laminin + chitosan group when compared to the plain chitosan nerve guides. After 12 weeks, factor + laminin + chitosan guides had comparable functional values to the laminin blended chitosan guides and autograft groups. Muscle weights indicated restoration of functional strength for the factor + laminin group, compared to the plain chitosan groups where atrophy was present. Moreover, behavioral testing demonstrated that the factor + laminin group regained sensation while the control groups did not. Thus, the factor + laminin + chitosan guides enhanced both functional and sensory recovery (Patel, Mao, Wu, & VandeVord, 2007).

A variety of bilayered chitosan tubes have been developed: for example a bilayered tube comprises an outer layer of chitosan film and an inner layer of electrospun chitosan non-woven. Glycine spacers were introduced into the CYIGSR sequence domain of laminin that enhances Schwann cells migration and attachment, as well as neural outgrowth, resulting in the aminoacid sequences CGGYIGSR and CGGGGGGYIGSR. These peptides were covalently bound to the nano/microfiber mesh surface of the chitosan tube. The efficacy of nerve regeneration into chitosan tubes with immobilized CGGGGGGYIGSR peptide was similar to that of the isograft (Wang, DeBoer, & DeGroot, 2008a; Wang et al., 2008b).

In addition to the above mentioned brilliant results, the sutureless *in vivo* anastomosis of rat tibial nerves was performed using a chitosan-based laser-activated adhesive sheet made of chitosan, indocyanine green, acetic acid and water, manufactured in thin sheets. Its adhesive strength was tested *in vitro* by bonding strips onto rat sciatic nerves and sheep intestine by laser activation with low fluence (50 J/cm^2). The adhesive bonded well to tissue with a tensile strength of 12.5 ± 2.6 kPa, so that *in vivo* anastomosed nerves were in continuity 3 days after surgery. The number and morphology of myelinated axons were normal (ca. 96%) compared with intact nerves (Lauto et al., 2008).

9. Regeneration of cartilage

In cartilage tissue engineering, chitosan has the ability to maintain the round morphology of chondrocytes, that is a normal phenotypic characteristic, and preserve their capacity to synthesize cell-specific extracellular matrix (Iwasaki, Yamane, & Majima, 2004; Lahiji, Sohrabi, Hungerford, & Frondoza, 2000; Risbud, Ringe, Bhonde, & Sittinger, 2001). In fact the chitosan delivered via intra-articular injection generated thicker epiphyseal cartilage in the tibial and femoral joints, with chondrocyte proliferation.

To increase the cell adhesion to chitosan, the chitosan-alginate-hyaluronan complexes were prepared with or without covalent attachment to RGD-containing protein. Once seeded with chondrocytes, these composites supported formation of new cartilage *in vitro*, and 1 month after implant into rabbit knee cartilage defects, partial repair was observed regardless of the presence of RGD indicating suitability of the complex for cartilage regeneration (Hsu et al., 2004). Chitosan was also conjugated with hyaluronan to obtain a biomimetic matrix for chondrocytes: chondrocyte adhesion and proliferation, and the synthesis of aggrecan and type II collagen were significantly higher on the mixed polysaccharides than on chitosan (Yamane et al., 2005). Furthermore, chitosan-based scaffolds were also used to deliver growth factors in a controlled fashion to promote the ingrowth and biosynthetic ability of chondrocytes. The porous collagen/chitosan/glycosaminoglycan scaffolds loaded with transforming growth factor β1 was reported to promote cartilage regeneration (Kim et al., 2003; Lee et al., 2004).

Upon addition of *n*-butanol to aqueous chitosan solution and subsequent sublimation, the formation of chitosan scaffolds was observed: the pore diameters were within the range 4–100 μm depending on the *n*-butanol concentration. The initial cell adhesion on the new prepared chitosan scaffold was 190% higher than that on control chitosan scaffold without addition of non-solvent. The proliferation rate of human dermal fibroblasts in the scaffold was nearly twice the control, after 3 days of culture, mainly because the specific surface area was large enough for cell attachment and tissue growth (Chun, Kim, & Kim, 2008).

A porous freeze-dried chitosan scaffold incorporating TGF-β1-loaded microspheres, was used for the treatment of cartilage defects (Kim et al., 2003). TGF-β1 was released in a sustained fashion, and promoted chondrocyte proliferation and matrix synthesis. The *N*,*N*-dicarboxymethyl chitosan associated with bone morphogenetic protein-7 was also found to repair femoral articular cartilage lesions in the rabbit, indicative of synergism of their respective biological properties (Mattioli-Belmonte et al., 1999a, 1999b). Indeed, BMP-7 enhanced the *in vivo* proliferation of chondrocytes, leading to partial healing of the articular surface of the lesions. Thus, a connection was established between chitosan and larger proliferation of fibro-vascular tissue, that is the initial process of ossification, and therefore chitosan was considered a crucial carrier for BMP-7.

The unexpected restorative effect of orally administered *N*-acetyl-D-glucosamine and glucuronic acid was brought to light in experimental cartilaginous injuries in rabbits. With respect to medial trochlear injury, one out of three holes were not cured in the control, but all were cured in the glucose, GlcNAc and GlcUA groups. With respect to the proximal hole, there was a significant difference between the control and GlcNAc or GlcUA. The injured parts were covered by fibrous connective tissues in the control and the glucose, whereas in the GlcNAc and GlcUA groups massive proliferation of matured cartilaginous tissues was observed, and the regenerated cartilaginous tissues were surrounded by the proliferation of chondroblasts (Tamai et al., 2003). These results mean that said monomers permit to reach satisfactory healing, even when they are administered *per os*; moreover, they confirm indirectly that the action of chitin/chitosan is mediated by hydrolases. They are in line with data by Zeng, Qin, Wang, Chi, and Li (2008) who used various chitosans labeled with fluorescein isothiocyanate to investigate the absorption and distribution of chitosan in mice after oral administration. The absorption of chitosan was inversely proportional to the molecular weight but directly dependent on the water solubility: the chitooligomers were promptly absorbed and metabolized, whilst the 52% acetylated chitosan lasted longer. The absorbed chitosans were present in all tested organs (liver, kidney, spleen, thymus, heart and lung).

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

10. Osteogenic chitosan composites for bone defects

Several injectable materials based on chitosan and its derivatives have been used as osteogenic bone substitutes. Chitosan-calcium phosphate composites and phosphorylated chitosan

composites have been used to fill defects in radius and tibia *in vivo*. Chemically modified hyaluronan-chitin and hyaluronan-chitosan materials were reported to be osteoinductive and exhibited rapid degradation and concomitant neovascularization *in vivo*. The combination of chitosan with antibiotics or growth factors is suitable for bone tissue engineering. The chitosan sponges incorporating platelet-derived growth factor induced new bone formation in rat calvarial defect (Lee et al., 2000a, 2000b).

In the context of current studies dealing with the roles of stem/progenitor cells in osteogenesis, it is remarkable that chitosan has the ability to promote osteogenic progenitor cell recruitment and attachment thus facilitating bone formation (Kim, Park, Kwon, Baik, & Cho, 2002). When cultured mesenchymal stem cells are treated *in vitro* with chitosan, the treated cells show significantly higher averages of colonies per well than the control, suggesting that chitosan may promote differentiation of osteoprogenitor cells and bone formation. The calcium phosphate/chitosan coating also showed an improved bone marrow stromal cell attachment. The chitosan-alginate gel/mesenchymal stem cells/bone morphogenetic protein-2 composites was found to stimulate new bone formation when injected into the mouse (Park et al., 2005).

The cationic nature of chitosan is primarily responsible for electrostatic interactions with anionic glycosaminoglycans, proteoglycans and other negatively charged molecules. A large number of cytokines/growth factors are linked to glycosaminoglycans (especially heparin and heparan sulfate), and a scaffold incorporating a chitosan-glycosaminoglycan complex may retain and concentrate growth factors most efficiently. For example, the layer-by-layer technique was used to assemble heparin (a strong polyanion) and chitosan: the polyelectrolyte complexes were characterized in terms of sensitivity of the polyelectrolyte composition and layer thickness to changes of processing parameters (Boddohi, Killingsworth, & Kipper, 2008).

Carbohydrate polymers exert a variety of biological actions in modulating the intra and extracellular environment. Substituted dextrans bind growth factors and protect them from enzymatic degradation. Heparin-like dextrans enhance the healing of bone in an environment where bone would otherwise not regenerate (Albo et al., 1996). The binding of heparin like polysaccharides to fibroblast growth factor (FGF) induces a conformational change in the latter, resulting in the formation of FGF dimers or oligomers, and this biologically active form becomes available to the FGF receptor for signal transduction (Venkataraman et al., 1996). Osteoinduction of the BMP–chitin complex was accompanied by excellent biocompatibility (Miyazawa, 1995).

Interactions between cell and extracellular matrix provide cells with information essential for controlling morphogenesis, migration, repair and death (Werb, 1997). Chitosan has been combined with a variety of materials such as alginate, hydroxyapatite, calcium phosphate, poly(methylmetacrylate), poly(L-lactic acid) and growth factors for potential application in orthopedics; hyaluronan shows morphogenetic activities suitable for a correct bone architecture. Chitosan improved cell adhesion, proliferation, biosynthetic activity and chondrocyte attachment to poly(L-lactic acid) and alginate (Cui et al., 2003).

About the bone tissue, it should be observed that the increase of extracellular Ca^{2+} is perceived by osteoblasts via specific receptors that lead to mutagenic and chemotactic action. Modified chitosans carrying calcium phosphate accelerate bone wound healing (Muzzarelli et al., 1998). In contact with bone, chitosan promotes direct endochondral ossification. Bone defects surgically produced in sheep and rabbit models have been treated with freeze-dried modified chitosans. Moreover, the pattern of bone regeneration has been studied in an osteoporotic experimental model with bone morphogenetic protein linked to chitosan (Muzzarelli et al., 1997).

6-Oxychitin, a modified chitin obtained via regiospecific oxidation of chitin at C6 and constituted by relatively short β(1–4) chains of 2-acetamido-2-deoxy glucuronic acid sodium salt, is functionally similar to hyaluronan and is endowed of anionic character and chelating ability (Muzzarelli, Cosani, & Terbojevich, 1999). For the preparation of 6-oxychitin, the stable nitroxyl radical 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo®) was used as a catalyst, together with NaBr, to regiospecifically oxidize chitin with aqueous NaOCl. 6-Oxychitin was fully characterized from the chemical and enzymatic standpoints and was sterilized by γ-ray irradiation at 25 kGy; the aspect was that of a white, soft, spongy and water-soluble material. The average molecular weight of 6-oxychitin was close to 10 kDa, and the degree of substitution was 1.0 (Muzzarelli et al., 2000). 6-Oxychitin, applied to femoral surgical defects for 3 weeks, produced a good histoarchitectural order in the newly formed bone tissue. When osteoblasts from newborn mouse calvariae were associated to the polysaccharide these preparations showed enhanced tissue mimicking capacity. The spongious trabecular architecture was restored in the defect site and the association of the chitin derivative with the osteoblasts seems to be one of the best biomaterials in term of bone tissue recovery; the addition of osteoblasts improved the performance of 6-oxychitin and the spongious trabecular architecture of the newly formed bone was superior to other remedies. 6-Oxychitin therefore represented an advance in the experimental study of the osteoinduction process and preluded to novel applications intended to reconstruct the correct morphology of bone tissues, even in the presence of important mechanical stress (Muzzarelli et al., 2001).

Rat calvarial osteoblasts were grown in porous chitosan sponges fabricated by freeze-drying. The prepared chitosan sponges had a porous structure with a 100–200 μm pore diameter, which allowed cell proliferation. Cell density, alkaline phosphatase activity and calcium deposition were monitored for up to 56 days. Cell numbers per g of sponge were 4×10^6 (day 1), 11×10^6 (day 28) and 12×10^6 (day 56); calcium depositions were 9 (day 1), 40 (day 28) and 48 (day 56) μg per g of sponge. Histological results corroborated the observed bone formation within the sponges (Seol et al., 2004).

Chitosan promotes growth and mineral-rich matrix deposition by osteoblasts in culture and allows osteoconduction (Muzzarelli et al., 1993). A 3D macroporous calcium phosphate bioceramic embedded with porous chitosan was developed: in this scaffold, a nested chitosan sponge enhanced the mechanical strength of the ceramic component via matrix reinforcement, and preserved the osteoblast phenotype (Zhang & Zhang, 2001; 2002a, 2002b). Similarly, gentamycin-conjugated macroporous chitosan scaffolds reinforced with β-tricalcium phosphate and calcium phosphate have been developed for bone engineering. Macroporous chitosan scaffolds incorporating hydroxyapatite or calcium phosphate glass with an interconnected porosity of approximately 100 μm have been synthesized (Zhang, Ni, Zhang, & Ratner, 2003).

Hydroxyapatite and chitosan are not indifferent to their reciprocal presence. In fact, the long-term aging of hydroxyapatite in chitosan acetate gel solutions resulted in changes in the surface chemistry, colloid stability and chemical composition of hydroxyapatite due to adsorption and solubility effects. Chitosan exhibited strong adsorption interactions with hydroxyapatite particles and improved the colloid stability of hydroxyapatite. At the thermogravimetric analysis, mass decreased by 3.3–6.5% as the chitosan concentration increased by 0–2.5%. The amount of chitosan adsorbed on hydroxyapatite was 2.8–3.1% based on elemental analysis. The specific surface area of hydroxyapatite doubled after aging in chitosan acetate solutions and attained 160 m^2/g (Wilson & Hull, 2008). As an alternative, finely dispersed hydroxyapatite in chitin solution (LiCl-dimethylacetamide solvent) was stirred for 4 days at 10 °C and cast into plastic molds to form hydroxyapatite–chitin

gels: they were immersed in water for 1 week to remove residual LiCl and dimethylacetamide. The hydrogels were freeze-dried or air-dried to yield films.

Mesenchymal stem cells harvested from rabbits were induced into osteoblasts *in vitro* and were cultured for a week, statically loaded onto the porous hydroxyapatite–chitin matrices and implanted into bone defects of the rabbit femur for 2 months. Histology of explants showed bone regeneration with biodegradation of the hydroxyapatite–chitin matrix. Similarly, green fluorescence protein transfected mesenchymal stem cells-induced osteoblasts were also loaded onto porous hydroxyapatite–chitin matrices and implanted into the rabbit femur. Mesenchymal stem cells-induced osteoblasts did not only proliferate but also recruited surrounding tissue to grow in. The hydroxyapatite–chitin matrix qualified for tissue engineered bone substitutes (Ge, Baguenard, Lim, Wee, & Khor, 2004).

When a chitosan-hydroxyapatite paste was applied to the surface of the tibia after periosteum removal, the formation of new bone was observed after a week: it continued during a 20-week follow-up indicating suitability of this paste as a bone filling material (Kawakami et al., 1992). The issue of mechanical resistance of chitosan-based composites was addressed by Hu, Jou, and Yang (2003), who reported a chitosan-hydroxyapatite multilayer nanocomposite with high strength and bending modulus suitable for fixation of long bone fractures. A macroporous chitosan/β-tricalcium phosphate composite scaffold for bone tissue engineering was developed by freeze-drying, and the effects of the composite concentration and of the freezing temperature on the ability to resist compression by the scaffold were studied (Leroux, Hatim, Freche, & Lacout, 1999; Yin et al., 2003). Because chitosan solutions gelify in response to a pH change from slightly acidic to neutral, the chitosan–calcium phosphate composites satisfy the need to develop bone fillers that set in response to physiological conditions, but not while mixing the components *in vitro*.

While the ability of chitosan to bind growth factors and release them in a controlled fashion is well demonstrated in the above cited articles, the cationicity of chitosan can be further enhanced by 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 apposition of trabecular bone (Muzzarelli et al., 1994). Chitosan has been used to modify the surface properties of prosthetic materials for enhancing the attachment of osteoblasts. Titanium coated with chitosan via silane–glutaraldehyde exhibited increased osteoblast attachment and proliferation. The bond strength of chitosan coating with Ti was in the range of 1.5–1.8 MPa and its full degradation took 8 weeks, thus supporting the hypothesis that chitosan promoted osseointegration of Ti devices commonly used for orthopedic implants, although chitosan bond strength was found to be less compared to calcium phosphate coatings (Bumgardner et al., 2003a, 2003b).

Because chitosan is interesting as a coating for dental/craniofacial and orthopedic implants, Wang et al. (2008a, 2008b, 2008c) used chitosan to increase the biocompatibility of electrolytically deposited apatite coatings on titanium alloys. This coating exhibited an improved bone marrow stromal cell attachment. Similar data were obtained for chitosan coupled with surface-immobilized cell-adhesive arginine-glycine-aspartic acid (RGD) peptide and for hyaluronan by Chua, Neoh, Kang, and Wang (2008). Likewise, titanium was coated with three chitosans of different degree of deacetylation and from different manufacturers via silane-glutaraldehyde. Coating bond strength was in the range 2.2–3.8 MPa regardless of degree of deacetylation. The coatings exhibited little dissolution over 5 weeks in media with or without lysozyme, and were judged to be osteocompatible *in vitro* (Yuan, Chesnutt, Wright, Haggard, & Bumgardner, 2008b). Cathodic electrophoretic deposition has been utilized for the fabrication of composite hydroxyapatite-chitosan coatings (up to 60 μm thick) on stainless steel. The coatings, whose composition was changed by altering the chitosan/hydroxyapatite ratio in the solutions, provided protection against corrosion (Pang & Zhitomirsky, 2007). The use of an antibiotic-loaded chitosan coating on stainless steel screws in contaminated bone fracture fixation was considered after optimization of antibiotic loading and release (Greene, Bumgardner, Yang, Moseley, & Haggard, 2008).

Improvements of the mechanical properties of chitosan-based composite biomaterials are essential for this type of application. Of great importance is the ability of chitosan to bind to anionic compounds such as growth factors, glycosaminoglycans and DNA. In fact, the combination of good biocompatibility, intrinsic antibacterial activity, ability to bind to growth factors and to be processed in a variety of different shapes makes chitosan a prominent scaffold material for cartilage, intervertebral disc and bone tissue engineering in clinical practice. Moreover, the formation of chitosan–DNA complexes renders chitosan a good support in orthopedic gene therapy (Di Martino, Sittinger, & Risbud, 2005).

The bone-regenerating efficacy of a nano-hydroxyapatite/collagen/poly(lactic acid) composite reinforced with chitosan fibers (83.8% deacetylated, 12.5 μm diameter, 550 MPa tensile strength) was evaluated in a goat shank model. Forty adult male goats with 40 mm defects in shank at the same anatomic site were divided into four groups. Besides the control group, three groups were implanted with porous poly(lactic acid), composite and reinforced composite, respectively. Composite implants, with and without chitin fibers, were more effective in repairing the defects than poly(lactic acid) alone. However, only the chitosan-reinforced implants provided perfect recovery (in 15 weeks) with appropriate strength and high mineral density (Li, Feng, Liu, Dong, & Cui, 2006).

New nanocomposites of chitosan–hydroxyapatite–polygalacturonan have been synthesized via a biomimetic approach. The chitosan–polygalacturonan complexes are well known (Muzzarelli, Stanic, Gobbi, Tosi, & Muzzarelli, 2004), thus the pectin polygalacturonan was expected to provide stronger interfacial interactions and improve the mechanical properties of the composite. Atomic force imaging of fractured and polished surfaces of the nanocomposite displayed chitosan-rich and polygalacturonan-rich domains made of smaller globular shaped particles in which, hydroxyapatite nanoparticles were embedded in the biopolymer matrix. The average size of the hydroxyapatite particles was found to be 34 nm; the elastic modulus of the composite was 23.62 GPa. Macro-mechanical tests showed significant enhancement of elastic modulus, strain to failure and compressive strength of said composite over those containing either chitosan–hydroxyapatite or galacturonan–hydroxyapatite only (Verma, Katti, Katti, & Mohanty, 2008).

BMPs induce differentiation of multipotential mesenchymal cells, pluripotent murine stem cell cultures and rat-bone-marrow stromal cell as well as proliferation and maturation in osteoblast populations Quantity and quality of newly formed bone, stimulated by recombinant human BMP-2 in combination with either monoolein or chitosan acetate gel as carriers, were evaluated in defects made in 36 Wistar rat mandibles. There were statistical differences between groups of animals receiving or not the rhBMP-2. Both carriers, monoolein and chitosan gels, were adequate for defect filling and control of protein release (Issa et al., 2008). Although rhBMP-2 seems to induce formation of new bone tissue by itself, association to an immobilizing carrier during a time sufficient to elicit a cellular response has a potentiating effect: the higher osteoinductive capacity of monoolein or chitosan gel associ-

ated to rhBMP-2, confirms observations by other investigators who considered that there is a direct relationship between the osteoinductive action of the morphogenetic protein and the carrier retention capacity. Many studies have shown that bone repair is optimized by association of the protein to a sustained release carrier. Sustained release dosages of rhBMP-2 from 0.5 to 115 mg were tested to find an optimal value around to 20 mg, not only in terms of efficacy but also cost (Abarrategi, Civantos, Ramos, Casado, & Lopez-Lacomba, 2008). Coated calcium sulfate pellets combined with rhBMP-2 facilitated new bone formation *in vivo* (Cui, Zhang, Wang, & Gao, 2008; Lee et al., 2002).

The effects of a combined chitosan and collagen matrix on osteogenic differentiation of rat-bone-marrow stromal cells was investigated on four study groups: collagen, chitosan, 1:1 chitosan–collagen and 1:2 chitosan–collagen sponges fabricated by freeze-drying. Bone-marrow stromal cells seeded on the sponges and cultivated in mineralized culture medium for 27 days attached to the sponges: the expression of alkaline phosphatase and osteocalcin (that reveal the progress of differentiation) in collagen and chitosan–collagen sponges was greater than on chitosan sponges; on the other hand, chitosan and chitosan–collagen sponges showed higher resistance to enzymatic degradation than collagen sponges. The best chitosan-collagen ratio was 1:1 because it promoted osteoblastic differentiation of bone-marrow stromal cells, and improved the mechanical and physical properties of the sponges (Arpornmaeklong, Pripatnanont, & Suwatwirote, 2008).

Mouse osteoblasts and fibroblasts were grown on chitosan in the presence of serum. Cell attachment and immuno-fluorescence analysis were done to analyse phenotypic profiles. Osteoblast attachment at 1 h was significantly greater than with fibroblasts. At 24 h, levels of cell attachment for fibroblasts increased and became similar to those in osteoblast cultures at 1 and 24 h. Fibroblasts showed heterogeneous population of round and semi-spread cells, but in comparison, osteoblasts displayed phenotypes that were well spread with a developed cytoskeleton (Fakhry, Schneider, Zaharias, & Senel, 2004).

11. Photo-chemistry and experimental surgery

For the preparation of photo-crosslinkable hydrogel, lactose moieties were introduced in chitosan (800 kDa, 0.80 deacetylated) by adding 2% lactobionic acid and promoting a condensation reaction with the amino groups of chitosan. Addition of 2.5% *p*-azidobenzoic acid provided the chemical functionality suitable for sensitivity to UV light and consequent photo-crosslinking in a matter of seconds. Fibroblast growth factor was optionally incorporated into the gel.

Ishihara et al. (2002) published several articles including a bilingual mini-review on the photo-crosslinkable chitosan bearing *p*-azidobenzoic acid and lactobionic acid: this derivative could be crosslinked by UV-irradiation (250 W lamp, 2 cm distance, 240–380 nm prevalent 340 nm), resulting in a rubber-like flexible and transparent hydrogel. The hydrogel showed a strong adhesion to tissues, significant induction of wound contraction, and acceleration of wound closure and healing, thus it is useful as a biological adhesive in surgical applications (Ishihara, 2002; Ono et al., 2000).

The chitosan hydrogel could completely stop bleeding from a cut mouse tail within 30 s of UV-irradiation and could firmly attach to each other two slices of skin. In order to evaluate its accelerating effect on wound healing, full-thickness incisions were made on the back of mice and subsequently an azido-lactose chitosan aqueous solution was applied to the wound and irradiated with UV light for 90 s. Application of the chitosan hydrogel significantly induced wound contraction and accelerated wound closure and healing. Histological examinations also demonstrated advanced granulation tissue formation and epithelialization in the chitosan hydrogel treated wounds. Thanks to its accelerating healing ability, this chitosan hydrogel was considered an excellent dressing for wound closure and tissue adhesion, to be preferred in emergencies (Ishihara et al., 2002); quantitative data were provided by Ono et al. (2001).

Fibroblast growth factor-2 stimulates the proliferation of fibroblasts and capillary endothelial cells, thus promoting correct wound repair via angiogenesis; however, this factor has short half life *in vivo* and high diffusibility. A way to keep it on the desired position is to use a photo-crosslinkable chitosan. The factors retained in the hydrogel remained biologically active and were released upon *in vivo* degradation of the chitosan. Wound contraction was induced and wound closure was accelerated in healing-impaired diabetic mice compared to controls. The application of a chitosan hydrogel as an occlusive dressing was recommended because chitosan would be more effective in this form in order to protect and contract the wound in a suitably moist healing environment (Obara et al., 2003).

To improve the adhesion and growth of epithelial cells on chitosan, the cell-adhesive peptide Gly-Arg-Gly-Asp (GRGD) was photo-chemically grafted to its surface. Grafting GRGD-(*N*-succinimidyl-6-[4′-azido-2′-nitrophenylamino]-hexanoate) from 25 mM solutions to chitosan and to anhydrous chitosan tripolyphosphate was performed by adsorption of the peptide and subsequent UV-irradiation, with grafting percentages of ca. 83 and 53, respectively. Human umbilical vein epithelial cells adhered firmly and grew on chitosan-GRGD and chitosan-TPP-GRGD surfaces after 36 h of incubation but not on chitosan and on chitosan-TPP. Moreover, the viability for the growth of epithelial cells on the chitosan-GRGD and chitosan-TPP-GRGD surfaces analysed by MTT assay also confirmed the efficacy of GRGD (Chung, Lu, Wang, Lin, & Chu, 2002).

In further remarkable experiments recalled below, the gastric submucosal layer of heparinized rats was injected with the photo-crosslinkable chitosan (which was then irradiated with ultraviolet light to form a hydrogel), or with sodium hyaluronate, or hypertonic saline and investigations were done using three sets of rats for the measurement of the thickness of the layer, the amount of bleeding induced by mucosal resection, and the histological effects of chitosan on wound healing. The gastric submucosal layers of chitosan-treated animals remained significantly thicker than those of other groups for at least 6 h after injection. The total amount of bleeding 20 min after mechanical mucosal resection was ca. 170, 678 and 1020 mg in the chitosan hydrogel, sodium hyaluronate and hypertonic saline groups, respectively. Histological study revealed that the site of bleeding was surrounded by chitosan hydrogel and that almost all the hydrogel was biodegraded within 4 weeks. The chitosan hydrogel produced mucosal elevation, and it significantly reduced bleeding after mucosal resection (Ishizuka et al., 2007).

Burn wound excision is necessary when preparing skin for grafting, and the success of graft "take" is thought to be dependent on the vascular supply to the wound. The photo-crosslinkable chitosan hydrogel in Dulbecco modified Eagle medium F12 promoted re-epithelialization and neovascularization. The latter occurred earlier in the group treated with azido-lactose chitosan compared with the collagen treated control group. The thickness of the granulation tissue in the test group was greater than that in the collagen treated group. The early degradative and angiogenic activities were deemed to be beneficial for granulation tissue formation in deep dermal burn wounds (Kiyozumi et al., 2007).

The same gel was tested for antibacterial activity in the case of infections of vascular protesic graft in the rabbit. Dacron graft fragments (2×3 mm) were coated with the hydrogel and their infection was provoked by topical inoculation of 10^6 colony forming units of *Escherichia coli* that was allowed to develop over 1 week

in vivo and *in vitro*. Infection of the graft was substantially inhibited by the photo-crosslinked hydrogel, as demonstrated by the biochemical analyses of white blood cells, crossreacting protein and lactate dehydrogenase (Fujita et al., 2004).

UV-curable chitosans were also synthesized using less toxic agents: *N*-selective introduction of a UV-curable side chain to chitosan was accomplished by reductive *N*-alkylation with photo-sensitive aldehydes. Six types of photo-sensitive aldehydes were prepared, including 3-methoxy-4-(2-hydroxy-3-methacryloyloxypropoxy) benzaldehyde. The photo-sensitive 2-hydroxy-3-methacryroyloxypropoxy group was introduced into vanillin or hydroxybenzaldehyde using glycidyl methacrylate to give the corresponding aldehydes. The modified vanillin, still an aldehyde, was the cheapest and was easily recovered in good yield by recrystallization: *N*-selective introduction of the latter aldehyde (0.8 mol equivalent relative to the glucosamine unit) to the chitosan (degree of deacetylation 0.97; molecular weight, 30–50 kDa) was accomplished by reductive *N*-alkylation, using $NaBH_3CN$ via Schiff base in pH 4.5 buffer (0.2 M acetic acid, 0.2 M sodium acetate). An implant into murine subcutaneous tissues, was completely cured by UV spot irradiation for 4 s: the implant was surrounded by thin fibrous granulating tissue with no inflammatory cellular infiltration and showed good biocompatibility (Renbutsu et al., 2005).

The photo-crosslinking technique is also suitable for the permanent coating of inorganic surfaces: for example the innovative 6-*O*-butyrylchitosan was used to coat a glass: γ-chloropropyltrimethoxy silane (0.6 g) in ethanol–water (20 ml, 95% v/v), adjusted to pH 4.5 with acetic acid, was stirred for 5 min in order to hydrolyse the trimethoxy silane to $-Si(OH)_3$, a reactive function towards the glass; the latter was dipped into the solution for 2 min and then rinsed. 4-Amino benzoic acid (0.050 mol) in HCl aqueous solution was reacted with aqueous sodium nitrite (0.055 mol) at −10 to 0 °C for 15 min, followed by aqueous sodium azide (0.060 mol) at room temperature for 1 h to yield 4-azidobenzoic acid (yield 80%). Chitosan (2.1 g) was added to methanesulfonic acid (11 ml) and the mixture was stirred at 0 °C for 15 min until homogeneous. Butyric anhydride (20 ml) was added dropwise and the total mixture stirred between 0 and 5 °C for 2 h: the resulting gel was poured into acetone to isolate the acylated chitosan that was dried in vacuo. Then *O*-butyrylchitosan was grafted to the silanized surface via the photo-crosslinking technique. The *O*-butyrylchitosan-coated glass depressed platelet adhesion and fibrinogen adsorption compared to the control surface, therefore, a biocompatible and blood anticoagulant glass surface was obtained (Mao et al., 2004).

Hydrogels based on water soluble methacrylated *O*-carboxymethyl chitosan and on poly(ethylene glycol) diacrylate, with degree of carboxymethylation 0.69–1.86, exhibiting different solubility, zeta potential (−52.7 to −12.8 mV) and decomposition temperature (260–283 °C), were reacted with glycidyl methacrylate to make UV crosslinkable compounds that were blended with poly(ethylene glycol) diacrylate, a photo-initiator and water and then photo-crosslinked. The porous structures of the hydrogels was modulated by the degree of methacrylation, as well as the *in vitro* biodegradation, the typical weight loss after 9 week being 15–19%. They supported attachment and proliferation of smooth muscle cells. In addition, the hydrogel based on the most anionic *O*-carboxymethyl chitosan showed higher adsorption of basic TGF-β1 than similarly modified agarose, dextran and chondroitin sulfate hydrogels (Poon, Bin Zhu, Shen, Chan-Park, & Ng, 2007).

Following the same approach, glycol chitosan, a derivative soluble at neutral pH, was converted into a photo-polymerizable prepolymer through graft methacrylation using glycidyl methacrylate in aqueous media at pH 9 with a view at obtaining cytocompatible hydrogels for use as scaffolds or drug delivery carriers. The degree of *N*-methacrylation, verified by H^1 NMR and C^{13} NMR, was easily varied in the range 1.5–25% by acting on reaction time and molar ratio of glycidyl methacrylate to glycol chitosan. By using a chondrocyte cell line, the *N*-methacrylated glycol chitosan was found to be cytocompatible up to 1 mg/ml. The prepolymer was crosslinked in solution by using UV light and the Irgacure 2959 photoinitiator to yield gels of low sol content (ca. 5%), high equilibrium water content (85–95%) and thickness up to 6 mm. Evidence of the complete conversion of the double bonds in the gel was obtained. Chondrocytes seeded directly onto the gel surface, populated the entirety of the gel and remained viable for up to 1 week. The hydrogels degraded slowly *in vitro* in the presence of lysozyme at a rate that increased with decreasing crosslink density (Amsden, Sukarto, Knight, & Shapka, 2007). In photo-active chitosans based on naphthyl chromophores the excitation energy was used to induce photo-chemical reactions via energy and electron transfer (Nowakowska, Moczek, & Szczubialka, 2008).

12. Conclusions

Recent progress in wound management is mainly in terms of physiologic support of healing. Chitin and chitosan substantially contribute to the advances in this direction, as testified by the sound knowledge of the biochemical mechanisms of healing promoted by various forms of these polysaccharides, and also by the absence of adverse data in the many articles published since the early observations 20 years ago. The modern clinical approach to improving the appearance and functionality of regenerated tissue in the healed wounds finds a valid basis in the versatility, functionality and efficacy of chitosans, whose performances underline the obsolescence of the cellulose-based medical items.

References

Abarrategi, A., Civantos, A., Ramos, V., Casado, J. V. S., & Lopez-Lacomba, J. L. (2008). Chitosan film as rhBMP2 carrier: Delivery properties for bone tissue application. *Biomacromolecules, 9*, 711–718.

Albo, D., Long, C., Jhala, N., Atkinson, B., Granick, MS., Wang, T., et al. (1996). Modulation of cranial bone healing with heparin-like dextran derivatives. *Journal of Craniofacial Surgery, 7*, 19–22.

Amsden, B. G., Sukarto, A., Knight, D. K., & Shapka, S. N. (2007). Methacrylated glycol chitosan as a photopolymerizable biomaterial. *Biomacromolecules, 8*, 3758–3766.

Arpornmaeklong, P., Pripatnanont, P., & Suwatwirote, N. (2008). Properties of chitosan-collagen sponges and osteogenic differentiation of rat-bone-marrow stromal cells. *International Journal of Oral and Maxillofacial Surgery, 37*, 357–366.

Bakkers, J., Semino, C. E., Stroband, H., Kijne, J. W., Robbins, P. W., & Spain, H. P. (1997). An important developmental role for oligosaccharides during early embryogenesis of cyprinid fish. *Proceedings of the National Academy of Sciences, 94*, 7982–7986.

Berthod, F., Sahuc, F., Hayek, D., Damour, O., & Collombel, C. (1996). Deposition of collagen fibril bundles by long-term culture of fibroblasts in a collagen sponge. *Journal of Biomedical Material Research, 32*, 87.

Bottomley, K. M. K., Bradshaw, D., & Nixon, J. S. (Eds.). (1999). *Metalloproteinases as targets for anti-Inflammatory drugs*. Basel: Birkhauser.

Boddohi, S., Killingsworth, C. E., & Kipper, M. J. (2008). Polyelectrolyte multilayer assembly as a function of pH and ionic strength using the polysaccharides chitosan and heparin. *Biomacromolecules, 9*, 2021–2028.

Bruyere, O., Pavelka, K., Rovati, L. C., Gatterova, J., Giacovelli, G., Olejarova, M., et al. (2008). Total joint replacement after glucosamine sulphate treatment in knee osteoarthritis: Results of a mean 8-year observation of patients from two previous 3-year, randomised, placebo-controlled trials. *Osteoarthritis and Cartilage, 16*, 254–260.

Bumgardner, J. D., Wiser, R., Elder, S. H., Jouett, R., Yang, Y., & Ong, J. L. (2003a). Contact angle, protein adsorption and osteoblast precursor cell attachment to chitosan coatings bonded to titanium. *Journal of Biomaterial Science Polymer Edition, 14*, 1401–1409.

Bumgardner, J. D., Wiser, R., Gerard, D., Bergin, P., Chestnutt, B., Marin, M., et al. (2003b). Chitosan: Potential use as a bioactive coating for orthopaedic and craniofacial/dental implants. *Journal of Biomaterial Science Polymer Edition, 14*, 423–438.

Burkatovskaya, M., Castano, A. P., Demidova-Rice, T. N., Tegos, G. P., & Hamblin, M. R. (2008). Effect of chitosan acetate bandage on wound healing in infected and noninfected wounds in mice. *Wound Repair and Regeneration, 16*, 425–431.

Chavez-Delgado, M. E., Mora-Galindo, J., Gomez-Pinedo, U., Feria-Velasco, A., Castro-Castaneda, S., Toral, F. A. L. D., et al. (2003). Facial nerve regeneration through chitosan prosthesis. *Journal of Biomedical Materials Research*, 702–711.

Chen, R. H., & Chen, H. C. (Eds.). (1998). *Advances in chitin science*. Taipei: Rita Advertising Co.
Chen, Q., Liu, S. Q., Du, Y. M., Peng, H., & Sun, L. P. (2006). Carboxymethyl-chitosan protects rabbit chondrocytes from interleukin-1 beta-induced apoptosis. *European Journal of Pharmacology, 541*, 1–8.
Chen, X. G., Wang, Z., Liu, W. S., & Park, H. J. (2002). The effect of carboxymethyl chitosan on proliferation and collagen secretion of normal and keloid skin fibroblasts. *Biomaterials, 23*, 4609–4614.
Cho, Y. W., Cho, Y. N., Chung, S. H., Yoo, G., & Ko, S. W. (1999). Water-soluble chitin as a wound healing accelerator. *Biomaterials, 20*, 2139–2145.
Cho, J. H., Kim, S. H., Park, K. D., Jung, M. C., Yang, W. I., Han, S. W., et al. (2004). Chondrogenic differentiation of human mesenchymal stem cells using a thermosensitive poly(*N*-isopropylacrylamide) and water-soluble chitosan copolymer. *Biomaterials, 25*, 5743–5751.
Chopra, S., Mahdi, S., Kaur, J., Iqbal, Z., Talegaonkar, S., & Ahmad, F. J. (2006). Advances and potential applications of chitosan derivatives as mucoadhesive biomaterials in modern drug delivery. *Journal of Pharmacy and Pharmacology, 58*, 1021–1032.
Chu, S. C., Yang, S. F., Lue, K. H., Hsieh, Y. S., Lee, C. Y., Chou, M. C., et al. (2006). Glucosamine sulfate suppresses the expressions of urokinase plasminogen activator and inhibitor, and gelatinases during the early stage of osteoarthritis. *Clinica Chimica Acta, 372*, 167–172.
Chua, P. H., Neoh, K. G., Kang, E. T., & Wang, W. (2008). Surface functionalization of titanium with hyaluronic acid/chitosan polyelectrolyte multilayers and RGD for promoting osteoblast functions and inhibiting bacterial adhesion. *Biomaterials, 29*, 1412–1421.
Chun, H. J., Kim, G. W., & Kim, C. H. (2008). Fabrication of porous chitosan scaffold in order to improve biocompatibility. *Journal of Physics and Chemistry of Solids, 69*, 1573–1576.
Chung, T. W., Lu, Y. F., Wang, S. S., Lin, Y. S., & Chu, S. H. (2002). Growth of human endothelial cells on photochemically grafted Gly-Arg-Gly-Asp (GRGD) chitosans. *Biomaterials, 23*, 4803–4809.
Clark, R. (1996). Wound repair overview and general considerations. In R. Clark (Ed.), *The molecular and cellular biology of wound repair* (pp. 3–50). New York: Plenum Press.
Crompton, K. E., Goud, J. D., Bellamkonda, R. V., Gengenbach, T. R., Finkelstein, D. I., Horne, M. K., et al. (2007). Polylysine-functionalised thermoresponsive chitosan hydrogel for neural tissue engineering. *Biomaterials, 28*, 441–449.
Cui, Y. L., Qi, A. D., Liu, W. G., Wang, X. H., Ma, D. M., & 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.
Cui, X., Zhang, B., Wang, Y., & Gao, Y. L. (2008). Effects of chitosan-coated pressed calcium sulfate pellet combined with recombinant human bone morphogenetic protein 2 on restoration of segmental bone defect. *Journal of Craniofacial Surgery, 19*, 459–465.
Cuy, J. L., Beckstead, B. L., Brown, C. D., Hoffman, A. S., & Giachelli, C. M. (2003). Adhesive protein interactions with chitosan: Consequences for valve endothelial cell growth on tissue-engineering materials. *Journal of Biomedical Material Research, A 67*, 538–544.
Dahiya, N., Tewari, R., & Hoondal, G. S. (2006). Biotechnological aspects of chitinolytic enzymes. *Applied Microbiology and Biotechnology, 71*, 773–782.
Dang, J. M., Sun, D. D., Shin-Ya, Y., Sieber, A. N., Kostuik, J. P., & Leong, K. W. (2006). Temperature-responsive hydroxybutyl chitosan for the culture of mesenchymal stem cells and intervertebral disk cells. *Biomaterials, 27*, 406–418.
Degim, Z. (2008). Use of microparticulate systems to accelerate skin wound healing. *Journal of Drug Targeting, 16*, 437–448.
Degim, Z., Celebi, N., Sayan, H., Babul, A., Erdogan, D., & Take, G. (2002). An investigation on skin wound healing in mice with a taurine-chitosan gel formulation. *Amino Acids, 22*, 187–198.
Di Martino, A., Sittinger, M., & Risbud, M. V. (2005). Chitosan, a versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials, 26*, 5983.
Englehart, M. S., Cho, S. D., Tieu, B. H., Morris, M. S., Underwood, S. J., Karahan, A., et al. (2008). A novel highly porous silica and chitosan-based hemostatic dressing is superior to HemCon and gauze sponges. *Journal of Trauma-Injury Infection and Critical Care, 65*, 884–890.
Fakhry, A., Schneider, G. B., Zaharias, R., & Senel, S. (2004). Chitosan supports the initial attachment and spreading of osteoblasts preferentially over fibroblasts. *Biomaterials, 25*, 2075–2079.
Felson, D. T. (2008). Glucosamine sulfate might have no effect on pain or structural changes associated with osteoarthritis. *Nature Clinical Practice Rheumatology, 4*(10), 518–519.
Ferguson, M. W., & O'Kane, S. (2004). Scar-free healing: From embryonic mechanisms to adult therapeutic intervention. *Philosophical Transactions of the Royal Society London, B 359*, 839–850.
Fischer, T. H., Bode, A. P., Demcheva, M., & Vournakis, J. N. (2007). Hemostatic properties of glucosamine-based materials. *Journal of Biomedical Materials Research, 80A*, 167–174.
Fischer, T. H., Thatte, HS., Nichols, T. C., Bender-Neal, D. E., Bellinger, A. D., & Vournakis, J. N. (2005). Synergistic platelet integrin signaling and factor XII activation in poly-*N*-acetyl glucosamine fiber-mediated hemostasis. *Biomaterials, 26*, 5433–5443.
Fischer, T. H., Valeri, C. R., Smith, C. J., Scull, C. M., Merricks, E. P., Nichols, T. C., et al. (2008). Non-classical processes in surface hemostasis: Mechanisms for the poly-*N*-acetyl glucosamine-induced alteration of red blood cell morphology and surface prothrombogenicity. *Biomedical Materials, 3*, 65–73.
Freier, T., Koh, H. S., Kazazian, K., & Shoichet, M. S. (2005a). Controlling cell adhesion and degradation of chitosan films by *N*-acetylation. *Biomaterials, 26*, 5872–5878.
Freier, T., Montenegro, R., Koh, H. S., & Shoichet, M. S. (2005b). Chitin-based tubes for tissue engineering in the nervous system. *Biomaterials, 26*, 4624–4632.
Fujita, M., Kinoshita, M., Ishihara, M., Kanatani, Y., Morimoto, Y., Simizu, M., et al. (2004). Inhibition of vascular prosthetic graft infection using a photo-crosslinkable chitosan hydrogel. *Journal of Surgical Research, 121*, 135–140.
Galiano, R. D., Tepper, O. M., Pelo, C. R., Bhatt, K. A., Callaghan, M., Bastidas, N., et al. (2004). Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. *American Journal of Pathology, 164*, 1935–1947.
Ge, Z., Baguenard, S., Lim, L. Y., Wee, A., & Khor, E. (2004). Hydroxyapatite chitin materials as potential tissue engineered bone substitutes. *Biomaterials, 25*, 1049–1058.
Gingras, M., Paradis, I., & Berthod, F. (2003). Nerve regeneration in a collagen-chitosan tissue-engineered skin transplanted on nude mice. *Biomaterials, 24*, 1653–1658.
Gorzelanny, C., Poppelmann, B., Strozyk, E., Moerschbacher, B. M., & Schneider, S. W. (2007). Specific interaction between chitosan and matrix metalloprotease 2 decreases the invasive activity of human melanoma cells. *Biomacromolecules, 8*, 3035–3040.
Greene, A. H., Bumgardner, J. D., Yang, Y., Moseley, J., & Haggard, W. O. (2008). Chitosan-coated stainless steel screws for fixation in contaminated fractures. *Clinical Orthopaedics and Related Research, 466*, 1699–1704.
Hong, Y. K., Lange-Asschenfeldt, B., Velasco, A., Hirakawa, S., Kunstfeld, R., Brown, L. F., et al. (2004). VEGF-A promotes tissue repair-associated lymphatic vessel formation via VEGFR-2 and the alpha1beta1 and alpha2beta1 integrins. *FASEB Journal, 18*, 1111–1113.
Hsieh, C. Y., Tsai, S. P., Wang, D. M., Chang, Y. N., & Hsieh, H. J. (2005). Preparation of gamma-PGA/chitosan composite tissue engineering matrices. *Biomaterials, 26*, 5617.
Hsu, S. H., Whu, S. W., Hsieh, S. C., Tsai, C. L., Chen, D. C., & Tan, T. S. (2004). Evaluation of chitosan–alginate–hyaluronate complexes modified by an RGD-containing protein as tissue-engineering scaffolds for cartilage regeneration. *Artificial Organs, 28*, 693–703.
Hu, S. G., Jou, C. H., & Yang, M. C. (2003). Protein adsorption, fibroblast activity and antibacterial properties of poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) grafted with chitosan and chitooligosaccharide after immobilization with hyaluronic acid. *Biomaterials, 24*, 2685–2689.
Huxtable, R. J., Azuma, J., Kuriyama, K., Nakagawa, M., & Baba, A. (Eds.). (1996). *Taurine, basic and clinical aspects*. New York: Plenum Press.
Huang, R. L., Deng, Z. Y., Yang, C. B., Yin, Y. L., Xie, M. Y., Wu, G. Y., et al. (2007). Dietary oligochitosan supplementation enhances immune status of broilers. *Journal of the Science of Food and Agriculture, 87*, 153–159.
Ishihara, M. (2002). Photo-crosslinkable chitosan hydrogel as a wound dressing and a biological adhesive. *Trends in Glycoscience and Glycotechnology, 14*, 331–341.
Ishihara, M., Nakanishi, K., Ono, K., Sato, M., Kikuchi, M., Saito, Y., et al. (2002). Photocrosslinkable chitosan as a dressing for wound occlusion and accelerator in the healing process. *Biomaterials, 23*, 833–840.
Ishizuka, T., Hayashi, T., Ishihara, M., Yoshizumi, Y., Aiko, S., Nakamura, S., et al. (2007). Submucosal injection for endoscopic mucosal resection, of photocrosslinkable chitosan hydrogel in DMEM/F12 medium. *Endoscopy, 39*, 428–433.
Issa, J. P. M., doNascimento, C., Bentley, M. V. L. B., DelBel, E. A., Iyomasa, M. M., Sebald, W., et al. (2008). Bone repair in rat mandible by rhBMP-2 associated with two carriers. *Micron, 39*, 373–379.
Itoh, S., Suzuki, M., Yamaguchi, I., Takakuda, K., Kobayashi, H., Shinomiya, K., et al. (2003). Development of a nerve scaffold using a tendon chitosan tube. *Artificial Organs, 27*, 1079–1088.
Iwasaki, N., Yamane, S. T., & Majima, T. (2004). Feasibility of polysaccharide hybrid materials for scaffolds in cartilage tissue engineering: Evaluation of chondrocyte adhesion to polyion complex fibers prepared from alginate and chitosan. *Biomacromolecules, 5*, 828–833.
Janvikul, W., Uppanan, P., Thavornyutikarn, B., Prateepasen, R., & Swasdison, S. (2007). Fibroblast interaction with carboxymethyl chitosan-based hydrogels. *Journal of Materials Science – Materials in Medicine, 18*, 943–949.
Jollès, P., & Muzzarelli, R. A. A. (Eds.). (1999). *Chitin and chitinases*. Basel: Birkhauser Verlag.
Jiang, T. D. (2001). *Chitosan*. Peking: Chemical Industry Publ..
Katrin, K., Yang, W., Harald, K., Perelman, L. T., Itzkan, I., & Dasari, R. R. (1997). Single molecule detection using surface enhanced Raman scattering. *Physics Reviews Letters, 78*, 1667–1670.
Kawakami, T., Antoh, M., Hasegawa, H., Yamagishi, T., Ito, M., & Eda, S. (1992). Experimental study on osteoconductive properties of a chitosan-bonded hydroxyapatite self-hardening paste. *Biomaterials, 13*, 759–763.
Kim, M. M., & Kim, S. K. (2006). Chitooligosaccharides inhibit activation and expression of matrix metalloproteinase-2 in human dermal fibroblasts. *FEBS Letters, 580*, 2661–2666.
Kim, S. E., Park, J. H., Cho, Y. W., Chung, H., Jeong, S. Y., Lee, E. B., et al. (2003). Porous chitosan scaffold containing microspheres loaded with transforming growth factor-beta1: Implications for cartilage tissue engineering. *Journal of Controlled Release, 91*, 365–374.
Kim, I. S., Park, J. W., Kwon, I. C., Baik, B. S., & Cho, B. C. (2002). Role of BMP, betaig-h3, and chitosan in early bony consolidation in distraction osteogenesis in a dog model. *Plastic and Reconstruction Surgery, 109*, 1966–1977.

Kiyozumi, T., Kanatani, Y., Ishihara, M., Saitoh, D., Shimizu, J., Yura, H., et al. (2007). The effect of chitosan hydrogel containing DMEM/F12 medium on full-thickness skin defects after deep dermal burn. *Burns, 33*, 642–648.

Kojima, K., Okamoto, Y., Kojima, K., Miyatake, K., Fujise, H., Shigemasa, Y., et al. (2004). Effects of chitin and chitosan on collagen synthesis in wound healing. *Journal of Veterinary Medical Science, 66*, 1595–1698.

Kumar, M. N. V. R., Muzzarelli, R. A. A., Muzzarelli, C., Sashiwa, H., & Domb, A. J. (2004). Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews, 104*, 6017–6084.

Kurita, K. (2006). Chitin and chitosan: Functional biopolymers from marine crustaceans. *Marine Biotechnology, 8*, 203–226.

Lahiji, A., Sohrabi, A., Hungerford, D. S., & Frondoza, C. G. (2000). Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. *Journal of Biomedical Material Research, 51*, 586–595.

Lauto, A., Foster, L. J., Avolio, A., Sampson, D., Raston, C., Sarris, M., et al. (2008). Sutureless nerve repair with laser-activated chitosan adhesive: A pilot in vivo study. *Photomedicine and Laser Surgery, 26*, 227–234.

Lee, J. E., Kim, K. E., Kwon, I. C., Ahn, H. J., Lee, S. H., Cho, H. C., et al. (2004). 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, K. Y., & Mooney, D. J. (2001). Hydrogels for tissue engineering. *Chemical Reviews, 101*, 1869–1879.

Lee, J. Y., Nam, S. H., Im, S. Y., Park, Y. J., Lee, Y. M., Seol, Y. J., et al. (2002). Enhanced bone formation by controlled growth factor delivery from chitosan-based biomaterials. *Journal of Controlled Release, 78*, 187–197.

Lee, Y. M., Park, Y. J., Lee, S. J., Ku, Y., Han, S. B., Choi, S. M., et al. (2000a). Tissue engineered bone formation using chitosan/tricalcium phosphate sponges. *Journal of Periodontology, 71*, 410–417.

Lee, Y. M., Park, Y. J., Lee, S. J., Ku, Y., Han, S. B., Klokkevold, P. R., et al. (2000b). The bone regenerative effect of platelet-derived growth factor delivered with a chitosan/tricalcium phosphate sponge carrier. *Journal of Periodontology, 71*, 418–424.

Leroux, L., Hatim, Z., Freche, M., & Lacout, J. L. (1999). Effects of various adjuvants (lactic acid, glycerol, and chitosan) on the injectability of a calcium phosphate cement. *Bone, 25*(Suppl. 2), 31S–34S.

Li, X. M., Feng, Q. L., Liu, X. H., Dong, W., & Cui, F. H. (2006). Collagen-based implants reinforced by chitin fibres in a goat shank bone defect model. *Biomaterials, 27*, 1917–1923.

Lin, Y. C., Liang, Y. C., Sheu, M. T., Lin, Y. C., Hsieh, M. S., Chen, T. F., et al. (2008). Chondroprotective effects of glucosamine involving the p38 MAPK and Akt signaling pathways. *Rheumatology International, 28*, 1009–1016.

Lu, S. Y., Gao, W. J., & Gu, H. Y. (2008). Construction, application and biosafety of silver nanocrystalline chitosan wound dressing. *Burns, 34*, 623–628.

Lu, N., Heuchel, R., Barczyk, M., Zhang, W. M., & Gullberg, D. (2006). Tandem Sp1/Sp3 sites together with an Ets-1 site cooperate to mediate alpha11 integrin chain expression in mesenchymal cells. *Matrix Biology, 25*, 118–129.

Lu, G. Y., Kong, L. J., Sheng, B. Y., Wang, G., Gong, Y. D., & Zhang, X. F. (2007). Degradation of covalently cross-linked carboxymethyl chitosan and its potential application for peripheral nerve regeneration. *European Polymer Journal, 43*, 3807–3818.

Maeda, Y., Jayakumar, R., Nagahama, H., Furuike, T., & Tamura, H. (2008). Synthesis, characterization and bioactivity studies of novel beta-chitin scaffolds for tissue-engineering applications. *International Journal of Biological Macromolecules, 42*, 463–467.

Mao, C., Zhu, J. J., Hu, Y. F., Ma, Q. Q., Qiu, Y. Z., Zhu, A. P., et al. (2004). Surface modification using photo-crosslinkable chitosan for improving hemo-compatibility. *Colloids and Surfaces, B-38*, 47–53.

Matsuda, A., Kobayashi, H., Itoh, S., Kataoka, K., & Tanaka, J. (2005). Immobilization of laminin peptide in molecularly aligned chitosan by covalent bonding. *Biomaterials, 26*, 2273–2279.

Mattioli-Belmonte, M., Gigante, A., Muzzarelli, R. A. A., Politano, R., DeBenedittis, A., Specchia, N., et al. (1999a). *N,N*-Dicarboxymethyl chitosan as delivery agent for bone morphogenetic protein in the repair of articular cartilage. *Medical and Biological Engineering and Computing, 37*, 130–134.

Mattioli-Belmonte, M., Nicoli-Aldini, N., DeBenedittis, A., Sgarbi, G., Amati, S., Fini, M., et al. (1999b). Morphological study of bone regeneration in the presence of 6-oxychitin. *Carbohydrate Polymers, 40*, 23–27.

Mendis, E., Kim, M. M., Rajapakse, N., & Kim, S. K. (2006). Carboxy derivatized glucosamine is a potent inhibitor of matrix metalloproteinase-9 in HT1080 cells. *Bioorganic and Medicinal Chemistry Letters, 16*, 3105–3110.

Mi, F. L., Wu, Y. Y., Lin, Y. H., Sonaje, K., Ho, Y. C., Chen, C. T., et al. (2008). Oral delivery of peptide drugs using nanoparticles self-assembled by poly(gamma-glutamic acid) and a chitosan derivative functionalized by trimethylation. *Bioconjugate Chemistry, 19*, 1248–1255.

Miller, M. C., & Nanchahal, J. (2005). Advances in the modulation of cutaneous wound healing and scarring. *BioDrugs, 19*, 363–381.

Minagawa, T., Okamura, Y., Shigemasa, Y., Minami, S., & Okamoto, Y. (2007). Effects of molecular weight and deacetylation degree of chitin/chitosan on wound healing. *Carbohydrate Polymers, 67*, 640–644.

Miyazawa, K. (1995). Osteoinduction of BMP-chitin complex. *Journal of Hard Tissue Biology, 4*, 70–81.

Mochizuki, M., Kadoya, Y., Wakabayashi, Y., Kato, K., Okazaki, I., Yamada, M., et al. (2003). Laminin-1 peptide-conjugated chitosan membranes as a novel approach for cell engineering. *FASEB Journal, 17*, 586–605.

Moon, J. S., Kim, H. K., Koo, H. C., Joo, Y. S., Nam, H. M., Park, Y. H., et al. (2007). The antibacterial and immunostimulating effects of chitosan-oligosaccharides against infection by Staphylococcus aureus isolated from bovine mastitis. *Applied Microbiology and Biotechnology, 75*, 989–998.

Mori, T., Murakami, M., Okumura, M., Kadosawa, T., Uede, T., & Fujinaga, T. (2005). Mechanism of macrophage activation by chitin derivatives. *Journal of Veterinary Medical Science, 67*, 51–55.

Mourya, V. K., & Inamdar, N. N. (2008). Chitosan-modifications and applications: Opportunities galore. *Reactive and Functional Polymers, 68*, 1013–1051.

Muzzarelli, R. A. A. (1977). *Chitin*. Oxford: Pergamon Press.

Muzzarelli, R. A. A. (2008a). Chitin and chitosan hydrogels. In G. O. Philips & A. Williams (Eds.), *Handbook of hydrocolloids* (2nd ed.). Cambridge, UK: Woodhead Publishing Ltd.

Muzzarelli, R. A. A. (2008b). Chitin chemistry and biochemistry. In M. Paoletti & S. Musumeci (Eds.), *Binomium chitin-chitinase: Emerging issues*. Hauppauge, NY: Nova Science.

Muzzarelli, R. A. A. (2008c). Chitin nanostructures in living organisms. In S. N. Gupta & D. Briggs (Eds.), *Chitin in the fossil record*. New York: Springer.

Muzzarelli, R. A. A., Biagini, G., DeBenedittis, A., Mengucci, P., Majni, G., & Tosi, G. (2001). Chitosan-oxychitin coatings for prosthetic materials. *Carbohydrate Polymers, 45*, 35–41.

Muzzarelli, R. A. A., Biagini, G., Mattioli-Belmonte, M., Talassi, O., Gandolfi, M. G., Solmi, R., et al. (1997). Osteoinduction by chitosan-complexed BMP. Morpho-structural response in an osteoporotic model. *Journal of Bioactive and Compatible Polymers*, 321–329.

Muzzarelli, R. A. A., Cosani, A., & Terbojevich, M. (1999). 6-Oxychitins, novel hyaluronan-like polysaccharides obtained by regioselective oxidation of chitins. *Carbohydrate Polymers, 39*, 361–367.

Muzzarelli, C., Stanic, V., Gobbi, L., Tosi, G., & Muzzarelli, R. A. A. (2004). Spray-drying of solutions containing chitosan together with polyuronans, and characterization of the microspheres. *Carbohydrate Polymers, 57*, 73–82.

Muzzarelli, R. A. A., Morganti, P., Morganti, G., Palombo, P., Palombo, M., Biagini, G., et al. (2007). Chitin nanofibrils/chitosan glycolate composites as wound medicaments. *Carbohydrate Polymers, 70*, 274–284.

Muzzarelli, R. A. A., & Muzzarelli, C. (2006). Chitosan, a dietary supplement and a food technology commodity. In C. G. Biliaderis & M. S. Izydorczyk (Eds.), *Functional food carbohydrates*. Orlando, USA: Francis and Taylor.

Muzzarelli, R. A. A., & Sipos, L. (1971). Chitosan for the collection from seawater of naturally occurring zinc, cadmium, lead and copper. *Talanta, 28*, 53–58.

Muzzarelli, R. A. A., Guerrieri, M., Goteri, G., Muzzarelli, C., Armeni, T., Ghiselli, R., et al. (2005). The biocompatibility of dibutyryl chitin in the context of wound dressings. *Biomaterials, 26*, 5844–5854.

Muzzarelli, R. A. A., Mattioli-Belmonte, M., Tietz, C., Biagini, R., Ferioli, G., Brunelli, M. A., et al. (1994). Stimulatory effect on bone formation exerted by a modified chitosan. *Biomaterials, 15*, 1075–1081.

Muzzarelli, R. A. A., Miliani, M., Cartolari, M., Genta, I., Perugini, P., Modena, T., et al. (2000). Pharmaceutical use of the 6-oxychitin-chitosan polyelectrolyte complex. *STP Pharma Sciences, 10*, 51–56.

Muzzarelli, R. A. A., Ramos, V., Stanic, V., Dubini, B., Mattioli-Belmonte, M., Tosi, G., et al. (1998). Osteogenesis promoted by calcium phosphate dicarboxyboxy-methyl chitosan. *Carbohydrate Polymers, 36*, 267–276.

Muzzarelli, R. A. A., Zucchini, C., Ilari, P., Pugnaloni, A., Mattioli-Belmonte, M., Biagini, G., et al. (1993). Osteoconductive properties of methylpyrrolidinone chitosan in an animal model. *Biomaterials, 4*, 925–929.

Nakade, T., Yokota, H., Taniyama, H., Hori, Y., Agata, N., Ikeda, T., et al. (2000). Matrix metalloproteinase 9 induced in skin and subcutaneous tissue by implanted chitin in rats. *Carbohydrate Polymers, 41*, 327–329 [corrected 42, 315].

Nowakowska, M., Moczek, L., & Szczubialka, K. (2008). Photoactive modified chitosan. *Biomacromolecules, 9*, 1631–1636.

Obara, K., Ishihara, M., Ishizuka, T., Fujita, M., Ozeki, Y., Maehara, T., et al. (2003). Photo-crosslinkable chitosan hydrogel containing fibroblast growth factor-2 stimulates wound healing in healing-impaired mice. *Biomaterials, 24*, 3437–3444.

Oda, N., Abe, M., & Sato, Y. (1999). Ets-1 converts endothelial cells to the angiogenic phenotype by inducing the expression of matrix metalloproteinases and integrin beta3. *Journal of Cell Physiology, 178*, 121–132.

Okamoto, Y., Inoue, A., Miyatake, K., Ogihara, K., Shigemasa, Y., & Minami, S. (2003). Effects of chitin/chitosan and their oligomers/monomers on migrations of macrophages. *Macromolecular Bioscience, 3*, 587–590.

Okamura, Y., Nomura, A., Minami, S., & Okamoto, Y. (2005). Effects of chitin/chitosan and their oligomers/monomers on release of type I collagenase from fibroblasts. *Biomacromolecules, 6*, 2382–2384.

Ono, K., Ishihara, M., Ozeki, Y., Deguchi, H., Sato, M., Saito, Y., et al. (2001). Experimental evaluation of photo-crosslinkable chitosan as a biologic adhesive with surgical applications. *Surgery, 130*, 844–850.

Ono, K., Saito, Y., Yura, H., Ishikawa, K., Kurita, A., Akaike, T., et al. (2000). Photo-crosslinkable chitosan as a biological adhesive. *Journal of Biomedical Material Research, 49*, 289–295.

Palmer, B. L., Gantt, D. S., Lawrence, M. E., Rajab, M. H., & Dehmer, G. J. (2004). Effectiveness and safety of manual hemostasis facilitated by the Syvek-Patch with 1 h of bedrest after coronary angiography using six-French catheters. *American Journal of Cardiology, 93*, 96–97.

Pang, X., & Zhitomirsky, I. (2007). Electrophoretic deposition of composite hydroxyapatite-chitosan coatings. *Materials Characterization, 58*, 339–348.

Park, D. J., Choi, B. H., Zhu, S. J., Huh, J. Y., Kim, B. Y., & Lee, S. H. (2005). Injectable bone using chitosan-alginate gel/mesenchymal stem cells/BMP-2 composites. *Journal of Cranio-Maxillofacial Surgery, 33*, 50–54.

Patel, M., Mao, L., Wu, B., & VandeVord, P. J. (2007). GDNF-chitosan blended nerve guides: A functional study. *Journal of Tissue Engineering and Regenerative Medicine, 1*, 360–367.

Pietramaggiori, G., Yang, H. J., Scherer, S. S., Kaipainen, A., Chan, R. K., Alperovich, M., et al. (2008). Effects of poly-N-acetyl glucosamine (pGlcNAc) patch on wound healing in db/db mouse. *Journal of Trauma, 64*, 803–808.

Polnok, A., Borchard, G., Verhoef, J. C., Sarisuta, N., Junginger, H. E., Borchard, G., et al. (2004). Influence of methylation process on the degree of quaternization of *N*-trimethylchitosan chloride. *European Journal of Pharmaceutics and Biopharmaceutics, 57*, 77–83.

Poon, Y. F., Bin Zhu, Y., Shen, J. Y., Chan-Park, M. B., & Ng, S. C. (2007). Cytocompatible hydrogels based on photocrosslinkable methacrylated *O*-carboxymethyl chitosan with tunable charge: Synthesis and characterization. *Advanced Functional Materials, 17*, 2139–2150.

Rajapakse, N., Mendis, E., Kim, M. M., & Kim, S. K. (2007). Sulfated glucosamine inhibits MMP-2 and MMP-9 expressions in human fibrosarcoma cells. *Bioorganic and Medicinal Chemistry, 15*, 4891–4896.

Renbutsu, E., Hirose, M., Omura, Y., Nakatsubo, F., Okamura, Y., Okamoto, Y., et al. (2005). Preparation and biocompatibility of novel UV-curable chitosan derivatives. *Biomacromolecules, 6*, 2385–2388.

Rhett, J. M., Ghatnekar, G. S., Palatinus, J. A., O'Quinn, M., Yost, M. J., & Gourdie, R. G. (2008). Novel therapies for scar reduction and regenerative healing of skin wounds. *Trends in Biotechnology, 26*, 173–180.

Riha, G. M., Lin, H., Lumsden, A. B., Yao, Q., & Chen, C. (2005). Application of stem cells for vascular tissue engineering. *Tissue Engineering, 11*, 1535–1540.

Rinaudo, M. (2006a). Characterization and properties of some polysaccharides used as biomaterials. *Macromolecular Symposia, 245*, 549–557.

Rinaudo, M. (2006b). Chitin and chitosan: Properties and applications. *Progress in Polymer Science, 31*, 603–632.

Risbud, M., Ringe, J., Bhonde, R., & Sittinger, M. (2001). In vitro expression of cartilage-specific markers by chondrocytes on a biocompatible. *Cell Transplantation, 10*, 755–763.

Roberts, A. B., & Sporn, M. B. (1996). Transforming growth factor beta. In R. Clark (Ed.), *The molecular and cellular biology of wound repair* (pp. 275–308). New York: Plenum Press.

Rodrigues, A. P., Sanchez, E. M. S., daCosta, A. C., & Moraes, A. M. (2008). The influence of preparation conditions on the characteristics of chitosan-alginate dressings for skin lesions. *Journal of Applied Polymer Science, 109*, 2703–2710.

Rosen, G. D., Barks, J. L., Iademarco, M. F., Fisher, R. J., & Dean, D. C. (1994). An intricate arrangement of binding sites for the Ets family of transcription factors regulates activity of the alpha 4 integrin gene promoter. *Journal of Biological Chemistry, 269*, 15652–15660.

Ruel-Gariepy, E., & Leroux, J. C. (2006). Chitosan: A natural polycation with multiple applications. In *Polysaccharides for drug delivery and pharmaceutical applications* (934, pp. 243–259). Washington, USA: American Chemical Society.

Seol, Y. J., Lee, J. Y., Park, Y. J., Lee, Y. M., Young-Ku Rhyu, I. C., Lee, S. J., et al. (2004). Chitosan sponges as tissue engineering scaffolds for bone formation. *Biotechnology Letters, 26*, 1037–1041.

Shi, C. M., Zhu, Y., Ran, X. Z., Wang, M., Su, Y., & Cheng, T. M. (2006). Therapeutic potential of chitosan and its derivatives in regenerative medicine. *Journal of Surgical Research, 133*, 185–192.

Shibata, Y., Foster, L. A., Metzger, W. J., & Myrvik, Q. N. (1997a). Alveolar macrophage priming by intravenous administration of chitin particles in mice. *Infection and Immunity*, 1734–1741.

Shibata, Y., Metzger, W. J., & Myrvik, Q. N. (1997b). Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan. Mannose receptor-mediated phagocytosis initiates IL-12 production. *Journal of Immunology, 159*, 2462–2467.

Short, B., Brouard, N., Occhiodoro-Scott, T., Ramakrishnan, A., & Simmons, J. (2003). Mesenchymal stem cells. *Archives of Medical Research, 34*, 565–570.

Sieval, A. B., Thanou, M., Kotze, A. F., Verhoef, J. C., Brussee, J., & Junginger, H. E. (1998). Preparation and NMR characterization of highly substituted *N*-trimethyl chitosan chloride. *Carbohydrate Polymers, 36*, 157–165.

Skotak, M., Leonov, AP., Larsen, G., Noriega, S., & Subramanian, A. (2008). Biocompatible and biodegradable ultrafine fibrillar scaffold materials for tissue engineering by facile grafting of L-lactide onto chitosan. *Biomacromolecules, 9*, 1902–1908.

Sogias, I. A., Williams, A. C., & Khutoryanskiy, V. V. (2008). Why is chitosan mucoadhesive? *Biomacromolecules, 9*, 1837–1842.

Suzuki, D., Takahashi, M., Abe, M., Sarukawa, J., Tamura, H., Tokura, S., et al. (2008). Comparison of various mixtures of beta-chitin and chitosan as a scaffold for three-dimensional culture of rabbit chondrocytes. *Journal of Materials Science – Materials in Medicine, 19*, 1307–1315.

Suzuki, M., Itoh, S., Yamaguchi, I., Takakuda, K., Kobayashi, H., Shinomiya, K., et al. (2003). Tendon chitosan tubes covalently coupled with synthesized laminin peptides facilitate nerve regeneration in vivo. *Journal of Neuroscience Research, 72*, 646–659.

Tajima, A., Miyamoto, Y., Kadowaki, H., & Hayashi, M. (2000). Mouse integrin alphaV promoter is regulated by transcriptional factors Ets and Sp1 in melanoma cells. *Biochimica et Biophysica Acta, 1492*, 377–384.

Tamai, Y., Miyatake, K., Okamoto, Y., Takamori, Y., Sakamoto, K., & Minami, S. (2003). Enhanced healing of cartilaginous injuries by *N*-acetyl-D-glucosamine and glucuronic acid. *Carbohydrate Polymers, 54*, 251–262.

Tanodekaew, S., Prasitsilp, M., Swasdison, S., Thavornyutikarn, B., Pothsree, T., & Pateepasen, R. (2004). Preparation of acrylic grafted chitin for wound dressing application. *Biomaterials, 25*, 1453–1460.

Terbojevich, M., & Muzzarelli, R. A. A. (2000). Chitosan. In G. Phillips & P. Williams (Eds.), *Handbook of hydrocolloids* (pp. 367–378). Cambridge, UK: Woodhead.

Thatte, H. S., Zagarins, S., Khuri, S. F., & Fischer, T. H. (2004). Mechanisms of poly-*N*-acetyl glucosamine polymer-mediated hemostasis: Platelet interactions. *Journal of Trauma, 57*, S13–S21.

Tonnesen, M. G., Feng, X., & Clark, R. A. (2000). Angiogenesis in wound healing. *Journal of Investigations in Dermatology, Symposium Proceedings, 5*, 40–46.

Turner, T. D., Schmidt, R. J., & Harding, K. G. (Eds.). (1986). *Advances in wound management*. Chichester, UK: Wiley.

Twu, Y. K., Chen, Y. W., & Shih, C. M. (2008). Preparation of silver nanoparticles using chitosan suspensions. *Powder Technology, 185*, 251–257.

Ueno, H., Mori, T., & Fujinaga, T. (2001a). Topical formulations and wound healing applications of chitosan. *Advanced Drug Delivery Reviews, 52*, 105–115.

Ueno, H., Nakamura, F., Murakami, M., Okumura, M., Kadosawa, T., & Fujinaga, T. (2001b). Evaluation effects of chitosan for the extracellular matrix production by fibroblasts and the growth factors production by macrophages. *Biomaterials, 22*, 2125–2130.

VanTa, Q., Kim, M. M., & Kim, S. K. (2006). Inhibitory effect of chitooligosaccharides on matrix metalloproteinase-9 in human fibrosarcoma cells (HT1080). *Marine Biotechnology, 8*, 593–599.

Varki, A. (1996). Does DG42 synthesize hyaluronan or chitin? *Proceedings of the National Academy of Sciences, 93*, 4523–4525.

Varlamov, V. P., Bykova, V. M., Vikhoreva, G. A., Lopatin, S. A., & Nemtsev, S. V. (Eds.). . *Modern perspectives in chitin and chitosan studies*. Moscow: Vniro.

Varma, A. J., Deshpande, S. V., & Kennedy, J. F. (2004). Metal complexation by chitosan and its derivatives: A review. *Carbohydrate Polymers, 55*, 77–93.

Venkataraman, G., Sasisekharan, V., Herr, A. B., Ornitz, D. M., Waksman, G., Cooney, C. L., et al. (1996). Preferential self-association of basic fibroblast growth factor is stabilized by heparin during receptor dimerization and activation. *Proceedings of the National Academy of Sciences, 93*, 845–850.

Verma, D., Katti, K. S., Katti, D. R., & Mohanty, B. (2008). Mechanical response and multilevel structure of biomimetic hydroxyapatite/polygalacturonic/chitosan nanocomposites. *Materials Science and Engineering, C-28*, 399–405.

Vournakis, J. N., Eldridge, J., Demcheva, M., & Muise-Helmericks, R. C. (2008). Poly-*N*-acetyl glucosamine nanofibers regulate endothelial cell movement and angiogenesis: Dependency on integrin activation of Ets1. *Journal of Vascular Research, 45*, 222–232.

Wang, J., DeBoer, J., & DeGroot, K. (2008a). Proliferation and differentiation of MC3T3-E1 cells on calcium phosphate/chitosan coatings. *Journal of Dental Research, 87*, 650–654.

Wang, W., Itoh, S., Matsuda, A., Aizawa, T., Demura, M., Ichinose, S., et al. (2008b). Enhanced nerve regeneration through a bilayered chitosan tube: The effect of introduction of glycine spacer into the CYIGSK sequence. *Journal of Biomedical Materials Research, 85A*, 919–928.

Wang, W., Itoh, S., Matsuda, A., Ichinose, S., Shinomiya, K., Hata, Y., et al. (2008c). Influences of mechanical properties and permeability on chitosan nano/microfiber mesh tubes as a scaffold for nerve regeneration. *Journal of Biomedical Materials Research, 84A*, 557–566 [Correction, 84, 557, 2008].

Wang, Y. C., Kao, S. H., & Hsieh, H. J. (2003). A chemical surface modification of chitosan by glycoconjugates to enhance the cell–biomaterial interaction. *Biomacromolecules, 4*, 224.

Watthanaphanit, A., Supaphol, P., Tamura, H., Tokura, S., & Rujiravanit, R. (2008). Fabrication, structure, and properties of chitin whisker-reinforced alginate nanocomposite fibers. *Journal of Applied Polymer Science, 110*, 890–899.

Werb, Z. (1997). ECM and cell surface proteolysis: Regulating cellular ecology. *Cell, 91*, 439–442.

Werner, S., & Grose, R. (2003). Regulation of wound healing by growth factors and cytokines. *Physiology Reviews, 83*, 835–870.

West, D. C., Shaw, D. M., Lorenz, P., Adzick, N. S., & Longaneker, M. T. (1997). Fibrotic healing of adult and late gestation fetal wounds correlates with increased hyaluronidase activity and removal of hyaluronan. *International Journal of Biochemistry and Cell Biology, 29*, 201–210.

Willoughby, D. A., & Tomlinson, A. (Eds.). (1999). *Inducible enzymes in the inflammatory response*. Basel, CH: Birkhauser Verlag.

Wilson, O. C., Jr., & Hull, J. R. (2008). Surface modification of nanophase hydroxyapatite with chitosan. *Materials Science and Engineering, C-28*, 434–437.

Witte, M. B. (1998). Metalloproteinase inhibitors and wound healing: A novel enhancer of wound strength. *Surgery, 124*, 464–470.

Xiao, Y. M., Li, D. X., Chen, X. N., Lu, J., Fan, H. S., & Zhang, X. D. (2008). Preparation and cytocompatibility of chitosan-modified polylactide. *Journal of Applied Polymer Science, 110*, 408–412.

Yamada, T., & Kawasaki, T. (2005). Microbial synthesis of hyaluronan and chitin: New approaches. *Journal of Bioscience and Bioengineering, 99*, 521–528.

Yamaguchi, I., Itoh, S., Suzuki, M., Sakane, M., Osaka, A., & Tanaka, J. (2003). The chitosan prepared from crab tendon: The characterization and the mechanical properties. *Biomaterials, 24*, 2031–2036.

Yamane, S., Iwasaki, N., Majima, T., Funakoshi, T., Masuko, T., Harada, K., et al. (2005). Feasibility of chitosan-based hyaluronic acid hybrid biomaterial for a novel scaffold in cartilage tissue engineering. *Biomaterials, 26*, 611–619.

Yin, Y., Ye, F., Cui, J., Zhang, F., Li, X., & Yao, K. (2003). Preparation and characterization of macroporous chitosan-gelatin/beta-tricalcium phosphate composite scaffolds for bone tissue engineering. *Journal of Biomedical Material Research, 67A*, 844–855.

Yuan, Y., Chestnutt, B. M., Haggard, W. O., Bumgardner, J. D., & Muzzarelli, R. A. A. (2008a). Genipin cross-linked chitosans for drug delivery applications. In R. Jayakumar & A. Prabaharan (Eds.), *Chitin and chitosan in biomaterial science*. Trivandrum, India: Research Signpost.

Yuan, Y., Chestnutt, B. M., Wright, L., Haggard, W. O., & Bumgardner, J. D. (2008b). Mechanical property, degradation rate, and bone cell growth of chitosan coated titanium influenced by degree of deacetylation of chitosan. *Journal of Biomedical Materials Research*, 245–252.

Yusof, N. L. B. M., Wee, A., Lim, L. Y., & Khor, E. (2003). Flexible chitin films as potential wound-dressing materials: Wound model studies. *Journal of Biomedical Materials Research, 66A*, 224–232.

Zeng, L. T., Qin, C. Q., Wang, W., Chi, W. L., & Li, W. (2008). Absorption and distribution of chitosan in mice after oral administration. *Carbohydrate Polymers, 71*, 435–440.

Zhang, Y., & Zhang, M. (2001). Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. *Journal of Biomedical Materials Research, 55*, 304–312.

Zhang, Y., & Zhang, M. (2002a). Calcium phosphate/chitosan composite scaffolds for controlled in vitro antibiotic drug release. *Journal of Biomedical Materials Research, 62*, 378–786.

Zhang, Y., & Zhang, M. (2002b). Three-dimensional macroporous calcium phosphate bioceramics with nested chitosan sponges for load bearing bone implants. *Journal of Biomedical Materials Research, 61*, 1–8.

Zhang, Y., Ni, M., Zhang, M., & Ratner, B. (2003). Calcium phosphate-chitosan composite scaffolds for bone tissue engineering. *Tissue Engineering, 9*, 337–345.

Zhu, X., Chian, K. S., Chan-Park, M. B., & Lee, S. T. (2005). Effect of argon plasma treatment on proliferation of human-skin-derived fibroblast on chitosan membrane in vitro. *Journal of Biomedical Materials Research, A-73*, 264–274.