Chitosan and Chitosan Derivatives in Tissue Engineering and Stem Cell Biology

by

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Abstract

Chitosan is a promising natural substances used in biomaterials research as it has several essential properties that can be applied in tissue engineering. This polymer can be easily combined with other biomaterials and it can be rapidly and economically processed to deliver growth factors and drugs. In the work presented in this thesis, the effect of natural, chitin-derived biomaterials on stem cell biology and osteogenic differentiation was determined and important properties of chitosan for tissue engineering applications were examined. Furthermore, it was evaluated how chitosan derivatives affect the expression and potentially regulate the chitinase-like protein YKL-40 in stem cells, which has been indicated to be involved in tissue remodeling, inflammation and disease pathogenesis.

In **paper I**, we investigated the biological effects of the aminosugar glucosamine, which is the smallest, completely deacetylated subunit of chitin. Glucosamine is best known as a dietary supplement for chondro-protection, yet we were able to demonstrate that it upregulates the expression of osteogenic marker genes, which was strongly correlated to YKL-40 expression. This proposes a so far unknown role for YKL-40 in late-stage osteogenic differentiation.

Chito-oligomers, derived from chitosan and chitin, are being increasingly studied owing to their bioactivity and water solubility. The biological potential is strongly dependent on the chemical properties and particularly hexamer and heptamer fractions are being considered most potent. The application of chito-oligomers is frequently limited to antitumor activity and inhibition of angiogenesis, but these chito-oligomers similarly affect gene expression and cytokine secretion, as described in **paper II**. The potency of hexamer fractions of chito-oligomers is strongly dependent on the degree of deacetylation, ultimately requiring the appropriate choice of chito-oligomer for any particular application.

Endotoxin contamination is difficult to avoid during the handling of natural substances, and the biological effects of endotoxins on the body are extensive. Strict regulations are in place to reduce the risk of adverse health effects induced by medical devices, yet these recommendations remain inadequate and insufficiently specified. In **paper III**, we showed that endotoxin contamination in chitosan derivatives can result in false-positive results, completely altering product performance *in vitro*.

In order to determine relevant properties of chitosan for tissue engineering applications, we prepared chitosan membranes as bioactive coatings. In **paper IV**, we compared chitosan membranes prepared from a wide range of degree of deacetylation and derived from different sources in terms of surface characteristics and bioactivity. This work resulted in **paper V** with the development of a standardized protocol for solution casting methods for chitosan membranes, in-house prediction of successful experimental outcome and long-term cell attachment comparable to commonly used tissue culture plastic.

Keywords:

Osteogenesis, YKL-40, Chitosan, Endotoxin, Titanium

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"Science is like a rollercoaster:
It goes up and down and up and down..."

Ólafur E. Sigurjónsson

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List of abbreviations

AFM .... atomic force microscopy
ALP .... alkaline phosphatase
AMCase .... Acidic Mammalian Chitinase
ANOVA .... analysis of variance
ATF4 .... activating transcription factor 4
BCA .... bicinchoninic acid
BMP2 .... bone morphogenic protein 2
BPI .... bactericidal/permeability increasing protein
ChOS .... chitooligosaccharide
CLP .... chitinase-like protein
COX-2 .... cyclooxygenase 2
DD .... degree of deacetylation
DMEM .... Dulbecco's modified eagle medium
DP .... degree of polymerization
ECM .... extracellular matrix
EGF .... epidermal growth factor
ELISA .... enzyme-linked immunosorbent assay
ERK .... extracellular signal-regulated kinase
FBS .... fetal bovine serum
FDA .... Food and Drug Administration
hBMSC .... human bone marrow-derived mesenchymal stem cells
HGF .... hepatocyte growth factor
HPLC .... high-performance liquid chromatography
HUVEC .... human umbilical vein endothelial cells
IFN-γ .... Interferon-gamma
IL .... interleukin
LBP .... LPS binding protein
LPS .... lipopolysaccharide
MAPK .... mitogen-activated protein kinase
M-CSF .... macrophage colony stimulating factor
MSC .... mesenchymal stem cells
OPG .... osteoprotegerin
PBS .... phosphate buffered saline
PGE_2 .... Prostaglandin E_2
PI-3K .... Phosphoinositide 3-kinase
qPCR .... quantitative polymerase chain reaction
RANKL .... receptor activator of nuclear factor kappa-B ligand
RUNX-2 .... runt-related transcription factor 2
TGF-β .... transforming growth factor-beta
TLR .... Toll-like receptor
TNF-α .... tumor necrosis factor-alpha
VEGF .... vascular endothelial growth factor

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List of papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-V):

I. **Lieder R**, Reynisdóttir ST, Thormódsson F, Ng CH, Einarsson JM, Gíslason J, Björnsson J, Gudmundsson S, Petersen PH, Sigurjónsson OE. Glucosamine increases the expression of YKL-40 and osteogenic marker genes in hMSC during osteogenic differentiation. Nat. Prod. Bioprospect. 2012; 2: 87-91.

II. **Lieder R**, Thormodsson F, Ng CH, Einarsson JM, Gislason J, Petersen PH, Sigurjonsson OE. Chitosan and Chitin Hexamers affect expansion and osteogenic differentiation of mesenchymal stem cells differently. Int. J. Biol. Macromol. 2012; 51: 675-680.

III. **Lieder R**, Gaware VS, Thormodsson F, Einarsson JM, Ng CH, Gislason J, Masson M, Petersen PH, Sigurjonsson OE. Endotoxins affect the bioactivity of chitooligosaccharides in cultures of human, bone marrow-derived mesenchymal stem cells. Acta Biomater.2013; 9: 4771-4778

IV. **Lieder R**, Darai M, Thor MB, Ng CH, Einarsson JM, Gudmundsson S, Helgason B, Gaware VS, Masson M, Gislason J, Örlygsson G, Sigurjonsson OE. *In vitro* bioactivity of different degree of deacetylation chitosan, a potential coating material for titanium implants. J. Biomed Mater. Res. Part A. 2012; 100A: 3392-3399.

V. **Lieder R**, Darai M, Örlygsson G, Sigurjonsson OE. Solution casting of chitosan membranes for *in vitro* evaluation of bioactivity. (submitted manuscript).

In addition, some unpublished data may be presented. All papers are reprinted with the kind permission of the publishers.

Further publications by the same author, not included in the thesis, are as follows:

- **Lieder R**, Petersen PH, Sigurjonsson OE. Endotoxins – the invisible companion in biomaterials research. Tissue Eng. Part B, 2013; doi:10.1089/ten.teb.2012.0636
- **Lieder R**, Sigurjonsson OE. Chitosan as a coating material for titanium implants. In: Chitin and Chitosan Derivatives: Advances in Drug Discovery and Developments. Se-Kwon Kim, 1 ed. CRC Press LLC, Boca Raton; 2014

“Nobody climbs mountains for scientific reasons.
Science is used to raise money for the expeditions,
but you really climb for the hell of it”

Sir Edmund Percival Hillary, New Zealand mountaneer (1919-2008)

1. Introduction

“Science is a way of thinking,
much more than it is a body of knowledge”

Carl Edward Sagan, American astrophysicist (1934-1996)

The human body, unlike that of fish and amphibians, cannot functionally regenerate organs and tissues lost due to injury or ageing (1). The main processes in response to tissue injury are repair mechanisms aimed at restoration by scar tissue formation, rather than regeneration of function and structure (2, 3). The objectives of promoting the body’s self-healing capability, restoration of normal cellular function, and the ability to recreate complex organs “at the bench”, are the basis of the interdisciplinary fields of tissue engineering and regenerative medicine (4, 5).

Because regeneration is by no means a spontaneous process, approaches aimed at replicating tissue function need to provide the essential microenvironment, - be it cells, biomaterials, or signaling molecules, - to support a body’s self-healing capabilities (2). While the field of regenerative medicine relies primarily on cues from stem cell biology to promote innate regeneration processes, tissue engineering sets out on solving the technical aspects, i.e., the support and restoration of function based on the principles of structural support (6). Ultimately, only a combination of the two approaches may succeed to fulfill the promise of recreating a process as complex as genuine regeneration.

1.1 Stem cells

Stem cells are a rare and diverse group of cells present at different developmental stages (7). These cells are not restricted to embryonic development, but are likewise present in adult tissues (somatic stem cells or adult stem cells), contributing to tissue remodeling and repair processes (8, 9). In general, stem cells are defined by the abilities of self-renewal, multipotency and clonogenicity (7). Self-renewal is a strictly controlled process involving symmetric and asymmetric division (10). Symmetric division results in the maintenance of a pool of undifferentiated stem cells by producing two identical daughter stem cells, whereas asymmetric division generates one mature cell and one stem cell (10). Multipotency describes the ability to differentiate into specialized tissues, which strongly depends on the developmental stage and the specific tissue location of the cells (11). Finally, clonogenicity is the ability of a single stem cell to produce genetically identical clones with indistinguishable properties (12).

Stem cells can be classified into four groups depending on the level of multipotency (9). The most potent cells are totipotent stem cells, i.e., the fertilized oocyte or the first blastomer, with the ability to generate an embryo and placenta (10). During early embryonic development, pluripotent stem cells emerge from the inner cell mass of the blastocyst and give rise to cells from all three germ layers, but

not the placenta (9). Multipotent stem cells, also called progenitor cells, are already partially committed and can only generate cells from one of the three germ layers, i.e., ectoderm, mesoderm, and endoderm (9). Monopotent stem cells are fully committed to the tissue they reside in and only give rise to cells of one specific lineage (10). The best characterized monopotent stem cells are hematopoietic stem cells that give rise to the blood cell lineage (13).

1.1.1 Mesenchymal stem cells (MSCs)

MSCs are multipotent progenitor cells residing as a heterogeneous cell population in the bone marrow stroma, adipose tissue, umbilical cord blood and other tissues (14-16). In fact, MSCs may even reside as pericytes in all tissues of the body to perform functions in tissue homeostasis and repair mechanisms (17). The quantity of MSCs in the bone marrow stroma is very low, i.e., 0.001-0.01% of nucleated cells, and it further decreases with age (18). This cell population is generally defined by the following criteria: 1) plastic adherence; 2) surface antigen expression of CD73, CD90, and CD105; 3) lack of expression of surface antigens associated with hematopoietic cells (CD45, CD34, CD14, and CD19); and 4) tri-lineage differentiation potential towards osteogenic, adipogenic, and chondrogenic lineages (19).

In the bone marrow, MSCs support the hematopoietic stem cell niche in providing the appropriate tissue framework for maintenance in a quiescent stage or activation by secreting hematopoietic cytokines and proteins associated with the extracellular matrix (ECM) (20). Furthermore, MSCs participate in the regeneration of tissue damage by migrating to the location of inflammation in response to damage-associated chemokines and cytokines, and they attract immune cells to the site of injury (21, 22). Tissue damage is decreased through secretion of paracrine mediators that favor angiogenesis and prevent apoptosis (23). In addition, MSCs mobilize tissue-resident progenitors to promote repair and decrease the development of scar tissue at the site of tissue damage (23, 24).

MSCs play an important role in immunity and can modulate immune responses by cell contact-dependent mechanisms and secretion of cytokines and growth factors, affecting regeneration and inflammation at the sites of tissue injury (24, 25). In particular, MSCs have been shown to suppress T-cell, B-cell, and natural killer cell proliferation by secreting soluble factors, i.e., transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), prostaglandin E_2 (PGE_2), interleukin-10 (IL-10), and others (26-29). In addition, dendritic cell differentiation and function are blocked, while regulatory T-cells and regulatory antigen-presenting cells are activated (30, 31). MSCs primarily affect T-helper 1 cell responses, resulting in the decrease of interferon-gamma (IFN-γ) secretion, which can adjust immune responses to anti-inflammatory T-helper 2 responses (32).

1.1.1.1 Osteogenesis

Osteoblasts are the major bone forming cells and originate from MSCs via tightly regulated expression of bone-specific transcription factors and matrix proteins (33, 34). *In vitro*, differentiation is induced by a cocktail of dexamethasone, ascorbic acid 2-phosphate, and β-glycerophosphate; however, it strongly depends on the donor-specific osteogenic differentiation potential (35). The mechanism of osteogenic differentiation can be divided into two stages: initiation and maturation phases (36). During initiation of

osteogenic differentiation, cells slowly proliferate, express several osteogenic marker genes, and secrete collagen type I, which is the main structural component of bone ECM (33). Mineralization of the matrix by deposition of calcium phosphate substituted hydroxyapatite characterizes the maturation phase, commonly initiated 2-3 weeks after induction of the differentiation process (37). During the temporal sequence from commitment to terminal differentiation, three cell maturation levels can be distinguished, i.e., mesenchymal osteoblast progenitors, osteoblast precursors, and active/mature osteoblasts (Figure 1) (34). Furthermore, cell shape changes mark the transition from mesenchymal progenitor cells (spindle-shaped) to mature osteoblasts (cuboidal) (38).

The three factors in the induction cocktail used for *in vitro* differentiation of MSCs play specific roles in the differentiation process and are essential components in the cell culture media. The glucocorticoide dexamethasone initiates cell shape changes during differentiation and induces the expression of alkaline phosphatase (ALP), an early marker of osteogenic differentiation (39, 40). Ascorbic acid 2-phosphate, a vitamin C derivative, participates in the induction of collagen type I matrix deposition, increases proliferation, and promotes matrix mineralization (41, 42). Finally, β-glycerophosphate provides essential phosphate ions for the deposition of hydroxyapatite during the mineralization phase (37, 43).

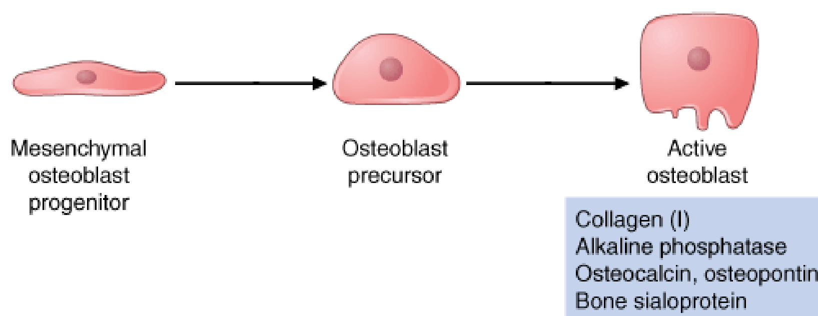

Figure 1. Cell maturation during osteogenic differentiation

The process of osteogenic differentiation is tightly regulated by the sequence of cell maturation from the mesenchymal osteoblast progenitor to the osteoblast precursor and finally to the active osteoblast. This is accompanied by cell shape changes from spindle shape morphology to a cuboidal cell shape in the active osteoblast. Functionally mature osteoblasts express major matrix proteins, i.e., collagen type I, osteocalcin, osteopontin, and bone sialoprotein. The transient expression of alkaline phosphatase provides phosphate ions for the deposition of hydroxyapatite during matrix mineralization. Adapted and modified from Fauci, AS et al. Harrison's principles of Internal Medicine, 17th Edition.

Successful osteogenic differentiation *in vitro* is accompanied by the sequential expression of functional matrix proteins, i.e., collagen type I, osteopontin, and osteocalcin (Figure 1) (33, 34). Collagen type I is secreted during the initiation phase of the differentiation process and is an essential part of the final mineralized ECM (33). Osteopontin, an intermediate stage marker expressed by immature osteoblasts, is believed to have functions in the stabilization of the matrix, whereas osteocalcin, a late-stage osteogenesis marker, may participate in the final mineralization of the ECM (33).

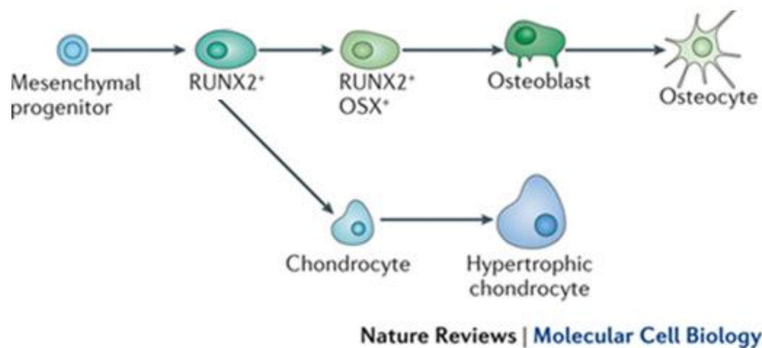

Figure 2. Control of MSC lineage commitment

The commitment of mesenchymal progenitor cells to the osteogenic lineage and the subsequent time-dependent expression of matrix proteins are dependent on the regulation by transcription factors. Runx-2 is expressed at the osteochondro-progenitor stage. Osterix acts downstream of runx-2 and directs differentiation of osteochondro-progenitor cells towards mature osteoblasts. ATF4 is only expressed in fully committed osteoprogenitor cells and regulates osteoblastic gene expression. Adapted and modified from Long, F. Building strong bones: molecular regulation of the osteoblast lineage. Nature Reviews. 2012, 13; 27-38

The appropriate expression of matrix proteins and the commitment of mesenchymal progenitor cells to the osteogenic lineage are dependent on the regulation by three main transcription factors: runt-related transcription factor 2 (runx-2), osterix, and activating transcription factor 4 (ATF4) (Figure 2) (34, 44). Runx-2, also known as the master regulator of osteogenic differentiation, is expressed in osteochondro-progenitor cells, which are MSCs that are not yet completely committed to osteogenic differentiation and may still undergo chondrogenesis (45, 46). Regulation of bone matrix formation is achieved by runx-2-mediated induction of the major matrix protein genes, i.e., collagen type I, osteopontin, and osteocalcin (47, 48). Downstream of runx-2, osterix is the main transcription factor directing the differentiation of osteochondro-progenitor cells toward the osteogenic lineage, and it may play a further role in mineralization processes (44). Finally, ATF4 is induced in completely committed osteoprogenitor cells and governs the expression of osteocalcin and receptor activator of NF-κB ligand (RANKL), based on interaction with runx-2 (49, 50).

1.1.1.2 Clinical application

MSCs have emerged as an attractive cell source of tissue engineering and regenerative medicine applications based on their favorable properties (51). Isolation protocols for MSCs are well-defined, and extensive cell numbers can be obtained due to their vast proliferative abilities (52). Furthermore, MSCs can be readily preserved by cryopreservation, and they do not pose the ethical issues commonly associated with the use of embryonic stem cells (51). In clinics, the use of MSCs is generally considered as safe and feasible, and indeed several reports have described the absence of adverse reactions to allogeneic and autologous MSC transplantation (6). This favorable lack of immune response, especially after allogeneic transplantation, is attributed to the exclusive presence of major histocompatibility complex (MHC) class I molecules, the absence of MHC class II surface markers, and the lack of co-stimulatory molecule expression (53).

MSCs have been proposed for the treatment of various diseases, predominantly based on their beneficial properties in tissue repair, i.e., tri-lineage differentiation potential, immune-modulatory properties, and secretion of growth factors and cytokines (27). Although the use of MSCs is considered safe and the formation of teratomas has been shown to be absent, there is reluctance

believing in the universal application of these cells (54, 55). In fact, there are indications that MSC transfusion might induce the development of malignant tumors and promote tumor growth based on the same properties that are generally attributed to their role in tissue repair (56-58).

Nevertheless, MSCs are considered for the treatment of orthopedic injuries, graft-versus-host disease, and myocardial infarction (59-61). The differentiation potential is particularly promising for applications in the treatment of non-union bone injuries, osteogenesis imperfecta, and craniotomy defects, both with and without the use of scaffolds (62-64). Many studies have also focused on the use of MSCs in the treatment of osteoarthritic conditions, promotion of spinal fusion, and provision of relief in autoimmune diseases (65-67). The only approved use of MSCs in the clinics today is in combination with hematopoietic stem cell transplantation after myeloablative therapy to promote engraftment and prevent secondary tissue injuries (68, 69).

1.2 Bone and the bone environment

Bone is a complex tissue defined by constant turn-over and remodeling in response to environmental and endogenous stimuli (70). In addition to maintaining the rigid structure of the skeletal system to allow movement and loading, bone needs to be both light and flexible (71). The sophisticated and tightly controlled interplay of several cell types is the basis for the regulation of this intricate process, providing appropriate cues for the maintenance of bone structure, function, and remodeling (Figure 3) (71).

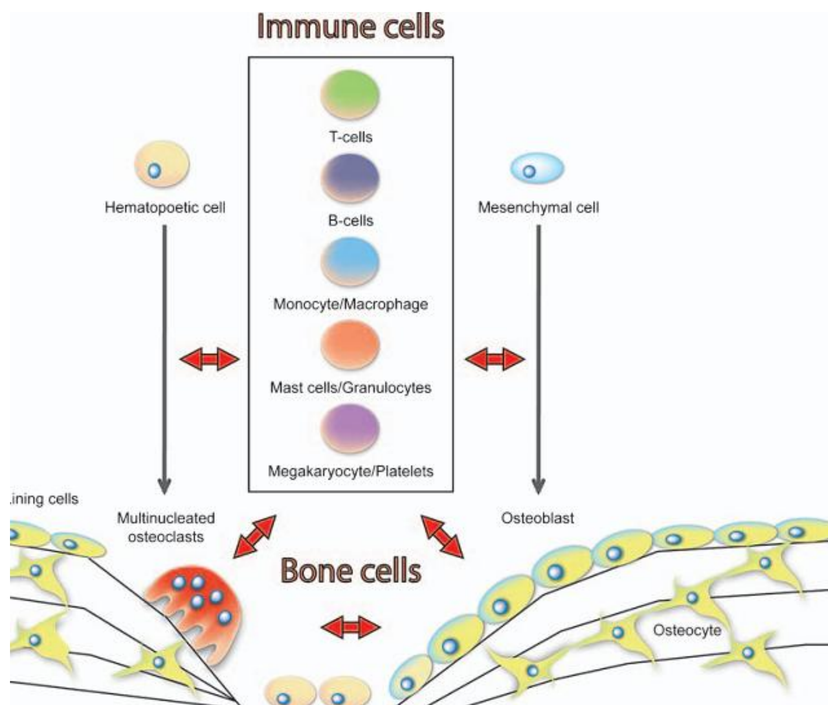

Figure 3. Interplay of cell types in the bone environment

The intricate processes of bone remodeling and turn-over are regulated by the tightly controlled interplay of several cell types. Osteoblasts are the major bone forming cells and originate from mesenchymal stem cells. Osteoclasts are derived from the hematopoietic lineage and mediate bone resorption. Osteoclasts secrete cytokines and chemokines that can regulate the action of immune cells and engage in tight reverse crosstalk with osteoblasts to maintain bone homeostasis. Adapted from Gruber R. Cell biology of osteoimmunology. Wien Med. Wochenschr. 2010, 160; 438-445

Among the vast number of cell types involved, the most important players are cells of the osteoblastic, osteoclastic, and endothelial lineages (72, 73). Osteoblasts, originating from MSC progenitors, are the major bone forming cells in the body and are responsible for ECM deposition and mineralization (33, 46). Antagonizing the action of osteoblasts is a bone-resorbing cell type, called osteoclast (72). Osteoclasts are derived from the hematopoietic lineage by NF-κB-mediated cell fusion of macrophages, giving rise to multinucleated cells (74, 75).

Several factors are known to promote osteoclastogenesis that can influence both the activation and migration of osteoclast precursor cells. Among these factors, RANKL and macrophage colony-stimulating factor (M-CSF) are considered as the two main regulators of osteoclastogenesis. However, ECM proteins, such as osteocalcin and collagen type I, have been similarly shown to participate in the activation process (74). In addition to bone resorption, osteoclasts regulate other cell types, especially hematopoietic stem cells and immune cells, by secreting cytokines, and engage in tight reverse cross-talk with osteoblasts to maintain bone homeostasis (76).

Because bone is metabolically active and requires transport of nutrients throughout the tissue, initiation of angiogenesis is essential during bone repair and remodeling (77). Furthermore, endothelial cells secrete growth factors, which provide essential cues for the regulation of osteoblast and osteoclast activation (78). This intercellular cross-talk occurs via paracrine interactions mediated by soluble factor release and direct cell-to-cell contact via gap junctions (79, 80). Endothelial cells secrete several cytokines known to play major roles in osteogenesis, i.e., RANKL, osteoprotegerin (OPG), bone morphogenic protein 2 (BMP2), and IL-6 (80, 81). The process of angiogenesis is tightly controlled by the microenvironment, direct cell contact-dependent interplay, and factors embedded in the ECM, ensuring the appropriate sequence of endothelial cell activation and migration required for vessel formation (82, 83). In addition, endothelial cells are known to participate in responses to inflammatory stimuli as well as wound healing and repair processes (84).

1.3 Innate immunity

The vertebrate immune system with its crucial role in protecting the organism against invading pathogens is composed of two elements: innate immunity and acquired/adaptive immunity (85, 86).

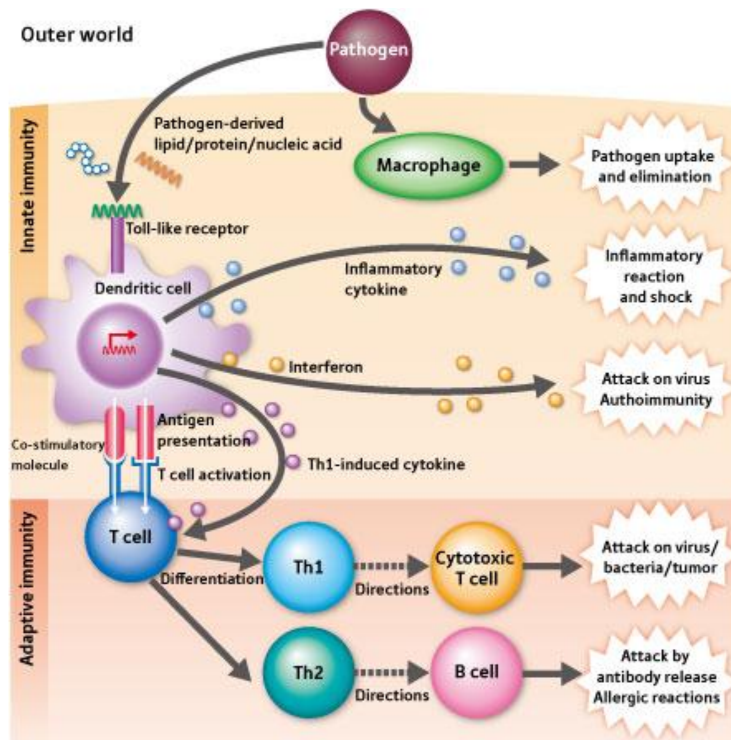

Figure 4. Role of Toll-like receptors in immunity

The activation of innate immune responses is based on the removal of invading pathogens by macrophages and the detection of conserved structural motifs via pattern-recognition receptors, e.g., Toll-like receptors. Ligand binding induces the secretion of cytokines and chemokines, in turn mobilizing acquired immunity. This involves the maturation of dendritic cells and antigen-presenting cells as well as the differentiation of naïve T-cells into T-helper 1 and T-helper 2 cells. Adapted from Kaisho T. Elucidating the mechanism behind immunity using dendritic cells. Riken Research. 2007; 2

Innate immunity represents an organism's initial defense device and acts by detecting evolutionary conserved microbial surface fragments, so-called pathogen-associated molecular patterns (Figure 4) (85, 87). Effective recognition together with distinction between self and non-self is mediated by pattern-recognition receptors on myeloid cells, e.g., Toll-like receptors (TLRs) (88, 89). The cellular component of innate immunity includes mononuclear and polymorphonuclear phagocytes responsible for the destruction of invading pathogens, cytokine secretion, and antigen presentation to cells of the acquired immune system (89). TLR-mediated recognition of invading pathogens induces cytokine and chemokine secretion, which is an essential requirement for the activation of acquired immune responses (90, 91).

In vertebrates, a second line of defense evolved as a prerequisite for the prevention of reinfections and offered further protection against invading microbial pathogens (92). Acquired immunity involves mobilization of antigen-presenting cells, maturation of dendritic cells, and differentiation of naïve T-cells into activated T helper 1 cells (Figure 4) (93). Specific, yet in comparison to innate immune responses delayed, recognition of invading pathogens is mediated by antigen receptors on T- and B-cells (94).

1.3.1 TLRs

Mammalian TLRs are evolutionary conserved, type I transmembrane proteins that are structurally related to the IL-1 receptor superfamily (95). The extracellular domain consists of leucine rich repeats that are believed to partially mediate ligand recognition, while the cytoplasmic domain is shared with the IL-1 receptor family, including three conserved boxes required for the induction of downstream signaling events (96, 97). Despite the evolutionary conserved structure, TLRs can sense pathogens of diverse origin and are expressed on several immune and non-immune cells (98). TLRs contribute essentially to innate and acquired immunity, where they may even exert crucial functions non-related to the recognition of pathogens (88, 95). In man, 11 members of the TLR family have been identified, each responsible for the recognition of different subsets of pathogen-associated molecular patterns of microbial surface fragments (96).

TLR2 is implicated in the cell-surface recognition of several structural components of gram-positive bacteria, including lipoproteins, peptidoglycan, and lipoteichoic acid, mediated by dimerization with related TLRs or extracellular proteins (99, 100). TLR3 recognizes viral double-stranded RNA and initiates antiviral processes through a unique signaling pathway resulting in the secretion of type I interferon-inducible genes (101, 102). The primary receptor activated after stimulation with lipopolysaccharides (LPS) is TLR4 (103, 104). This receptor has also been implicated in the recognition of endogenous ligands, e.g., heat-shock proteins, oligosaccharides of hyaluronic acid, and fibronectin; however, the assumption of the immune system responding to signals that signify potential harm rather than non-self molecules remains highly controversial (105-108). TLR5 and TLR9 are associated with responses to bacteria, whereas TLR7 and TLR8 recognize single-stranded virus RNA and react to several synthetic compounds (109, 110).

TLR ligand recognition induces intracellular signaling cascades resulting in the activation of transcription factors and subsequent NF-κB-mediated induction of proinflammatory cytokines, including IL-6, tumor necrosis factor-alpha (TNF-α), and IL-12 (95). Signaling is transmitted by either one of the two pathways: MyD88-dependent signaling associated with the initiation of cytokine secretion and MyD88-independent signaling/TRIF signaling responsible for the induction of type I interferon-inducible genes and delayed activation of NF-κB in response to LPS (96, 111).

Because uncontrolled production of inflammatory cytokines is associated with poor clinical prognosis, TLRs have been linked to the development and/or maintenance of disease state in several inflammatory and immune system-related pathologies, including Crohn’s disease, rheumatoid arthritis, and atherosclerosis (112, 113). Apart from their approved role in innate and acquired immunity, TLRs are believed to maintain epithelial homeostasis and participate in fibroblast maturation processes (114, 115).

1.3.1.1 Effect of TLRs on MSCs

MSCs have been shown to actively express TLR1-6 under *in vitro* conditions, whereas the expression of TLR7-10 remains controversial and may depend on specific experimental settings (116, 117). Based on the crucial role of TLRs in innate and adaptive immune responses, the participation in MSC-mediated immune-modulatory functions, roles in tissue remodeling after injury and functions in stem

cell biology have been proposed (118, 119). The role of TLRs in MSC biology has been studied extensively in cells derived from different tissue locations and species; however, the consequences of TLR stimulation remain contradictory (117, 120). Particularly TLR-agonist concentration and duration of exposure can strongly affect the experimental outcome (112, 121). Generally, activation of TLR3-mediated signaling is believed to promote anti-inflammatory actions and participate in MSC stress responses, whereas TLR4 activation enhances the secretion of proinflammatory cytokines (119). TLR4 stimulation was shown to prevent oxidative stress-mediated apoptosis and promote survival in a rat model of myocardial infarction (120, 122).

Overall, the activation of TLR signaling has been associated with the modulation of MSC migration, differentiation, and immune-modulatory properties, but it does not affect proliferation in the human system (116, 119). In human bone marrow-derived MSCs (hBMSCs), only long-term exposure to LPS, a TLR4 ligand, can enhance osteogenic differentiation, whereas the short-term exposure to any TLR agonist did not affect the tri-lineage differentiation potential (121, 123). Immune-modulatory properties were demonstrated to be either enhanced or inhibited, depending on the T-cell population and the experimental setting (117, 123). Regarding adipose tissue-derived MSCs, the general consensus indicates a role of TLR stimulation in promoting osteogenic differentiation while inhibiting adipogenesis, yet no effect on MSC-mediated suppression of T-cell proliferation was observed (116, 119).

1.3.2 Endotoxins

Endotoxins, a characteristic feature of gram-negative bacteria, were found to consist of LPS linked to proteins and other cell membrane-related components (124). Endotoxins are an integral part of the outer membrane of gram-negative bacteria, but are only secreted in minute amounts during cell division and normal life cycle (124). Substantial amounts of endotoxins can be released during the destruction of the bacterial cell wall either by antibiotics or the actions of the host immune system (125).

The toxicity inducing substance in endotoxins, LPS, is built of three main parts: a lipid moiety, called Lipid A, linked to an oligosaccharide, the core oligosaccharide, which in turn is attached to a sequence of repetitive subunits, named O-specific antigen (Figure 5) (126). The Lipid A moiety is characterized by a disaccharide backbone with two negatively charged phosphate groups and can carry as much as six acyl chains with 14-16 carbon atoms (127). The amphiphilic nature of Lipid A, having both hydrophobic and hydrophilic features, causes aggregation in aqueous solutions to form three-dimensional supra-molecular structures, called micelles (128). The O-specific antigen is used to classify the bacterial serotype and enables bacteria to evade the attack of serum complements from the host immune system (129, 130). The general properties of endotoxins include that they are 1) negatively charged in solutions, 2) thermally stable up to 180°C, 3) UV-resistant, and 4) insoluble in methanol and ethanol (131).

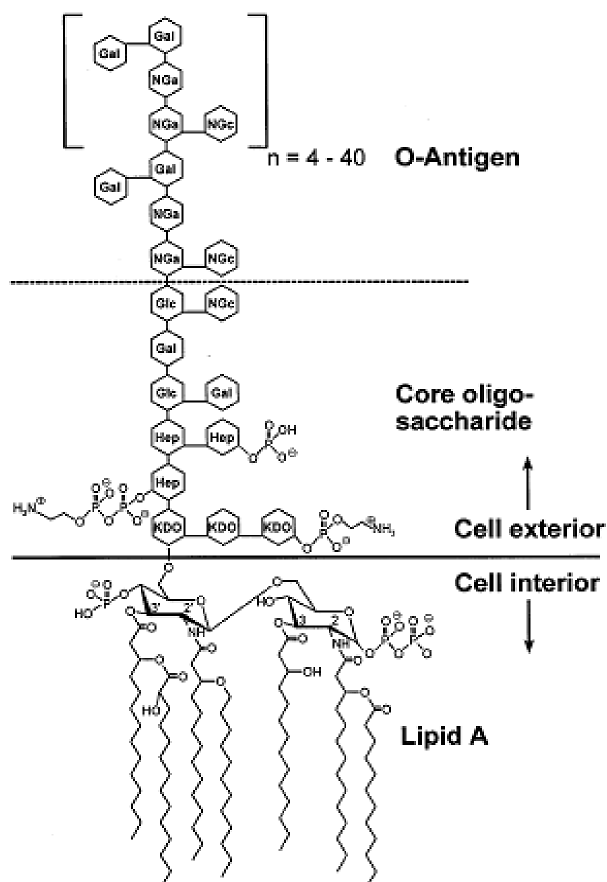

Figure 5. Bacterial endotoxin from *E.coli* O111:B_4

The chemical structure of lipopolysaccharides is composed of three main parts, i.e., Lipid A, core-oligosaccharide, and O-antigen. The Lipid A moiety contains a disaccharide backbone with two negatively charged phosphate groups and is linked to the core-oligosaccharide. The O-specific antigen is built of repetitive subunits and is used to classify the bacterial serotype.

Hep (L-Glycero-D-manno-heptose), Gal (galactose), Glc (glucose), KDO (2-keto-3-deoxyoctonic acid), NGa (N-Acetyl-galactosamine), NGc (N-Acetyl-glucosamine). Adapted from Ohno, N et al. Lipopolysaccharide interactions with lysozyme differentially affect lipopolysaccharide immunostimulatory activity. Eur. J. Biochem.1989, 186; 629-636

The immune system is able to detect and react to LPS at levels of < 1 ng/ml (132). Even without signs of bacterial infection, LPS can be detected in human tissues. Low levels of LPS may even aid in the development of several chronic diseases, but higher concentrations are known to induce high fever, hypotension, and septic shock (133, 134). Multi-organ failure, adult respiratory distress syndrome, and disseminated intravascular coagulation are further consequences of increased endotoxin levels in the blood (134).

The term sepsis describes a medical condition in which an overwhelming bacterial infection can promote an inflammatory state of the whole body, accompanied by fever, increased heart, and respiratory rate (135). Antibiotics, one of the most common treatments for sepsis, can deteriorate the disease by destroying the bacterial cell wall and releasing even higher quantities of endotoxins into the blood stream (136). Highly sensitive bioassays are needed to determine the presence of LPS in human plasma, since the compound has a very short serum half-life, is poorly immunogenic, and exhibits extreme potency (135, 137).

1.3.2.1 Aseptic implant loosening

The role of endotoxins in the clinical setting of aseptic implant loosening is controversial, but increasingly more evidence is being accumulated that indicates endotoxins as at least partially responsible for osteolysis and the loosening of medical devices (138-140). LPS was detected in the tissue surrounding the implant in patients diagnosed with aseptic implant loosening, who did not show any signs of microbial infection (141). In addition, the prophylactic use of antibiotics, systemically or as part of the implant, resulted in a 50% reduction in the incidence of aseptic implant loosening (142).

Furthermore, endotoxins have been associated with 1) inflammation in response to wear particles, 2) acceleration of the foreign body reaction, 3) proinflammatory cytokine release, and 4) macrophage activation (143, 144). This assumption was based on the notion that macrophages at the bone-biomaterial interface express endotoxin receptors, markedly TLRs, which are highly sensitive to even minute amounts of endotoxins and could be responsible for the induction of cytokine secretion, impairing osseo-integration and, finally, causing the failure of the implant (145, 146).

There are a number of potential sources for endotoxins in the peri-prosthetic tissue, including the bacterial biofilm on the surface of the implant, endotoxin contamination during the implant manufacture, and endotoxins derived from wear particles, absorbing LPS from systemic infections or the intestinal flora (147). This notion also reinforces the call for strict monitoring of endotoxin contamination during the evaluation of wear debris in *in vitro* models (148).

1.3.2.2 Endotoxin-induced signaling pathways in vitro

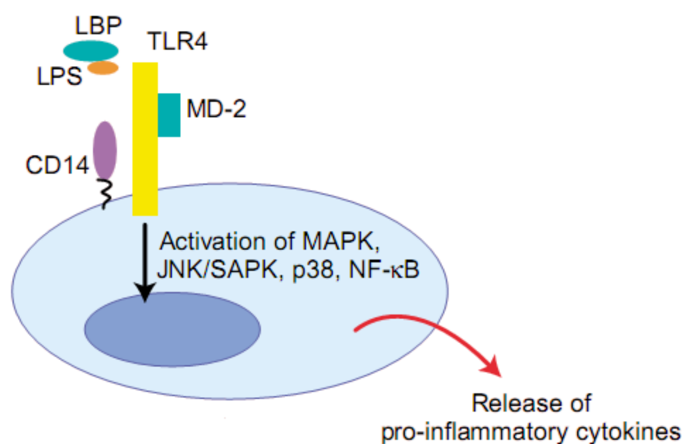

Figure 6. Simplified endotoxin-mediated signaling *in vitro*

LBP binds LPS aggregates and converts them to monomers, which are presented to the glycoprotein CD14. In turn, CD14 transfers LPS monomers to the adaptor protein MD-2. The resulting LPS-MD-2 complex interacts with cell-surface bound TLR4, the primary receptor for bacterial LPS. Activation of TLR4 results in the translocation of NF-κB into the nucleus, which is crucial for the secretion of proinflammatory cytokines and chemokines.
LPS (lipopolysaccharide), LBP (LPS binding protein), TLR4 (Toll-like receptor 4), MAPK (mitogen-activated protein kinase), JNK/SAPK (c-Jun N-terminal kinase signaling pathways). Adapted and modified from Triantafilou M et al. Sepsis: molecular mechanisms underlying lipopolysaccharide recognition. ERMM. 2004, 6;1-18

The human body is unusually responsive to the biological effects of LPS as compared to other species, and it is the response to LPS rather than the biological potency itself that causes severe inflammatory reactions (135). The recognition of LPS and the downstream activated cell machinery varies between human cells and mouse cells, which greatly affects the comparability of studies (149).

The possible development and course of sepsis is dependent on the activation of the innate immune system and the subsequently induced cellular machinery (150). In the human body, TLR4 was identified as the primary receptor for bacterial LPS (104).

The correct presentation of LPS to its receptor is mediated by two proteins (Figure 6) (132). LPS binding protein (LBP), an acute phase plasma protein derived from the liver, binds LPS aggregates and delivers them in the form of monomers to a second protein, CD14 (137). The biological activity of LPS is based on the interaction in the form of aggregates, since the encounter with monomer structures of LPS does not induce cytokine production *in vivo* (128). Furthermore, LBP acts in a concentration-dependent manner with low concentrations enhancing the activation of downstream

receptors and high concentrations inhibiting stimulation (151, 152). The glycoprotein CD14, expressed by myelo-monocytic cells, transfers the LPS monomers to the extracellular adaptor protein MD-2 and can be present either in a soluble state or membrane bound as glycosylphosphatidylinositol-anchored (GPI-anchored) molecule (153). The resulting LPS-MD-2 complex then interacts with cell-surface bound TLR4 through the formation of dimeric structures (135).

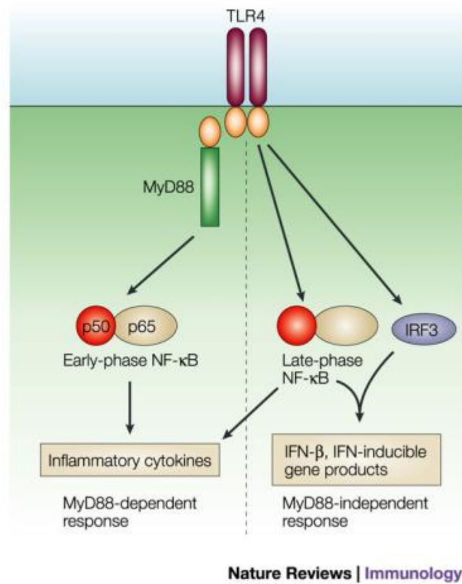

Figure 7. TLR4 signaling cascade

Unlike any other TLR, the activation of TLR4 results in the induction of both the MyD88-dependent and the TRIF-dependent/MyD88-independent pathway. Ligand binding initiates the translocation of NF-κB into the nucleus and the induction of IFN-inducible genes, which is crucial for the expression of cytokines, chemokines, and acute phase proteins in response to LPS stimulation. Adapted from Akira S. et al. Toll-like receptor signaling. Nature Reviews Immunology. 2004, 4; 499-511

Activation of TLR4 results in the induction of two distinctive signaling pathways, the MyD88-dependent and the TRIF-dependent pathway, subsequently causing translocation of NF-κB into the nucleus (Figure 7) (104). This translocation is crucial to the activation of LPS responsive genes, i.e., cytokines, chemokines, and acute phase proteins (135).

To prevent the full induction of biological responses, susceptibility can be reduced by innate agonists in the blood stream (149). These antagonists include 1) bactericidal/permeability increasing protein (BPI), which can neutralize LPS, 2) apolipoproteins that reduce the production of pro-inflammatory cytokines, and 3) lactoferrin, which inhibits the binding of LPS to CD14 through competitive interaction (149, 154).

1.3.2.3 Biological effects of endotoxins

The chemical structure of the Lipid A moiety and the final concentration of LPS directly affect the interaction with its receptor TLR4 and influence the biological potency (155). High concentrations of LPS induce toxic effects in the body, including irreversible shock and pyrogenicity, whereas low concentrations can act as stimulants for the immune system to withstand general bacterial and viral infections (155, 156).

In the body, LPS can affect cell proliferation and the secretion of cytokines and chemokines along with delaying wound healing. The secretion of bioactive mediators can be altered, and endotoxins were shown to directly inhibit collagen production in osteoblasts (157, 158). The primary biological effect of endotoxins includes the activation of immune cells but subsequently also affects bone homeostasis and the performance of biomaterials *in vivo* (159, 160). Bone resorption during the bone

remodeling process can be strongly influenced by proinflammatory cytokines secreted from immune cells, modifying both survival and activation of osteoclasts. The same cytokines are known to similarly affect osteoblasts *in vitro* (161, 162). Several regulatory mechanisms are shared between the immune and the musculoskeletal system, and they modulate not only osteoblast function but also bone formation (163).

1.3.2.4 Regulations for medical devices

In human application of medical devices and parenteral drugs, endotoxins constitute a major health threat (131, 164). The Food and Drug Administration (FDA) has therefore established recommendations for endotoxin measurements and safety limits that describe the allowed amount of contamination depending on the application site of the medical device (131, 164). The current protocols include the immersion of the medical device in endotoxin-free water for at least 1 h at room temperature and then measuring the endotoxin content in the extract (131). The FDA guidelines recommend endotoxin levels < 0.5 EU/ml for medical devices and levels < 0.06 EU/ml for devices in contact with cerebrospinal fluid (131, 164).

The use of extracts instead of the actual medical device raises concern over the accuracy of endotoxin levels (131, 164-166). The amount of LPS that is released into solution during the immersion of a medical device is strongly dependent on the properties of the biomaterial, and the dissolution of hydrophobic LPS into a polar solvent such as water is slow (166, 167).

The difficulty in accurately measuring endotoxin levels exponentially increases with specimens that are not transparent or have complex shapes, making direct determination of endotoxin levels on medical devices challenging (164). This creates serious issues in the field of biomaterials research, because the biological response to the presence of endotoxins *in vitro* can overrule the actual effect of the biomaterial (164, 165).

Despite the concern about the measurement of endotoxins in extracts of water-immersed biomaterials based on the unknown amount of endotoxins that is released into the solution, the regulations have proven sufficient over the years for *in vivo* applications (168). Nevertheless, these recommendations may be inadequate for the *in vitro* evaluation of biomaterials (131). Depending on the cell type used, the amount of endotoxin that can be tolerated without biological response can be different and this is not sufficiently specified at present (157, 169). Furthermore, there is a lack of standard guidelines in the *in vitro* evaluation of biomaterials, including the need for regular endotoxin testing and publication of all values along with biomaterial characteristics (131).

1.4 Chitinases and chitinase-like proteins (CLPs)

Chitinases (EC 3.2.1.14) and CLPs belong to the family 18 glycosyl hydrolases and have only recently been discovered in mammals (170, 171). Two active chitinases, namely, acidic mammalian chitinase (AMCase) and chitotriosidase, as well as four CLPs (YKL-40, YKL-39, oviductin, and stabilin-1 interacting CLP) have been identified in men (172-177).

Structurally, chitinases contain a chitin-binding domain and a catalytic domain, capable of digesting chitin polymers and preventing the enrichment of chitin in the environment (170). A glutamate residue

in the catalytic domain acts as proton donor on the glycosidic bond, catalyzing the hydrolysis of the β-(1-4) linkage between the N-acetyl-glucosamine residues (173, 178). Although the catalytic domain is highly conserved, CLPs lack enzymatic activity because of the substitution of the essential glutamic acid residue by leucine, which is evolutionary favored as determined by phylogenetic analysis (179). The binding cleft is composed of six solvent-exposed cysteine residues and is typically absent in CLPs (177, 178). Despite the lack of the typical chitin-binding domain, CLPs can still bind chitin and chito-oligosaccharides (ChOS) with high affinity (180).

1.4.1 Biological role

Chitinases and CLPs have first been described 25 years ago, yet biological function and putative endogenous substrates remain speculative (181, 182). Roles in defense mechanisms against chitin-containing particles and participation in host immune response by attracting cells of the acquired immune system to the site of infection, have been proposed (170, 183). Since CLPs are strongly secreted during inflammatory and allergic conditions, it is assumed that the proteins have a function in tissue remodeling, inflammation and disease pathogenesis (183-185).

Expression and secretion of chitinases can have both advantages and disadvantages, as is the case for AMCase and chitotriosidase in human pathologies. AMCase has been suggested to participate in the development of human asthma and allergic airway inflammation by adjusting T-helper 2 inflammation and remodeling processes (186); whereas chitotriosidase is thought to have essential fungistatic effects, participating in defense mechanisms (187). Whether the proposed biological functions are mediated directly by the chitinases and CLPs or are dependent on the induction of downstream cytokines and growth factors, remains elusive (183); however, the *in vivo* scenario is most likely represented by a combination of the two mechanisms.

1.4.2 The CLP YKL-40

YKL-40 (also known as chitinase 3-like protein 1 or HCgp39) is a secreted, 40 kDa mammalian glycoprotein, expressed by articular chondrocytes, differentiated macrophages, synoviocytes, and osteoblasts (188, 189). Even though *in vivo* biological function remains controversial, crystallographic analysis showed that binding of a putative ligand can induce conformational changes in the protein, indicating a potential signaling role (180). Moreover, two distinct binding sites, interacting with either long or short ChOS, in addition to possible binding sequences for heparin and hyaluronan, have been identified (180, 190).

Upregulation of YKL-40 in several inflammatory and degenerative diseases, including rheumatoid arthritis, osteoarthritis, and certain cancers, implies that YKL-40 in particular might prevent damage to the ECM by reducing the deleterious effects of proinflammatory cytokines (191, 192). Furthermore, YKL-40 has been linked to the process of fibrosis, based on its expression during cirrhosis of the liver and scleroderma (185, 193). Since YKL-40 is expressed in normal bone marrow, a connection between the host response to inflammation and the process of tissue repair seems plausible (191, 194).

Several studies indicate YKL-40 as a macrophage and chondrocyte differentiation marker; in the latter promoting the expression of the essential transcription factor SOX9 and synthesis of collagen type II (195, 196). Furthermore, roles in tissue remodeling, inflammation, mitogenesis, and differentiation of various cell types have been proposed (195, 197). *In vivo*, stimulation of proliferation was shown for synoviocytes and skin fibroblasts, and was correlated to the induction of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI-3K) signaling cascades (198, 199). Based on the promotion of endothelial cell adhesion, migration, and tubulogenesis, YKL-40 was linked to the processes of angiogenesis (200, 201).

In addition, YKL-40 is expressed during embryonic development with proposed function in proliferation, differentiation, and tissue morphogenesis; particularly in the embryonic heart and the musculoskeletal system (202). Expression in undifferentiated human embryonic stem cells and progenitor cells in all three germ layers has only recently been demonstrated, and could indicate a role for YKL-40 in the differentiation of pluripotent stem cells towards more differentiated lineages (203).

1.5 Chitin

Chitin, a polymer of β-(1→4)-linked poly-N-acetyl D-glucosamine, is after cellulose the second most abundant organic compound in nature, but is not enriched in the environment due to enzymatic degradation by chitinases (204, 205). The structural difference between cellulose and chitin is limited to the substitution of the C2 hydroxyl group in cellulose by an acetamido group (Figure 8) (204). Chitin is a structural component in the cell walls of bacteria, fungi, crustaceans, and insects (206, 207). Characterization and application are challenging due to the insolubility in aqueous solutions (208, 209). In nature, chitin occurs in alpha and beta crystalline polymorphic forms, with alpha chitin being the commonly used form; it is characterized by repeating units of parallel and anti-parallel chains (210, 211).

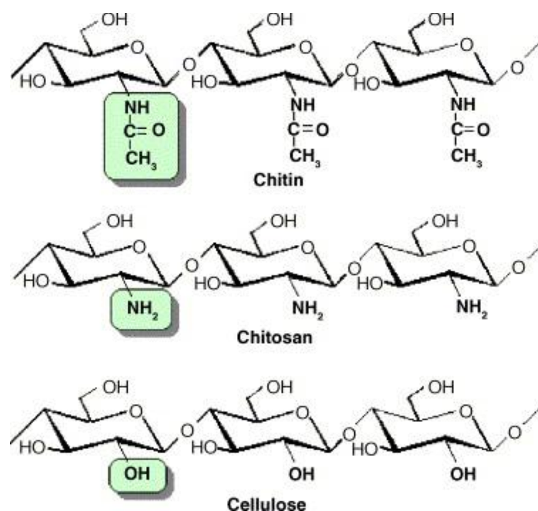

Figure 8. Structural comparison of chitin, chitosan, and cellulose

The structural difference between cellulose and chitin is limited to C2, where the hydroxyl group of cellulose is substituted by an acetamido group in chitin. Chitosan is derived from chitin by removal of at least 50% of N-acetyl groups in aqueous alkali. Adapted from Nosal W.H. et al. UV-vis-infrared optical and AFM study of spin-cast chitosan films. Colloids and Surfaces B: Biointerfaces, 2005, 43; 131-137

Despite the historical assumption that chitin may only have structural properties, the polymer was found to positively affect wound healing by influencing platelet function and alternatively activated macrophages (212, 213). Furthermore, chitin was demonstrated to mediate immune responses depending on the size of its fragments (214, 215). Large polymers are processed by endogenous chitinases and oxidants into smaller-sized fragments, rather than directly stimulating immune responses, resulting in cytokine-mediated modulation of type 2 immune responses (214). However,

chitin can also induce the migration of neutrophils and stimulate non-specific reactivity to bacterial and viral pathogens (216, 217).

Since processing of chitin is laborious, the polymer has mainly been used to obtain chitosan, ChOS and the aminosugar glucosamine, by deacetylation and/or hydrolysis (218). Chitin derivatives are interesting for the use in regenerative medicine and tissue engineering applications, based on their favorable properties, i.e., non-toxic, biodegradable, and biocompatible (206, 219). Other applications of chitin include 1) the immobilization of enzymes in food industry; 2) affinity chromatography columns; 3) waste water treatment based on the adsorption of metal cations, dyes, and aromatic hydrocarbons; 4) biosensors; and 5) dietary supplements to reduce the uptake of lipids during digestion (220-222).

1.5.1 Chitosan

Partial deacetylation of the chitin acetamido group by aqueous alkali yields chitosan, a heterogeneous polymer of N-acetyl-glucosamine and glucosamine components (Figure 8) (209). Chitosan is characterized by three functional groups, i.e., an amino and acetamido group, and hydroxyl groups in primary and secondary formation, which are reactive to chemical modifications (223, 224). The deacetylation process introduces a cationic charge to the polymer and favors interactions with negatively charged cytokines and growth factors, as well as the negatively charged phosphate groups in LPS (208, 225). The affinity for endotoxins is in fact so strong that cross-linked chitosan microfiltration membranes have been used for the removal of endotoxin contamination from medical preparations (226). Furthermore, the cationic nature of chitosan is the basis for its solubility in dilute aqueous acids, as opposed to the insolubility of chitin, favoring its versatile use in tissue engineering applications (204, 209).

Since chitosan is a heterogeneous polymer, its properties are strongly dependent on the number of charged groups (degree of deacetylation; DD), the molecular weight or its distribution (polydispersity index), and the successive order of acetylated and deacetylated residues in the chain (227). As a general rule, chitosan is considered to have $< 50\%$ N-acetyl-glucosamine units, however, there is no conclusive definition on the DD (227).

In addition to the general properties attributed to chitin derivatives, the key properties of chitosan include that it is fungicidal, bactericidal, and has immune-enhancing properties (204, 228). The bactericidal activity is related to the cationic charge with high affinity for the microbial cell wall, inhibiting nutrient flow into the microorganism while at the same time mediating the release of cytoplasmic components (229). Concerning its role in enhancing immune responses, chitosan was shown to stimulate cytokine secretion by macrophages and fibroblasts, modulate migration of immune cells, and attract polymorphonuclear cells to aid in tissue regeneration processes (230-232). By attracting macrophages and neutrophils, chitosan is exerting positive effects on wound healing and skin regeneration, both in early and late stages of regeneration processes (232, 233). Chitosan acts as natural hemostat and analgesic, and may even induce healing without scar tissue formation (234, 235). Inconsistency in reports concerning the biological performance of chitosan is frequently due to the lack of detailed information on source, sample preparation, and chemical properties of the

polymer, which ultimately causes unnecessary difficulties in the comparison of results from different studies (236).

Chitosan is a versatile polymer with diverse applications in many fields, including agriculture, food industry, tissue engineering, waste water treatment, and gene therapy (237, 238). Tissue engineering approaches are based on the favorable effects of chitosan on osteogenesis *in vitro* and *in vivo*, where it promotes cell attachment and supports the formation of the natural ECM, critically aiding in bone regeneration (239, 240). In gene therapy, chitosan is considered for the delivery of DNA, providing a feasible replacement for traditional viral gene-transfer systems (237). Finally, chitosan can also be used as raw material for the production of water-soluble low molecular weight chitosan and highly deacetylated ChOS preparations (223, 241).

1.5.2 ChOS

ChOS can be derived from chitosan and chitin either by chemical or regioselective enzymatic degradation (209, 242). However, mass production of sufficient quantities of pure oligomer fractions is laborious and economically undesirable, which is why many studies focus on the use of ChOS mixtures to determine biological function (243, 244). Because the use of ChOS mixtures instead of purified fractions of oligomers introduces a higher level of complexity, rigorous characterization to ensure reproducibility is essential (209). Despite advanced analysis methods, biological potency can be impaired by the simultaneous presence of active and inhibitory oligomer fractions (209). However, the application of ChOS in the development of potential drugs for the treatment of asthma, vectors in gene therapy, and as wound dressings is attractive, since chitin and chitosan can hardly be dissolved under aqueous, acid-free conditions (170, 245, 246).

The biological potential strongly depends on the chemical properties, including DD, degree of polymerization (DP), molecular weight, and the distribution pattern of acetylated and deacetylated residues in the macromolecule chain (247, 248). In general, ChOS can be easily adsorbed and is considered non-toxic as it is quickly eliminated from the tissue (249, 250). Notably, both hexamer and heptamer fractions of ChOS, independent of the DD, have been associated with strong biological activities (247, 248). Currently, the most appealing properties of ChOS include antitumor activity, inhibition of angiogenesis, immune-stimulatory effects, and the promotion of osteogenesis (243, 249, 251, 252).

The inhibitory effect on tumor activity and progression is associated with the cationic and hydrophobic properties of the macromolecule as well as the inhibition of angiogenesis (243, 244). Strongest antitumor activity was shown for completely acetylated hexamers, which can decrease angiogenesis by regulating endothelial cell growth, migration, and vascular endothelial growth factor (VEGF) mRNA expression (243, 248). In fact, antitumor potency is strongly dependent on the DP, with both pentamers and fractions higher than hexamers proven ineffective (244, 253).

Immune-stimulation and beneficial effects on inflammatory processes are related to the promotion of macrophage migration to the sites of inflammation, induction of chemotaxis, and the stimulation of immune cells (254, 255). In addition, ChOS has been shown to size-dependently enhance the synthesis of nitric oxide and TNF-α in RAW 264.7 macrophages after stimulation with LPS or IFN-γ

(256, 257). Clinical treatment of sepsis could benefit from the use of ChOS, based on the decrease of circulating proinflammatory cytokines and the prevention of oxidative tissue damage by free radical scavenging activity (258, 259). In particular, ChOS has been shown to decrease IL-6 production in human umbilical vein endothelial cells (HUVECs) stimulated with LPS, which is mediated by the down-regulation of NF-κB activity (260).

In the bone environment, ChOS has been impressively shown to promote the differentiation of MSC progenitors into osteoblasts, stimulate calcium deposition by increasing calcium-ion bioavailability, and enhance bone strength and decrease bone turn-over in models of osteoporosis (249, 261, 262).

1.5.3 Glucosamine

Glucosamine is widely known as a dietary supplement for chondro-protection and is commercially produced by hydrolysis of crustacean chitin (263). In the human body, glucosamine is naturally found as an aminosugar in connective tissues and as a subunit in glycosaminogylcans and proteoglycans of the cartilage ECM (264). Glucosamine is easily absorbed in the intestines and is generally considered safe (265, 266). Its potential biological functions include cartilage protection, anti-inflammatory activities, and beneficial effects on the balance of bone remodeling (267, 268). However, the exact mechanism of biological efficacy is only poorly understood (264, 269).

In cartilage and osteoarthritis models, glucosamine was shown to enhance proteoglycan synthesis, prevent proteoglycan and collagen degradation, and promote mitogenesis (270, 271). Furthermore, catabolic downstream events of IL-1β induction in osteoarthritic chondrocytes was ameliorated by glucosamine, including 1) positive effects on matrix gene expression, 2) reduced NF-κB activation, and 3) downregulation of nitric oxide production and cyclo-oxygenase 2 (COX-2) expression (267, 272, 273). In cell culture models of MSC chondrogenic differentiation, glucosamine was linked to the promotion of differentiation, enhanced chondrogenic marker expression, and the prevention of dedifferentiation in the presence of serum (267).

Glucosamine has been studied repeatedly for potential clinical use in the treatment of osteoarthritis, where it is associated with decreased joint space loss and analgesic activities (269, 274). Particularly, the anti-inflammatory properties of glucosamine were linked to modulating the disease pathology by inhibiting neutrophil function, decreasing the activation of immune cells, and preventing the expression of inducible nitric oxide synthase (275-277).

Bone resorption and remodeling were similarly shown to be beneficially affected by glucosamine. Osteogenic differentiation of dental pulp stem cells was enhanced *in vitro*, mediated by increased osteogenic marker gene expression and stimulation of matrix mineralization (278). At the same time, glucosamine has been linked to the downregulation of osteoclast differentiation in late-stage osteoarthritis, directly resulting in decreased bone resorption (279).The inhibitory effect on osteoclasts is mediated primarily by decreased RANKL expression, attenuation of IL-6 levels, and the increase in anti-inflammatory cytokine IL-10 (279). In addition, glucosamine is believed to enhance the healing of dental pulp wounds, suggesting potential applications in dentistry (232, 280).

1.6 Bone tissue engineering and biomaterials

The field of tissue engineering focuses on the technical aspects of regenerative medicine, particularly on supporting and restoring the function of injured tissues by using a structural support matrix, i.e., biomaterials (2). A biomaterial per definition can be any implantable construct or scaffold, intended at providing the microenvironment necessary to promote the replication of natural tissue function (2). In bone tissue engineering, autograft and allograft bone materials have been extensively used in the clinics; however, the limitations include the potential for infection at the site of graft harvest (donor-site morbidity), rejection in the case of allograft transplantation, and the restricted quantity of graft material (281, 282). Alternatively, inorganic, natural, or synthetic biomaterials are frequently used and offer the potential for chemical modifications to meet the requirements of any particular application (283, 284).

1.6.1 Bone-biomaterial interface

The integration of an implant and its interaction with the surrounding tissue at the bone-biomaterial interface crucially determine the success of an orthopedic fixation (Figure 9) (285, 286). The successful integration crucially depends on the surface characteristics of the material, i.e., surface chemistry, topography, charge, and wettability (285, 286). Moreover, mechanical properties such as stiffness, limit of fatigue, and degradation rate affect the biological performance (285, 286). Loose integration of the implant can result in the failure of the fixation by causing micromotion at the bone-biomaterial interface, favoring the formation of a fibrous tissue capsule around the implant (286, 287).

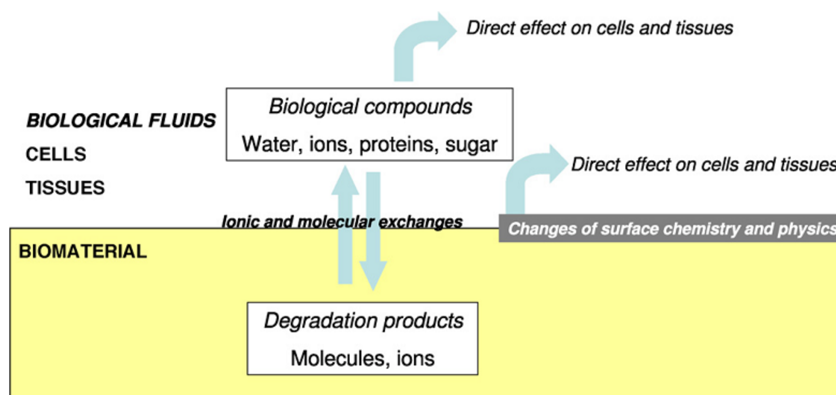

Figure 9. Interaction of biological environment at the bone-biomaterial interface

After implantation, it is the biological environment that primarily interacts with the biomaterial by unspecific adsorption of matrix molecules, release of wear particles, and surface-mediated changes in cell responses. Adapted from Barrère F. et al. Advanced biomaterials for skeletal tissue regeneration: Instructive and smart functions. Mat Sci Eng R, 2008, 59; 38-71

After implantation, the biological environment strongly influences the interaction of the biomaterial with the surrounding tissue and approaching cells, which is mediated by the unspecific adsorption of matrix proteins (Figure 9) (288). A material's surface characteristics critically determine the initial amount and conformation of proteins adsorbed to the implant surface, and it is this outermost atomic layer of adhesive proteins that

primarily affects integrin signaling, cell responses, and tissue regeneration (289, 290). In addition, friction at the bone-implant interface or between parts of the metallic implant can lead to the generation of wear particles, which negatively affect the quality and the life-time of the fixation (291, 292).

Fibronectin is one of the main adhesion proteins at the bone-biomaterial interface, and it contains an integrin-specific binding sequence (RGD-sequence) (293). This protein is particularly important for the attachment and differentiation of osteoblastic cells (294, 295). The conformation of fibronectin at the biomaterial surface strongly depends on the surface wettability, with generally higher bioactivity on hydrophilic surfaces due to decreased denaturation of the secondary structure (296, 297). The negatively charged protein is frequently used to coat biomaterials in order to promote cell attachment *in vitro*, and it has been shown that the structural changes upon surface interaction mediate the protein's bioactivity (298, 299).

The geometrical architecture of the surface (surface topography) is one of the parameters that strongly affect initial protein adsorption and cell interactions (286, 300). Generally, rougher surfaces are associated with enhanced osteoblastic differentiation and can be created by mechanical and chemical processes, though with questionable reproducibility (301-303). In terms of wettability, moderately hydrophilic surfaces are considered to favor cell attachment, mediated by surface-induced changes in protein conformation (304, 305).

The role of surface charge in modulating cellular functions is controversial, as both positive and negative charges are associated with increased bone formation (306, 307). A strong positive charge at the biomaterial surface can induce unnaturally strong focal adhesion and integrin binding; however, from a chemical point of view, the cell membrane is negatively charged, thus favoring electrostatic interactions with positively charged surfaces (308-310). In addition, the osteoblast-mediated secretion of a mineralized matrix has been shown to be particularly enhanced on cationic surfaces (311, 312). Furthermore, the effect of surface charge is closely related to the chemical groups present at the biomaterial surface. Amino functionalities ($-NH_2$) at the surface are especially desirable as they are associated with 1) increased cell adhesion and proliferation, 2) high adsorption of matrix proteins, 3) stimulation of integrin binding, and 4) enhanced mineralization and osteogenic gene expression (313, 314).

Although the general parameters affecting cell responses and protein adsorption are known, the optimal surface characteristics and the detailed processes at the bone-biomaterial interface are only partially understood (286). The use of two-dimensional *in vitro* cell culture models to gain knowledge about the intricate mechanisms at the bone-biomaterial interface is advantageous, as it allows for straight-forward control of experimental conditions and identification of factors contributing to cell activation and gene expression (315, 316). The *in vivo* environment can be much more closely represented by three-dimensional models, accounting for variations in integrin-mediated cell adhesion and the dynamic physiologic environment (317, 318). Conclusions derived from the evaluation of two-dimensional *in vitro* cell culture models can be only partially translated to understand the processes occurring *in vivo* (317).

1.6.2 Titanium implants

Clinical treatment of orthopedic tissue injuries often requires fixation via bone implant material (319). Implants produced from titanium and titanium alloys have been the gold standard in load-bearing orthopedic applications for many years because of their favorable biological and mechanical properties (320, 321). The advantages of titanium and its alloys used for biomedical devices include 1) high corrosion resistance, 2) biocompatibility due to the spontaneous formation of oxide layers, 3) high ratio of tensile strength to density (specific strength) and, 4) lack of toxicity (321, 322). However, there is still room for improvement, particularly concerning the stabilization of the implant, osseointegration at the bone-implant interface, and the prevention of bone stress shielding (323, 324).

Successful integration and stabilization of the implant critically depends on the surface characteristics, i.e., surface chemistry, roughness, topography, and wettability (285). Increasing surface roughness and modifying surface topography by sandblasting, plasma spraying, or acid etching has been extensively used to enhance initial stabilization of the implant and promote bone formation at the peri-implant region (325, 326). Osseointegration is another factor critically determining the life-time of the implant, and it describes the direct interaction of the implant with the bone tissue, resulting in bone growth on the implant surface (285). During implantation, damage to the bone environment and the direct contact of the implant with body fluids can induce the formation of a fibrous tissue capsule, preventing osteoblastic cell attachment to the implant surface (286, 327). This may ultimately lead to the loosening of the implant and decreases the patient’s quality of life (286).

A major drawback of metallic implants is associated with the high modulus of elasticity, resulting in stress shielding of the bone and ultimately leading to implant failure or complications (328). Stress shielding is defined as the translation of stress through the implant rather than the bone, which is mediated by a higher stiffness (Young’s modulus) of implant materials as compared to physiologic bone tissue (321, 329). Bone remodeling and repair relies on environmental stimuli, such as mechanical loading, to appropriately adjust for movement and flexibility; particularly, the lack of mechanical stimulation can result in bone resorption (71). Generally, the Young’s modulus of titanium implants is 3-10 times higher than that of natural bone, yet it remains more suitable for biomedical applications than stainless steel or cobalt/chrome-based alloys (321, 328).

Titanium has a high affinity for oxygen, which makes the implant surface sufficiently reactive for chemical modification, including the deposition of bioactive coatings that enhance bioactivity, osseointegration, and implant stabilization (284). Frequently used surface modifications include 1) coating with calcium phosphate, hydroxyapatite, or bioactive glass; 2) anodization; or 3) immobilization of integrin recognition sequences (e.g., RGD-sequence) (330-332). Currently, the performance of ceramic coatings (e.g., calcium phosphate, bioactive glass) is insufficient because the materials are too brittle to obtain adequate strength at the interface between the implant and the coating (333, 334).

1.6.3 Chitosan membranes

One particular group of materials under investigation as bioactive coatings for titanium and its alloys are polysaccharides, i.e., chitosan, starch, and mucopolysaccharides (335). For applications in various systems, chitosan can be pressed into various forms and combined with other biomaterials; excellent basic properties for application as bioactive coating and scaffolds in tissue engineering (204). Chitosan membranes can be prepared by solution casting methods, but bioactivity and surface properties strongly depend on the chemical properties of the starting material (236, 336). A higher DD is associated with 1) increased wettability (higher hydrophilicity), 2) higher degree of crystallinity, 3) enhanced tensile strength and elastic modulus, 4) increased adsorption of negatively charged proteins, and 5) decreased surface roughness (335, 337). The appropriate choice of substrate for the solution casting process critically determines the final degree of crystallinity, as the crystal structure is crucially influenced by the surface structure of the substrate (338).

Cross-linking methods are commonly used in biomaterials research to prolong the stability and improve the properties of biomaterials, i.e., degradation, chemical resistance, porosity, and mechanical properties (339, 340). Chitosan materials with a DD of around 50% are soluble in aqueous solutions and require cross-linking to improve stability (341). Solubility of these materials is attributed to the degradation of the secondary structure and the subsequent increase in hydrophilicity (341).

Before use in cell culture experiments and animal models, chitosan membranes require sterilization; however, even the commonly used sterilization methods can induce chemical modifications in the polymer structure and ultimately affect the biological performance (340, 342, 343). Autoclaving and dry heat have been shown to decrease the molecular weight and negatively affect aqueous solubility (342, 343). Sterilization via gamma irradiation or ethylene oxide is associated with chain scission; thereby, decreasing mechanical properties and increasing degradation susceptibility (340, 342).

1.6.3.1 Biological performance

For ease of handling and simplicity, the evaluation of chitosan membrane bioactivity is generally performed on tissue culture plastic before coating titanium implants, which allows initial characterization of surface properties and biological performance. The coating of titanium is attempted only after ensuring that the general properties of the membranes meet the requirements for coated implants in tissue engineering applications.

The bioactivity of any chitosan membrane is crucially affected by the surface characteristics and the DD, which was reported repeatedly to alter cellular behavior depending on the cell type (344). A higher DD is generally considered to increase cell attachment and proliferation, yet a lower DD has the promising ability to induce healing without scar tissue formation (234, 336). The cationic charge has been proposed to mediate the attachment of osteoblasts rather than fibroblasts, which would be remarkably useful for the prevention of fibrous tissue capsule formation around medical implants (345).

Chitosan membranes as biological substrates have been studied for a variety of cell types, including osteoblastic and pre-osteoblastic cells as well as non-osteoblastic cell lines and primary cells

. Generally, osteoblastic cell attachment and proliferation is favored on high DD chitosan membranes, supporting differentiation and secretion of ECM molecules (239, 261). Attachment and proliferation of MSC seems to be more complex, strongly depending on the thickness of the membrane and requiring a DD of at least 96% (338, 346). Non-osteoblastic cell lines and primary rat hepatocytes were demonstrated to adhere to chitosan membranes, but no induction of differentiation was observed (338, 347).

1.6.3.2 Chitosan-coated titanium

Coating of titanium and titanium alloys with chitosan membranes can be achieved by a number of methods, e.g., solution casting, silanization, electrophoretic deposition, and layer-by-layer self-assembly (348, 349). The use of solution casting methods is limited to non-complex shapes of titanium and is associated with insufficiently low bonding strength of the coating to the medical device (350). Silanization is based on the reaction of APTES, an aminosilane, with the titanium oxide layer, followed by secondary ketimine formation with glutaraldehyde and, finally, cross-linking between glutaraldehyde and chitosan (351, 352). This chemical process provides significantly increased bonding strength of the coating (350). Electrophoretic deposition is an inexpensive, fast, and easily-scalable technique for biomaterial processing based on the principles of electrophoresis (353, 354). Finally, layer-by-layer self-assembled coatings are generated by alternate solution casting with positively charged chitosan followed by a negatively charged biomaterial solution (355, 356).

Titanium coated with chitosan via silanization was shown to improve osteoblast attachment in comparison to uncoated titanium, remain stable for more than 8 weeks and positively affect integration of the implant at the bone-biomaterial interface (239, 348). Similarly, chitosan membranes deposited using layer-by-layer self-assembly methods, performed consistently better than uncoated titanium films in terms of cell attachment and stimulation of osteogenic differentiation (355, 356). However, numerous challenges remain before chitosan-coated titanium implants can be successfully translated into the clinics.

2. Aims

The general aim of this thesis was to analyze the role of natural biomaterials derived from chitin, i.e., chitosan and chitosan derivatives, in stem cell biology (Part 1) and tissue engineering applications (Part 2).

Part 1 was intended at identifying the impact of chitosan derivatives (glucosamine, hexa-oligomers, and a heterogeneous mixture of ChOS) on osteogenesis and the expression of the CLP YKL-40 in human MSCs. YKL-40 has been strongly indicated to play a role in tissue regeneration and remodeling, yet its expression and regulation by chitosan derivatives in MSCs had not been determined.

Part 2 was focused on identifying important properties of chitosan for tissue engineering applications, including the analysis of surface characteristics and three-dimensional clues that may enhance osteogenesis and the interaction with titanium implant surfaces. Based on the lack of consistent literature concerning casting methods for chitosan membranes, the studies in this part included the development of standard protocols for solution casting of chitosan membranes onto tissue culture plastic.

The specific aims for respective papers in each part of this thesis are summarized below.

2.1. Part 1 – Chitosan derivatives in stem cell biology

I. Study the effect of the aminosugar glucosamine on expansion, osteogenic differentiation, and expression of the CLP YKL-40 in human MSCs.

II. Compare the biological effect of chitin-derived hexamers (N-acetyl chitohexaose) and chitosan-derived hexamers (chitohexaose) on osteogenesis, cytokine secretion, and expression of the CLP YKL-40 in human MSCs.

III. Investigate the potential impact of endotoxin contamination in ChOS preparations on bioactivity, including effects on osteogenic differentiation, gene expression, and cytokine secretion in human MSCs.

2.2. Part 2 – Chitosan in tissue engineering

IV. Relate surface characteristics of chitosan membranes prepared from chitosan starting material with a wide range of DD to *in vitro* bioactivity of the mouse pre-osteoblastic cell line MC3T3-E1.

V. Develop standard protocols for solution casting of chitosan membranes, enabling the use of chitosan starting material from different sources, with different DD and chemical modifications.

"A fact is a simple statement that everyone believes. It is innocent until found guilty.
A hypothesis is a novel suggestion that no one wants to believe. It is guilty, until found effective"

Edward Teller, nuclear physicist (1908-2003)

3. Materials and methods

3.1. Cell culture models

Studying separate cell populations in *in vitro* experiments has impressively contributed to the understanding of numerous signaling pathways and the regulation of cell differentiation and maturation (357). Regarding the investigation of complex tissues, including the bone environment, the approximation by studying single-population cell culture models cannot sufficiently represent the *in vivo* situation (357, 358). The physiological processes underlying bone remodeling and fracture repair are dependent on an elaborate network of different cell types and tightly controlled cross-talk (81).

Nevertheless, the *in vitro* study of single-population cell cultures offers several enticing advantages, including 1) reduced complexity, 2) stable cell metabolism, 3) homogeneity, 4) easy characterization, and 5) induction of specialized differentiation programs. Thereby, *in vitro* cell culture studies can create invaluable models for the screening of potential drug compounds and innovative coating materials for implants in orthopedic research, as well as for advancement in the understanding of regulatory processes during differentiation. This holds true as far as the limitations of a single-cell population model are recognized (357).

3.1.1. hBMSCs

Stem cells hold a great promise as an attractive cell source for tissue engineering and regenerative medicine applications. Potential therapeutic utility is based on migratory abilities, immune-modulatory functions, and promotion of tissue repair and regeneration (359). In the first part of this thesis (papers I-III) hBMSCs were used as model cell culture system to evaluate the effect of chitosan derivatives on stem cell biology and the process of osteogenic differentiation.

Low passage (2-5) cells were used in all experiments and cell surface antigen expression was analyzed in compliance with the criteria posed by the International Society for Cellular Therapy (19). All experiments included control cultures without the addition of chitosan derivatives. Stock solutions of chitosan derivatives were stored in aliquots at - 20° C to prevent degradation.

In paper I, a single donor was used in all experiments and supplemented with 200 µg/ml glucosamine (YSK – Yaizu Suisankagaku Industry, Japan). Glucosamine stock solutions were prepared by dissolving D-glucosamine hydrochloride salt in sterile phosphate buffered saline (PBS) and subsequent filtration through 0.45 µm and 0.22 µm filters.

In paper II, two independent donors were supplemented with 200 µg/ml chitosan hexamers (chitohexaose 6HCl; 1203.73 g/ml, 100% DD, and 96% purity per high performance liquid chromatography (HPLC); Carbosynth Limited, UK) and one donor received 200 µg/ml chitin hexamers (hexa-N-acetyl chitohexaose; 1237.1 g/ml, 0% DD, and 95% purity per HPLC; IsoSep AB, Sweden). Before use in cell culture systems, hexamer preparations were run through Detoxi-Gel Endotoxin Removing Columns with immobilized Polymixin B.

In paper III, three independent donors were supplemented with 10 ng/ml LPS, 400 µg/ml ChOS (ChOS mixture; 60% DD; Genis ehf, Iceland) or a combination of the two. ChOS preparations were run through the Detoxi-Gel Endotoxin Removing Columns with immobilized Polymixin B and were independently endotoxin tested by Lonza (Belgium).

3.1.2. Mouse preosteoblastic cell line, MC3T3-E1

The mouse preosteoblastic cell line MC3T3-E1 is a spontaneously immortalized, clonal cell line derived from mouse calvariae, which can be induced to mineralize in response to ascorbic acid and β-glycerophosphate (360, 361). This cell line was shown to express osteogenic marker genes and react with osteotropic hormones in a manner similar to that of primary calvarial osteoblasts, resulting in a well-suited cell model to reliably study osteogenic differentiation processes, transcriptional regulation and ECM deposition (362, 363). MC3T3-E1, subclone 4, cells were used in papers IV and V to determine the bioactivity of chitosan membranes prepared from chitosan with different DD and for the development of standard protocols for solution casting methods.

3.2. Validation of osteogenic phenotype

Successful osteogenic differentiation was determined by several methods: 1) Alizarin red staining (specific for calcification) and quantitation, 2) von Kossa staining (staining of mineral deposits), 3) ALP activity assay, and 4) gene expression analysis of osteogenic marker genes.

In papers I-III, calcium deposition was assessed by incubation with a 2% alizarin red solution at pH 4.1, which in paper I was additionally quantified after incubation with 10% cetyl-pyridinium chloride and analysis at 562 nm in a spectrometer. In papers IV and V, alizarin red staining was used to assess homogeneous membrane casting. Based on the chemical characteristics of chitosan, acidic dyes, including alizarin red, are strongly retained.

In addition, mineral deposition in papers I and III was determined by von Kossa staining following standard protocols. The silver nitrate stain reacts with mineral deposits to form dark brown precipitates.

In papers III and IV, ALP activity was determined in cell lysates by incubation in 1 mg/ml p-nitrophenyl phosphate solution. After 30 min of incubation, optical density was measured at 400-405 nm in a spectrometer. The conversion of p-nitrophenyl phosphate to p-nitrophenol by ALP was calculated using the general Beer-Lambert law. In paper IV, ALP activity was further normalized to protein content, determined by bicinchoninic acid (BCA) assay, to account for differences in cell proliferation.

Finally, osteogenic differentiation was, without exception, validated by the expression of osteogenic marker genes, including human osteocalcin (paper I), human osteopontin (papers I and III), human ALP (papers I-III), human runx-2 (papers I-III), human collagen type I (papers II and III), mouse ALP (paper IV), mouse runx-2 (paper IV), and mouse osteopontin (paper IV).

3.3. Molecular biology

RNA isolation was performed using Qiagen BioRobot workstation and the EZ-1 RNA Cell Mini Kit. Samples from osteogenic differentiation experiments in papers II and III were homogenized in a FastPrep 24-instrument using Lysing Matrix D tubes containing 1.4 mm ceramic spheres before RNA isolation. RNA was transcribed with the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. A total of 10 µl of RNA sample was added to a master mix containing 2.0 µl 10x RT

buffer, 0.8 µl 25x dNTP (100 mM), 2.0 µl 10x Random Primers RT, 1.0 µl Multiscribe Reverse Transcriptase, 1.0 µl RNase Inhibitor, and 3.2 µl Nuclease-free H_2O. Cycling conditions were the following: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 sec, and then 4 °C until transferred to the -20 °C freezer for storage until use.

Selected genes were quantified in a 7500 Real Time PCR System with 9 µl of 1:10 diluted sample cDNA, 1 µl Taqman assay, and 10 µl Taqman master mix. Data were analyzed using GenEX 5.3.2.13 software and included preprocessing to identify outliers and handle missing data. Raw data was averaged based on quantitative polymerase chain reaction (qPCR) repeats, normalized to the expression of a reference gene or globally normalized (paper III), and relative quantities were determined.

3.4. Solution casting of chitosan membranes

A standard protocol was developed for the solution casting of chitosan membranes, detailed in paper V. Briefly, a 1% (w/w) chitosan solution in deionized H_2O with 50 mM glacial acetic acid (100%) was stirred on a magnetic stirrer until fully dissolved. Homogeneous membrane casting and removal of undissolved particles was asserted by 1 h centrifugation at 5000 rpm before solution casting. A total of 0.1 ml chitosan solution/cm^2 was cast into tissue culture-treated plates and dried over-night in an incubator at 37°C. Next, chitosan membranes were neutralized with 0.1 M NaOH, sterilized with 70% ethanol, and further sterilized under UV-light. To improve attachment, membranes were incubated with a 5 µg/ml fibronectin solution in sterile PBS for 3 h at 37°C. Equilibration was performed in Dulbecco's modified eagle medium (DMEM/F12) media supplemented with penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS).

To improve stability of low DD chitosan membranes during long-term cultures of osteogenic differentiation, respective membranes were internally cross-linked using 0.02% glutaraldehyde solution (papers IV and V).

3.5. Surface characterization of chitosan membranes

In paper IV, chitosan membranes of different DD were analyzed regarding their surface characteristics, including 1) water contact angle measurements to estimate surface wettability, 2) atomic force microscopy (AFM) to evaluate surface topography and to calculate average surface roughness, and 3) fibronectin adsorption studies to determine the amount of protein retained after the initial fibronectin-coating procedure.

Water contact angle measurements were performed in an optical contact angle meter with a droplet of 5 µl distilled H_2O and calculated using the Laplace and Young equation. Surface topography was determined in an XE-100 atomic force microscope, operating at a scan size of 5 µm and a scan rate of 0.15 Hz in noncontact mode. The average mean surface roughness was derived from at least 8 measurements. Fibronectin adsorption studies were performed with in-house enzyme-linked immunosorbent assay (ELISA) using rabbit anti-fibronectin antibody and goat anti-rabbit IgG ALP-conjugated antibody. After incubation with the substrate p-nitrophenyl phosphate, optical density was measured at 405 nm in a spectrometer.

3.6. Statistical analysis

Statistical analysis was performed using Prism 5.01 software and GenEx 5.3.2.13 software (for qPCR analysis). For direct comparison between control and treatment groups, t-test was used (papers I-IV). In addition, one-way analysis of variance (ANOVA; papers II-IV) with Tukey's Multiple Comparison Post Test, two-way ANOVA (papers II and III), and three-way ANOVA (paper III) were practiced wherever applicable. Heatmaps generated from qPCR data were based on hierarchical clustering of autoscaled, preprocessed relative fold changes (papers I and III). The gene expression data visualized in the heatmap set-up was further validated by Spearman Correlation analysis (paper I); $p < 0.05$ was considered statistically significant.

"No amount of experimentation can ever prove me right;
a single experiment can prove me wrong"

Albert Einstein, theoretical physicist (1879-1955)

4. Summaries and discussion of individual papers

4.1. Paper I – The effect of glucosamine on osteogenic marker genes

Glucosamine is a chitin-derived aminosugar that naturally occurs in connective tissues and as subunit in the cartilage ECM; however, it is best known as a dietary supplement for chondro-protection (264). In the bone environment, glucosamine has been shown to reduce excessive bone formation and bone remodeling while simultaneously decreasing bone resorption (279). Chemically, glucosamine can be described as a subunit of the natural substrate for chitinases and CLPs, with the latter being indicated to participate in tissue remodeling, inflammation, and disease pathogenesis (181, 219). YKL-40 is the most studied CLP in men and recent reports have implied particular roles in preventing damage to the ECM and promoting tissue repair in normal bone marrow (191). Crystallographic analysis revealed that binding of a putative ligand could induce conformational changes in the YKL-40 protein, indicating a potential signaling role; yet, biological function and possible endogenous substrates remain speculative (190, 364). Therefore, we studied the expression of *YKL-40* during expansion and osteogenic differentiation of hBMSCs and determined the effect of glucosamine on *YKL-40* expression and its impact on bone biology.

A single donor of hBMSCs was treated with 200 µg/ml D-glucosamine hydrochloride salt (in PBS) during short-term (7 days) expansion and osteogenic differentiation (28 days). In the presence of glucosamine, no changes in the expression of cell surface markers, pluripotency, morphology, and proliferation were observed during cell expansion, proving that basic cell characteristics remained unaltered. *YKL-40* was expressed in control cultures at similar levels after 3 days and 7 days of short-term culture, without significant effects of glucosamine on the gene expression levels. During osteogenic differentiation, a trend of increased *YKL-40* expression in the presence of glucosamine was observed, yet statistical significance was not met (p=0.083).

Osteogenic marker genes followed the expected expression profile during the course of 28 days of differentiation in control cells. Glucosamine treatment significantly increased the expression of *ALP*, osteocalcin (*OCN*) and *RUNX-2* after 28 days in culture. Osteopontin (*OPN*) expression was not significantly different between the two groups (p=0.097), yet a trend towards higher gene levels was observed in the presence of glucosamine. Clustering by means of a heatmap was applied to visualize gene expression correlations between *YKL-40* and osteogenic marker genes. Thereby, late stage osteogenesis (28 days) was characterized by peak expression of *YKL-40*, *OCN*, *RUNX-2,* and *ALP*, in combination with low *OPN* expression. Spearman correlation coefficient (r^2) was calculated to be 77.64% for *YKL-40/RUNX-2*, 90.45% for *ALP/RUNX-2,* and 78.87% for *OCN/RUNX-2*. This correlation suggests that *YKL-40* in combination with increased osteogenic marker gene expression might play a role during the late stages of osteogenic differentiation of hBMSCs and was successfully modified in the presence of glucosamine. The active chitinases, *AMCase,* and chitotriosidase (*CHIT*) were not detected in any of the experimental conditions applied.

Overall, mineralization was impaired following treatment with glucosamine as compared to the control cultures, most likely due to acidosis of the cell culture media. However, the observed effect of glucosamine on osteogenic marker gene expression was not affected by the general effects attributed to acidosis during osteogenic differentiation (365). Other groups have reported similar changes in

gene expression following glucosamine treatment (268) and control experiments adjusting glucosamine culture pH to 7.4 by sodium bicarbonate solution resolved the delay in mineralization.

The secretion of ten selected cytokines was not affected by glucosamine over 28 days of osteogenic differentiation. No expression of IL-1β, IL-12p(40), epidermal growth factor (EGF), and RANTES was observed in any of the cultures. The anti-inflammatory cytokines IL-4, IL-10, and TNF-α were expressed at low levels, whereas the growth factor VEGF was secreted at intermediate levels. Comparatively high secretion of the proinflammatory cytokines IL-6 and IL-8 was observed, yet no effect could be attributed to the presence of glucosamine.

In conclusion, we showed that *YKL-40* was expressed both during short-term (7 days) expansion and osteogenic differentiation in hBMSCs, whereas the active chitinases could not be detected under any of the experimental conditions. Glucosamine did not affect basic cell characteristics, including proliferation, pluripotency, and surface antigen expression. During osteogenic differentiation, glucosamine increased the expression of *YKL-40*, yet expression during short-term expansion was unaltered. Glucosamine significantly amplified osteogenic marker gene expression, while mineralization was delayed in response to acidosis of the cell culture media. The strong correlation of *YKL-40* and osteogenic marker gene expression proposes a yet unknown role for YKL-40 in late stage osteogenic differentiation of hBMSCs.

4.2. Paper II – Chitooligomers have different bioactivity

Chitosan derivatives, particularly chitooligomers prepared from chitosan and chitin, are appealing as potential drugs for asthma, vectors in gene therapy, and as wound dressings, owing to their water solubility and promising bioactivity (243, 246). The biological potential is strongly dependent on the chemical properties, particularly the molecular weight, size of the oligomers, and the DD (247, 248). The general features attributed to chitooligomers include 1) antitumor activity, 2) inhibition of angiogenesis, 3) immune-stimulatory effects, 4) lack of toxicity, and 5) rapid elimination from the tissue (251, 252). Particularly, hexamer and heptamer chitooligomers, independent of the DD, display highest bioactivity; however, mass production of industrial quantities of pure oligomer fractions is challenging and expensive (243, 244). Because the use of chitooligomers has been increasing over the last decades, mostly focusing on antitumor and anti-angiogenesis potency, we compared the effect of 200 µg/ml chitosan hexamers (chitohexaose, 100% DD) and 200 µg/ml chitin hexamers (N-acetyl chitohexaose, 0% DD) on expansion and osteogenic differentiation potential of hBMSCs.

Basic hBMSC cell characteristics, i.e., proliferation, morphology, and attachment, remained unaltered in the presence of chitooligomers. Although both hexamer chitooligomers were prepared to guarantee the absence of endotoxins, we estimated innate immune system activation by evaluating the expression of TLRs. These first line defense receptors of the innate immune system recognize molecular patterns in substances that could pose potential harm to the host. Activation of these receptors induces signaling cascades, resulting in the secretion of cytokines and chemokines that are essential for the subsequent progression of acquired immune responses (96). TLR4 is the primary receptor recognizing endotoxins, whereas TLR3 is believed to participate in hBMSC stress responses (119). During short-term expansion, no effect on the expression of *TLR4* was detected, whereas chitin

hexamers significantly increased the expression of *TLR3* on day 7. Similarly, the expression of *YKL-40* was elevated at the end of the expansion period after treatment with chitin hexamers, however, chitosan hexamers also caused a significant increase, though smaller than the fully acetylated chitooligomer fraction. TLRs were shown to participate in epithelial homeostasis, proposing a potential role in tissue remodeling similar to the CLPs (123). Analysis of cytokine secretion patterns displayed increased levels of IL-6 and IL-8 following treatment with hexamer preparations, despite the absence of endotoxins. Hexamer chitooligomers have been shown previously to cause elevated IL-8 levels, which was linked to promoting wound healing process by attracting cells of the acquired immune system (231).

The expression of selected osteogenic marker genes, i.e., *RUNX-2* and collagen type I (*COL1A2*), was significantly higher in the presence of chitin hexamers, whereas chitosan hexamers had no effect. Despite the increase in osteogenic marker gene expression, mineralization and morphology were comparable in all groups during the culture period. YKL-40 protein and gene expression did not differ in any of the samples, regardless of the initial increase observed during short-term expansion. This could indicate a short-lived effect of hexamer preparations on *YKL-40* expression; however, there have been publications proposing that ligand interaction is not required for YKL-40 activation (199). In contrast, the increase in *TLR3* expression was prolonged and could be observed for chitin hexamers, as initially described, yet additionally also for chitosan hexamers during the differentiation period. However, the potency of chitin hexamers was again significantly higher. Finally, cytokine secretion patterns followed the same trend as observed during short-term expansion, showing significantly higher levels of IL-6 and IL-8 secretion after treatment with either hexamer chitooligomers. The induction of proinflammatory cytokines is tightly linked to bone biology, promoting osteogenesis in single-population cell cultures, yet inducing increased rates of bone turn-over in co-cultures that mimic the *in vivo* environment more closely (366, 367).

In this study, we showed that chitooligomers can significantly affect gene expression and cytokine secretion, both during short-term expansion (7 days) and osteogenic differentiation; however, the observed potency of hexamer preparations is strongly dependent on the DD. Chitin hexamers (N-acetyl chitohexaose) induced significantly higher levels of gene expression, i.e., *YKL-40*, *TLR3,* and selected osteogenic marker genes, which is why the choice of chitooligomer strongly depends on the intended application.

4.3. Paper III – Endotoxins feign bioactivity of chitosan derivatives

Metal implants and polymeric devices applied in the clinical treatment of orthopedic tissue injuries are increasingly being coated with bioactive materials derived from natural substances to induce favorable biological effects (337, 368). Since the field of biomaterials research has been expanding over the last decade, an increasing number of non-chemists and non-biologists have entered the field, often lacking the necessary knowledge to understand the complex chemical interaction mechanisms and the presence of contaminating biota found in most natural substances. One of the materials under investigation as potential bioactive coating for metal implants is chitosan, the partly deacetylated form of chitin (369). The deacetylation process introduces a cationic charge to the polymer and favors interactions with the negatively charged phosphate groups of endotoxins (225, 370). The affinity for endotoxins is in fact so strong that cross-linked chitosan microfiltration membranes have been used for the removal of endotoxin contamination from medical preparations (226). Endotoxins are an integral part of the outer membrane of gram-negative bacteria, but are only secreted in minute amounts during cell division and the normal life cycle of the bacteria (124). However, substantial quantities of endotoxins can be released during the destruction of the bacterial cell wall by antibiotics or the actions of the host immune system (125). The biological effects of endotoxins on the human body are extensive and strict regulations are in place to reduce the risk of adverse health effects induced by medical devices (131, 371). However, these recommendations may be inadequate for the *in vitro* evaluation of biomaterials and at the moment the amount of endotoxins that may be tolerated without biological response is insufficiently specified (157, 169). In addition, there is a lack of standard guidelines for *in vitro* evaluation of biomaterials, including the need for regular endotoxin testing and publication of these values along with other biomaterial characteristics (131).

In this study, we used 10 ng/ml LPS –applied here as a synonym for endotoxins– in combination with 400 µg/ml of water-soluble chitosan derivatives (ChOS; DD = 60%; DP 6-12 = 78.5%; 0.38 ± 0.13 EU/mg endotoxin) to evaluate the effect of endotoxin contamination on *in vitro* bioactivity studies. Prior to the application in cell culture systems, ChOS was subjected to endotoxin removal and independent endotoxin testing. Initial characterization by HPLC, Infrared spectroscopy, and ^{1}H-nuclear magnetic resonance (NMR) proved that the endotoxin removal procedure had no effect on material properties, i.e., DD, DP, and structure.

During short-term expansion (7 days), the presence of LPS did not affect proliferation, morphology, or the expression levels of selected genes, i.e., *TLR3* (hBMSC stress responses), *TLR4* (endotoxin receptor), and *YKL-40* (CLP indicated in innate immune responses). However, the evaluation of cytokine secretion patterns indicated the presence of LPS as early as 3 days after culture initiation via significantly increased secretion of IL-6, IL-8, IL-12(p40), and RANTES. The proinflammatory cytokines IL-6 and IL-8 belong to the innate repertoire of host defense, yet similarly affect bone metabolism (366). Similarly, IL-12(p40) plays a role in cell-mediated immune responses and participates in the activation of acquired immune system progression (372). RANTES is generally expressed at sites of inflammation and was identified as a key player in the uncontrolled expression of proinflammatory cytokines during inflammatory immune responses (373).

The evaluation of osteogenic marker genes after 21 days of differentiation revealed increased expression of collagen type I (*COL1A2*) after treatment with ChOS, which was completely abrogated in the presence of LPS. The effect on *COL1A2* expression might be explained by the heparin binding site in this particular protein, as the ChOS mixture applied in this study is structurally similar to heparin (374). Because ChOS can strongly interact with LPS by the formation of hydrogen bonds and electrostatic interaction, the thus formed stable complex could sterically hinder the interaction with the collagen type I protein (370). However, the biological potency of LPS would remain unaffected, because approximately 30% of the endotoxin will remain free to interact with cellular receptors (375).

In the presence of LPS, the expression of *ALP* and *RUNX-2* was significantly upregulated, accompanied by increased ALP activity and calcium hydroxyapatite deposition. The increase in osteogenic marker gene expression was highly correlated to increased expression of *TLR3* and *YKL-40*, as determined by hierarchical clustering. The effect of LPS on osteogenic differentiation in hBMSCs has been described previously and was linked to the activation of the extracellular signal-regulated kinase (ERK) pathway (116, 376).

The presence of LPS in ChOS preparations could not be conclusively determined from the quality of osteogenic differentiation, yet it required the analysis of cytokine secretion patterns. Several cytokines were strongly secreted in the presence of LPS, including IL-6, IL-8, IL-12p(40), RANTES, IL-4, and TNF-α. Because osteoclasts and immune cells are both derived from the hematopoietic lineage, the former can strongly react to cytokines secreted during inflammation and consequently activate bone remodeling pathways *in vivo* (377). Particularly, IL-6 induced after the ligation of endotoxins with TLR4 was tightly linked to osteoclast-induced bone resorption (367).

In conclusion, we have shown that endotoxin contamination in chitosan derivatives can result in false-positive results, altering product performance *in vitro*. The presence of endotoxins promoted osteogenic differentiation, yet it did not elicit cytotoxicity. Improvement of osteogenic differentiation was completely abrogated after appropriate endotoxin removal. Endotoxin contamination is difficult to avoid during the handling of natural substances, which in combination with sensitive cell-based assays could lead to inaccurate evaluation of biomaterials. Hence, there should be a genuine concern for the possible effects of endotoxin contamination, including the implementation of simple quality control procedures.

4.4. Paper IV – Potential coating material for titanium implants

Clinical treatment of orthopedic tissue injuries often requires fixation via bone implant material (321). Implants produced from titanium and titanium alloys are recommended for a number of load-bearing applications, yet improving the performance at the bone-biomaterial interface could further increase the success of orthopedic fixations (324). Successful integration and stabilization of the implant critically depend on surface characteristics and osseointegration (285). During implantation, damage to the bone environment and the direct contact of the implant with bodily fluids can promote the formation of a fibrous tissue capsule, ultimately resulting in the loosening of the implant (286). Chitosan is one of the natural materials under investigation in order to improve implant osseointegration and cellular attachment due to its favorable key properties (350). The main

characteristics attributed to chitosan include 1) lack of toxicity, 2) biocompatibility, 3) degradation *in vivo*, 4) fungistatic properties, and 5) bacteriostatic effects (204). In addition, the straight-forward use of chitosan in combination with an ample amount of different biomaterials and the easy molding abilities make this polymer an attractive tool for tissue engineering applications (378). The biological properties of chitosan are strongly dependent on the number of charged groups (DD) and the molecular weight and its distribution (polydispersity index) (227). Cellular behavior of any cell type is crucially affected by the surface characteristics and the DD, with distinct effects depending on the cell type (336). Generally, cell attachment and proliferation are superior on membranes prepared from higher DD chitosan, yet lower DD chitosan coatings hold the promising ability to induce healing without scar tissue formation (379). The cationic charge of the polymer is indicated in mediating the attachment of osteoblasts, rather than fibroblasts, offering a remarkably useful strategy for the prevention of fibrous tissue capsule formation around medical implants (345). However, the detailed mechanism of how chitosan membranes affect bone cell attachment and proliferation remain insufficiently understood, and, particularly, attachment rates to lower DD chitosan coatings remain unsatisfactorily low for clinical applications (380).

Consequently, in this study, we evaluated the surface characteristics and bioactivity of different DD chitosan in the form of chitosan membranes. Crab shell chitosan with 87% DD served as positive control for the innovatively prepared range of shrimp shell chitosan (47% DD, 68% DD, 87% DD, and 94% DD). However, a direct comparison of physicochemical properties of chitosan preparations from different natural sources is challenging. Crab shell-derived chitosan requires longer deacetylation processes, causing a decrease in molecular weight, subsequently affecting bioactivity (381).

Prior to bioactivity studies, chitosan membranes were coated with fibronectin to improve initial cell attachment. The interaction of a biomaterial with approaching cells is mediated by the unspecific adsorption of matrix proteins and fibronectin is one of the main adhesion proteins at the bone-biomaterial interface, containing an integrin-specific binding sequence (290). The negatively charged protein is particularly important for attachment and differentiation of osteoblastic cells and is, therefore, frequently used to coat biomaterials (294, 382). Finally, since chitosan membranes with a DD close to 50% are particularly soluble in aqueous solutions, they require cross-linking to improve stability. The solubility of these materials is attributed to the degradation of the secondary structure and the subsequent increase in hydrophilicity (341). Cross-linking methods are commonly used in biomaterials research to prolong material stability and improve chemical resistance (339). Glutaraldehyde is by far the most widely used cross-linking reagent for chitosan membranes and acts via the formation of an imine bond between the primary amino group of chitosan and the aldehyde group (383). In the present study, a low degree (0.02%) of glutaraldehyde cross-linking was applied to 47% DD and 68% DD chitosan membranes to improve stability during long-term cultures of osteogenic differentiation.

Surface characteristics and the biological microenvironment surrounding any biomaterial, strongly influence the interactions with approaching cells and determine bioactivity (384). Therefore, membranes were characterized in terms of surface topography, wettability and fibronectin adsorption. AFM showed that there was no significant difference in surface roughness between chitosan membranes and the tissue culture plastic control. Yet, surface patterns diverged, since chitosan

membranes displayed a valley and hill-like topography, whereas tissue culture plastic presented evenly distributed fibers. Rougher surfaces are associated with enhanced osteoblastic differentiation and cell attachment, while fibroblastic cells prefer smooth surfaces (302). The geometrical architecture at the micrometer and millimeter scale is important for the successful integration of a biomaterial; however, the nano surface roughness mediates the cell-specific interactions (385). Concerning wettability, a trend of increase of the contact angle from lowest to highest DD was observed. Water contact angle measurements are used as an estimation of the hydrophilicity and hydrophobicity of a material surface (386). Low water contact angles are generally associated with superior cell responses, yet matrix protein adsorption may be thermodynamically undesirable. Hydrophobic surfaces attract high levels of matrix proteins, but strong adsorption may damage the protein conformation and hence reduce bioactivity (387). Despite the statistical differences in contact angle measurements between high and low DD chitosan membranes, either surface is still considered hydrophilic. The interaction of fibronectin with chitosan membranes is based on the hydrophilicity and the cationic charge, which plays an important role in decreasing the denaturation process (296). In the present study, high DD chitosan membranes were shown to adsorb similar or even higher amounts of fibronectin to their surface than tissue culture plastic, while low DD chitosan membranes displayed significantly reduced fibronectin retention. These results are in agreement with previously published studies, showing a direct correlation of fibronectin adsorption to increases in the DD (346).

The influence of chitosan membranes on the bioactivity of the preosteoblastic, mouse cell line MC3T3-E1 was determined in terms of attachment, proliferation, and osteogenic differentiation. Cell attachment was observed on all chitosan membranes and could be sustained for extended periods of time (at least 24 days in culture). Cell morphology varied at early time points depending on the DD, but all cell layers were confluent after 1 week of culturing. Compared to the tissue culture plastic controls, proliferation was decreased but similar to that of the fibronectin-coated controls, except for minimal proliferation on 47% DD and 68% DD chitosan membranes. Fibronectin coating increases cell attachment, which in turn decreases the ability of cells to proliferate. Regarding osteogenic differentiation, no spontaneous induction of osteogenic differentiation was observed, but 94% DD chitosan membranes caused significantly higher ALP activity even in the absence of an osteogenic stimulus. Osteogenic marker gene expression revealed no adverse effects of chitosan membranes on osteogenesis, although morphological changes associated with osteogenic differentiation could be observed during the culture period. An increase in ALP activity was observed with higher DD chitosan membranes and the tissue culture plastic control, whereas lower DD chitosan membranes did not secrete active ALP enzyme, independent of the presence of an osteogenic stimulus. However, since the analysis of ALP activity was only a momentary snapshot, upregulation of enzyme activity at different time points could not be excluded.

In conclusion, we showed that higher DD chitosan membranes are associated with increased surface roughness, increased fibronectin adsorption, and improved bioactivity. Osteogenic differentiation was not affected and membranes could not spontaneously induce differentiation processes in the absence of an osteogenic stimulus. Furthermore, cell attachment on low DD chitosan membranes was successfully achieved and cross-linking protocols established to enable long-term

cultures. Much research has focused on the use of chitosan membranes as bioactive coatings, but the opportunities in tissue engineering applications are far from being exhausted. An increase of implant biocompatibility and early strength development at the bone-biomaterial interface could significantly improve the existing practices in the clinical treatment of orthopedic tissue injuries.

4.5. Paper V – Standard protocol for solution casting of chitosan membranes

Implants produced from titanium and titanium alloys have been the gold standard in load-bearing orthopedic applications for several years because of their beneficial biological and mechanical properties. The advantages of titanium and its alloys for biomedical devices include 1) high corrosion resistance, 2) biocompatibility due to spontaneous formation of oxide layers, 3) high specific strength, and 4) lack of toxicity (321). High affinity for oxygen makes the surface of titanium implants sufficiently reactive for chemical modifications, including the deposition of bioactive coatings to enhance bioactivity, osseointegration, and implant stabilization (284). One of the materials under investigation as a bioactive coating for titanium and its alloys is chitosan (378). The prerequisite for the preparation of coatings and membranes is the cationic nature of chitosan, which is the premise for its solubility in dilute aqueous acids. Following dissolution, membranes can be cast on virtually any substrate, and the resulting coating becomes insoluble in aqueous solutions after a simple neutralization step (388). For ease of handling, the validation of a new coating procedure or starting material and subsequent evaluation of bioactivity are generally performed on tissue culture plastic rather than on the final substrate. This allows straightforward characterization of surface properties and biological performance.

The biological properties of chitosan are strongly dependent on the DD, the molecular weight, the polydispersity index, and the successive order of acetylated and deacetylated residues in the chain (227). Inconsistency in reports describing the biological performance of chitosan coatings are frequently due to the lack of detailed information on source, sample preparation, and chemical properties of the polymer, which strongly affects the comparability of results from different studies (236). Furthermore, there are a vast number of protocols available for solution casting methods of chitosan membranes, but they are often restricted to the use of a specific DD (338, 346, 383). In addition, the often scarcely detailed methodological sections in biomaterial-related publications strongly impede reproducibility.

Consequently, we developed a standardized and easily applied protocol for the solution casting of chitosan membranes. This protocol is suitable for chitosan material derived from different sources, spanning a wide DD, and even for the use of chitosan derivatives with innovative properties. The protocol includes a detailed description of preparation steps and quality control methods that ultimately result in long-term attachment and bioactivity similar to that of tissue culture plastic. Furthermore, simple in-house methods for the early prediction of successful experimental outcome were included. This protocol, therefore, allows selection of promising chitosan materials in accordance to the general requirements for coated implants in tissue engineering applications.

5. Concluding Remarks

In this thesis, I determined the effect of natural, chitin-derived biomaterials on stem cell biology and examined important properties of chitosan for tissue engineering applications. I accomplished this task by comparing the effect of three chitosan derivatives on osteogenesis and the expression of the CLP YKL-40 in hBMSCs. The chitosan derivatives in question included 1) the aminosugar glucosamine, which is the smallest, completely deacetylated subunit of chitin; 2) pure hexamer chitooligomer preparations derived either from chitin or chitosan; and 3) a heterogeneous, well-characterized ChOS mixture, mimicking commonly used ChOS preparations in previously reported studies. I evaluated how chitosan derivatives affect the expression of and potentially regulate YKL-40 in hBMSCs, which has not been attempted before. I also demonstrated that strong consideration needs to be placed upon the choice of chitosan derivatives depending on the intended application. Finally, I raise awareness concerning endotoxin contamination in biomaterials derived from natural substances, because current recommendations for *in vitro* evaluation of bioactivity remain inadequate and insufficiently specified. Following a different approach, I determined relevant properties of chitosan for tissue engineering applications based on chitosan membranes as bioactive coatings with potential applications in the surface modification of titanium implants. I compared chitosan membranes prepared from a wide range of DD (47% - 94%) and derived from different sources (crab shell versus shrimp shell) in terms of surface characteristics and bioactivity. This work resulted in the development of a standardized protocol for solution casting processes of chitosan membranes, in-house prediction of successful experimental outcome, and long-term cell attachment comparable to that of commonly used tissue culture plastic.

I would like to emphasize that the biomaterial community is well aware of the effects of endotoxins on ***in vivo*** validation assays, especially due to the stringent guidelines imposed by governing bodies such as the FDA. However, if the biomaterial community was generally aware of the effects of endotoxins on ***in vitro*** assays, I would expect a more open discussion about this topic in the literature. To the best of my knowledge, there is only a handful of groups that report endotoxin testing or endotoxin removal prior to the use of natural substances, and I am not aware of any standard guidelines, to date, that request the analysis of endotoxin levels prior to *in vitro* evaluation of natural substances and more importantly prior to publication of the results of such evaluations.

Furthermore, I would like to point out that because tissue engineering, biomaterial development, and evaluation are interdisciplinary fields, the potential for misunderstandings and negligence is eminent. The evaluation of a biomaterial not only requires knowledge in engineering and materials science for development and production but also demands skills in sterile cell culturing techniques, the understanding of molecular biology, and the ability to appropriately interpret biological results. Scientists working in this field come from a variety of backgrounds, which is one of the reasons why endotoxins in biomaterials research should be more openly discussed. Scientists in this field need to be made aware of the difficulties in detecting endotoxins in natural substances during *in vitro* evaluation of bioactivity.

However, inflammation, as among other stimuli induced by endotoxins, is and stays an important part of wound healing; however, the inflammation process needs to be controlled. Bone is a complex tissue that strongly reacts to environmental and endogeneous stimuli (70). The regulation of bone turn-over and remodeling is regulated by the tightly controlled interplay of several cell types in order to provide appropriate cues for the maintenance and repair of bone structure and function (71). These cell types include osteoblastic cells as well as cells derived from hematopoietic and endothelial lineages (73). Endotoxins are well known for their effects on cellular functions, including the activation of immune cells, but subsequently also the modulation of bone homeostasis (169). Bone resorption during the bone remodeling process can be strongly affected by proinflammatory cytokines secreted from immune cells that modify survival and activation of osteoclasts (389). The same cytokines are also known to similarly affect osteoblasts *in vitro* (390). Several regulatory mechanisms are shared between the immune and the musculoskeletal system and were reported not only to influence osteoblast function but also bone formation (163). Concerning the endothelial cell lineages, these cells are also strongly influenced by endotoxins, upregulating NF-κB - mediated secretion of inflammatory cytokines, and the expression of surface adhesion molecules (391, 392). Furthermore, endotoxins can affect actin organization, monolayer barrier function, and cell attachment to the ECM (393, 394). The response of endothelial cells may in fact contribute to the pathogenesis of systemic inflammation, because endotoxins induce a non-tolerant inflammatory response and the upregulation of adhesion molecules, which may result in injury to the tissue (395). There are a number of potential sources for endotoxins found in the peri-prosthetic tissue, including the bacterial biofilm on the surface of the implant, endotoxin contamination during the implant manufacture, and endotoxins derived from wear particles that can adsorb endotoxins from systemic infections or the intestinal flora (148).

The role of endotoxins in the clinical setting of aseptic implant loosening may remain controversial but more and more evidence is accumulated to indicate that endotoxins are at least partially responsible for osteolysis and the loosening of a medical device (138, 165). Endotoxins were detected in the tissues surrounding implants in patients diagnosed with aseptic implant loosening, although no signs of microbial infections were detected (141). In addition, the prophylactic use of antibiotics, systemically or as part of the implant, resulted in 50% reduction in the incidence of aseptic implant loosening (142). Furthermore, endotoxins have been associated with inflammation in response to wear particles, accelerating the foreign body reaction, proinflammatory cytokine release, and macrophage activation (143). This hypothesis is based on the notion that macrophages at the bone-biomaterial interface express TLRs, which are highly sensitive to even minute amounts of endotoxins (139, 146). Consequently, the activation of these receptors could be responsible for the induction of cytokine secretion, impairing osseointegration, and causing the failure of the implant (165). However, I would like to stress that the removal of endotoxins has been shown to have greater impact in *in vitro* cell culture studies than in *in vivo* studies (143).

Chitosan is one of the most promising natural substances used in biomaterials research and provides several essential key properties for the use in tissue engineering applications (206). The polymer can be easily combined with other biomaterials for virtually any application, rapidly processed; cost-effectively with the possibility of delivering growth factors and drugs to the site of implantation (204). An increase of implant biocompatibility and early strength development at the bone-biomaterial interface could significantly improve existing practices in the clinical treatment of orthopedic tissue injuries.

Much research has focused on the use of chitosan membranes as bioactive coatings, however, numerous challenges remain prior to the successful translation into the clinics. The batch-to-batch variability of chitosan starting materials complicates the prediction of the clinical outcome, and the bonding strength of chitosan-coated implants often remains insufficiently low to sustain the pressure during implantation (206). Furthermore, the effects of long-term storage of coated titanium implants prior to implantation remain unknown and current coating procedures require a more profound understanding of the relationship between processing parameters and the structure of the final coating (396, 397).

Personally, I hope to see an increased understanding and application of the parameters that are involved in the design of chitosan membranes as bioactive coatings for titanium implants. The optimization of these coatings requires intricate knowledge of mechanisms that influence bioactivity, surface properties, and bonding strength. In addition, more extensive *in vivo* evaluation of chitosan-coated titanium implants is necessary prior to clinical applications. I strongly believe that the use of innovative chitosan derivatives could revolutionize bioactive coatings prepared from chitosan. However, certain standards need to be established for publishing reports on the use of chitosan, which should at the very least include the DD, molecular weight, source, detailed processing parameters, and potential contaminants.

References

1. Tanaka EM. Regeneration: if they can do it, why can't we? Cell. 2003 May;113(5):559-62. PubMed PMID: 12787496. eng.
2. Williams DF. To engineer is to create: the link between engineering and regeneration. Trends Biotechnol. 2006 Jan;24(1):4-8. PubMed PMID: 16289395. eng.
3. Martin P, Leibovich SJ. Inflammatory cells during wound repair: the good, the bad and the ugly. Trends Cell Biol. 2005 Nov;15(11):599-607. PubMed PMID: 16202600. eng.
4. Yabut O, Bernstein HS. Human embryonic stem cells in regenerative medicine. In: Bernstein HS, editor. Tissue engineering in regenerative medicine. Springer New York Dordrecht Heidelberg London: Humana Press; 2011. p. 17-38.
5. Daly KA, Brown BN, Badylak SF. Regenerative medicine and the foreign body response. In: Bernstein HS, editor. Tissue engineering in regenerative medicine. Springer New York Dordrecht Heidelberg London: Humana Press; 2011. p. 353-78.
6. Malgieri A, Kantzari E, Patrizi MP, Gambardella S. Bone marrow and umbilical cord blood human mesenchymal stem cells: state of the art. Int J Clin Exp Med. 2010;3(4):248-69. PubMed PMID: 21072260. Pubmed Central PMCID: PMC2971538. eng.
7. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. Cell. 2000 Jan;100(1):157-68. PubMed PMID: 10647940. eng.
8. Jackson L, Jones DR, Scotting P, Sottile V. Adult mesenchymal stem cells: differentiation potential and therapeutic applications. J Postgrad Med. 2007 2007 Apr-Jun;53(2):121-7. PubMed PMID: 17495381. eng.
9. Ratajczak MZ, Zuba-Surma EK, Wysoczynski M, Wan W, Ratajczak J, Wojakowski W, et al. Hunt for pluripotent stem cell -- regenerative medicine search for almighty cell. J Autoimmun. 2008 May;30(3):151-62. PubMed PMID: 18243661. Pubmed Central PMCID: PMC2692479. eng.
10. Lemoli RM, Bertolini F, Cancedda R, De Luca M, Del Santo A, Ferrari G, et al. Stem cell plasticity: time for a reappraisal? Haematologica. 2005 Mar;90(3):360-81. PubMed PMID: 15749669. eng.
11. Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol. 2004 Apr;36(4):568-84. PubMed PMID: 15010324. eng.
12. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell. 2003 Sep;114(6):763-76. PubMed PMID: 14505575. eng.
13. Shizuru JA, Negrin RS, Weissman IL. Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system. Annu Rev Med. 2005;56:509-38. PubMed PMID: 15660525. eng.
14. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002 Jul;418(6893):41-9. PubMed PMID: 12077603. eng.
15. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001 Apr;7(2):211-28. PubMed PMID: 11304456. eng.
16. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol. 2000 Apr;109(1):235-42. PubMed PMID: 10848804. eng.
17. Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res. 2003 Apr;18(4):696-704. PubMed PMID: 12674330. eng.
18. Stenderup K, Justesen J, Clausen C, Kassem M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone. 2003 Dec;33(6):919-26. PubMed PMID: 14678851. eng.
19. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7. PubMed PMID: 16923606. eng.
20. Dazzi F, Ramasamy R, Glennie S, Jones SP, Roberts I. The role of mesenchymal stem cells in haemopoiesis. Blood Rev. 2006 May;20(3):161-71. PubMed PMID: 16364518. eng.
21. Chapel A, Bertho JM, Bensidhoum M, Fouillard L, Young RG, Frick J, et al. Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome. J Gene Med. 2003 Dec;5(12):1028-38. PubMed PMID: 14661178. eng.

22. Fox JM, Chamberlain G, Ashton BA, Middleton J. Recent advances into the understanding of mesenchymal stem cell trafficking. Br J Haematol. 2007 Jun;137(6):491-502. PubMed PMID: 17539772. eng.
23. Caplan AI. Why are MSCs therapeutic? New data: new insight. J Pathol. 2009 Jan;217(2):318-24. PubMed PMID: 19023885. eng.
24. Ortiz LA, Gambelli F, McBride C, Gaupp D, Baddoo M, Kaminski N, et al. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. Proc Natl Acad Sci U S A. 2003 Jul;100(14):8407-11. PubMed PMID: 12815096. Pubmed Central PMCID: PMC166242. eng.
25. Bernardo ME, Locatelli F, Fibbe WE. Mesenchymal stromal cells. Ann N Y Acad Sci. 2009 Sep;1176:101-17. PubMed PMID: 19796238. eng.
26. Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. Blood. 2007 Nov;110(10):3499-506. PubMed PMID: 17664353. eng.
27. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002 May;99(10):3838-43. PubMed PMID: 11986244. eng.
28. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human mesenchymal stem cells modulate B-cell functions. Blood. 2006 Jan;107(1):367-72. PubMed PMID: 16141348. eng.
29. Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells. 2006 Jan;24(1):74-85. PubMed PMID: 16099998. eng.
30. Jiang XX, Zhang Y, Liu B, Zhang SX, Wu Y, Yu XD, et al. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. Blood. 2005 May;105(10):4120-6. PubMed PMID: 15692068. eng.
31. Maccario R, Podestà M, Moretta A, Cometa A, Comoli P, Montagna D, et al. Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. Haematologica. 2005 Apr;90(4):516-25. PubMed PMID: 15820948. eng.
32. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005 Feb;105(4):1815-22. PubMed PMID: 15494428. eng.
33. Caetano-Lopes J, Canhão H, Fonseca JE. Osteoblasts and bone formation. Acta Reumatol Port. 2007 2007 Apr-Jun;32(2):103-10. PubMed PMID: 17572649. eng.
34. Long F. Building strong bones: molecular regulation of the osteoblast lineage. Nature Reviews: Molecular Cell Biology. 2012;13:27-38.
35. Rattner A, Sabido O, Massoubre C, Rascle F, Frey J. Characterization of human osteoblastic cells: influence of the culture conditions. In Vitro Cell Dev Biol Anim. 1997 1997 Nov-Dec;33(10):757-62. PubMed PMID: 9466680. eng.
36. Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. J Bone Miner Res. 1992 Jun;7(6):683-92. PubMed PMID: 1414487. eng.
37. Bellows CG, Heersche JN, Aubin JE. Inorganic phosphate added exogenously or released from beta-glycerophosphate initiates mineralization of osteoid nodules in vitro. Bone Miner. 1992 Apr;17(1):15-29. PubMed PMID: 1581703. eng.
38. Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells. 2007 Nov;25(11):2739-49. PubMed PMID: 17656645. eng.
39. Cheng SL, Zhang SF, Avioli LV. Expression of bone matrix proteins during dexamethasone-induced mineralization of human bone marrow stromal cells. J Cell Biochem. 1996 May;61(2):182-93. PubMed PMID: 9173083. eng.
40. Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV. Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. Endocrinology. 1994 Jan;134(1):277-86. PubMed PMID: 8275945. eng.
41. Franceschi R. The role of ascorbic acid in mesenchymal differentiation. Nutr Rev. 1992;50:60-5.
42. Takamizawa S, Maehata Y, Imai K, Senoo H, Sato S, Hata R. Effects of ascorbic acid and ascorbic acid 2-phosphate, a long-acting vitamin C derivative, on the proliferation and differentiation of human osteoblast-like cells. Cell Biol Int. 2004;28(4):255-65. PubMed PMID: 15109981. eng.

43. Chung CH, Golub EE, Forbes E, Tokuoka T, Shapiro IM. Mechanism of action of beta-glycerophosphate on bone cell mineralization. Calcif Tissue Int. 1992 Oct;51(4):305-11. PubMed PMID: 1422975. eng.
44. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell. 2002 Jan;108(1):17-29. PubMed PMID: 11792318. eng.
45. Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. Nature. 2003 May;423(6937):349-55. PubMed PMID: 12748654. eng.
46. Komori T. Regulation of osteoblast differentiation by transcription factors. J Cell Biochem. 2006 Dec;99(5):1233-9. PubMed PMID: 16795049. eng.
47. Kern B, Shen J, Starbuck M, Karsenty G. Cbfa1 contributes to the osteoblast-specific expression of type I collagen genes. J Biol Chem. 2001 Mar;276(10):7101-7. PubMed PMID: 11106645. eng.
48. Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, et al. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev. 1999 Apr;13(8):1025-36. PubMed PMID: 10215629. Pubmed Central PMCID: PMC316641. eng.
49. Xiao G, Jiang D, Ge C, Zhao Z, Lai Y, Boules H, et al. Cooperative interactions between activating transcription factor 4 and Runx2/Cbfa1 stimulate osteoblast-specific osteocalcin gene expression. J Biol Chem. 2005 Sep;280(35):30689-96. PubMed PMID: 16000305. eng.
50. Yang X, Matsuda K, Bialek P, Jacquot S, Masuoka HC, Schinke T, et al. ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. Cell. 2004 Apr;117(3):387-98. PubMed PMID: 15109498. eng.
51. Brooke G, Cook M, Blair C, Han R, Heazlewood C, Jones B, et al. Therapeutic applications of mesenchymal stromal cells. Semin Cell Dev Biol. 2007 Dec;18(6):846-58. PubMed PMID: 18024097. eng.
52. Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells. 2002;20(6):530-41. PubMed PMID: 12456961. eng.
53. Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringdén O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp Hematol. 2003 Oct;31(10):890-6. PubMed PMID: 14550804. eng.
54. Shi Y, Su J, Roberts AI, Shou P, Rabson AB, Ren G. How mesenchymal stem cells interact with tissue immune responses. Trends Immunol. 2012 Mar;33(3):136-43. PubMed PMID: 22227317. eng.
55. Prockop DJ, Brenner M, Fibbe WE, Horwitz E, Le Blanc K, Phinney DG, et al. Defining the risks of mesenchymal stromal cell therapy. Cytotherapy. 2010 Sep;12(5):576-8. PubMed PMID: 20735162. eng.
56. Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood. 2003 Nov;102(10):3837-44. PubMed PMID: 12881305. eng.
57. Jeong JO, Han JW, Kim JM, Cho HJ, Park C, Lee N, et al. Malignant tumor formation after transplantation of short-term cultured bone marrow mesenchymal stem cells in experimental myocardial infarction and diabetic neuropathy. Circ Res. 2011 May;108(11):1340-7. PubMed PMID: 21493893. Pubmed Central PMCID: PMC3109741. eng.
58. Spaeth EL, Dembinski JL, Sasser AK, Watson K, Klopp A, Hall B, et al. Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. PLoS One. 2009;4(4):e4992. PubMed PMID: 19352430. Pubmed Central PMCID: PMC2661372. eng.
59. Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, et al. Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med. 2001 Feb;344(5):385-6. PubMed PMID: 11195802. eng.
60. Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet. 2004 May;363(9419):1439-41. PubMed PMID: 15121408. eng.
61. Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, et al. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet. 2003 Jan;361(9351):45-6. PubMed PMID: 12517467. eng.

62. Arinzeh TL, Peter SJ, Archambault MP, van den Bos C, Gordon S, Kraus K, et al. Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect. J Bone Joint Surg Am. 2003 Oct;85-A(10):1927-35. PubMed PMID: 14563800. eng.
63. Horwitz EM, Prockop DJ, Gordon PL, Koo WW, Fitzpatrick LA, Neel MD, et al. Clinical responses to bone marrow transplantation in children with severe osteogenesis imperfecta. Blood. 2001 Mar;97(5):1227-31. PubMed PMID: 11222364. eng.
64. Krebsbach PH, Mankani MH, Satomura K, Kuznetsov SA, Robey PG. Repair of craniotomy defects using bone marrow stromal cells. Transplantation. 1998 Nov;66(10):1272-8. PubMed PMID: 9846508. eng.
65. Wakitani S, Imoto K, Yamamoto T, Saito M, Murata N, Yoneda M. Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. Osteoarthritis Cartilage. 2002 Mar;10(3):199-206. PubMed PMID: 11869080. eng.
66. Muschler GF, Nitto H, Matsukura Y, Boehm C, Valdevit A, Kambic H, et al. Spine fusion using cell matrix composites enriched in bone marrow-derived cells. Clin Orthop Relat Res. 2003 Feb(407):102-18. PubMed PMID: 12567137. Pubmed Central PMCID: PMC1425047. eng.
67. Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, et al. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood. 2005 Sep;106(5):1755-61. PubMed PMID: 15905186. eng.
68. Ball LM, Bernardo ME, Roelofs H, Lankester A, Cometa A, Egeler RM, et al. Cotransplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. Blood. 2007 Oct;110(7):2764-7. PubMed PMID: 17638847. eng.
69. Macmillan ML, Blazar BR, DeFor TE, Wagner JE. Transplantation of ex-vivo culture-expanded parental haploidentical mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: results of a phase I-II clinical trial. Bone Marrow Transplant. 2009 Mar;43(6):447-54. PubMed PMID: 18955980. eng.
70. Walkley CR, Shea JM, Sims NA, Purton LE, Orkin SH. Rb regulates interactions between hematopoietic stem cells and their bone marrow microenvironment. Cell. 2007 Jun;129(6):1081-95. PubMed PMID: 17574022. Pubmed Central PMCID: PMC2768301. eng.
71. Seeman E, Delmas PD. Bone quality--the material and structural basis of bone strength and fragility. N Engl J Med. 2006 May;354(21):2250-61. PubMed PMID: 16723616. eng.
72. Ducy P, Schinke T, Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance. Science. 2000 Sep;289(5484):1501-4. PubMed PMID: 10968779. eng.
73. Pogoda P, Priemel M, Rueger JM, Amling M. Bone remodeling: new aspects of a key process that controls skeletal maintenance and repair. Osteoporos Int. 2005 Mar;16 Suppl 2:S18-24. PubMed PMID: 15551056. eng.
74. Malone JD, Teitelbaum SL, Griffin GL, Senior RM, Kahn AJ. Recruitment of osteoclast precursors by purified bone matrix constituents. J Cell Biol. 1982 Jan;92(1):227-30. PubMed PMID: 6976967. Pubmed Central PMCID: PMC2112000. eng.
75. Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, De Smedt T, et al. RANK is essential for osteoclast and lymph node development. Genes Dev. 1999 Sep;13(18):2412-24. PubMed PMID: 10500098. Pubmed Central PMCID: PMC317030. eng.
76. Boyce BF, Yao Z, Xing L. Osteoclasts have multiple roles in bone in addition to bone resorption. Crit Rev Eukaryot Gene Expr. 2009;19(3):171-80. PubMed PMID: 19883363. Pubmed Central PMCID: PMC2856465. eng.
77. Street J, Bao M, deGuzman L, Bunting S, Peale FV, Ferrara N, et al. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc Natl Acad Sci U S A. 2002 Jul;99(15):9656-61. PubMed PMID: 12118119. Pubmed Central PMCID: PMC124965. eng.
78. Collin-Osdoby P. Role of vascular endothelial cells in bone biology. J Cell Biochem. 1994 Jul;55(3):304-9. PubMed PMID: 7962161. eng.
79. Villars F, Bordenave L, Bareille R, Amédée J. Effect of human endothelial cells on human bone marrow stromal cell phenotype: role of VEGF? J Cell Biochem. 2000 Sep;79(4):672-85. PubMed PMID: 10996857. eng.
80. Guillotin B, Bourget C, Remy-Zolgadri M, Bareille R, Fernandez P, Conrad V, et al. Human primary endothelial cells stimulate human osteoprogenitor cell differentiation. Cell Physiol Biochem. 2004;14(4-6):325-32. PubMed PMID: 15319536. eng.

81. Schindeler A, McDonald MM, Bokko P, Little DG. Bone remodeling during fracture repair: The cellular picture. Semin Cell Dev Biol. 2008 Oct;19(5):459-66. PubMed PMID: 18692584. eng.
82. Berthod F, Germain L, Tremblay N, Auger FA. Extracellular matrix deposition by fibroblasts is necessary to promote capillary-like tube formation in vitro. J Cell Physiol. 2006 May;207(2):491-8. PubMed PMID: 16453301. eng.
83. Elbjeirami WM, West JL. Angiogenesis-like activity of endothelial cells co-cultured with VEGF-producing smooth muscle cells. Tissue Eng. 2006 Feb;12(2):381-90. PubMed PMID: 16548696. eng.
84. Peters K, Unger RE, Brunner J, Kirkpatrick CJ. Molecular basis of endothelial dysfunction in sepsis. Cardiovasc Res. 2003 Oct;60(1):49-57. PubMed PMID: 14522406. eng.
85. Janeway CA, Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002;20:197-216. PubMed PMID: 11861602. eng.
86. Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. Microbes Infect. 2004 Dec;6(15):1382-7. PubMed PMID: 15596124. eng.
87. Janeway CA. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol. 1989;54 Pt 1:1-13. PubMed PMID: 2700931. eng.
88. Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol. 2005 Jan;17(1):1-14. PubMed PMID: 15585605. eng.
89. Beutler B. Innate immunity: an overview. Mol Immunol. 2004 Feb;40(12):845-59. PubMed PMID: 14698223. eng.
90. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol. 2001 Aug;2(8):675-80. PubMed PMID: 11477402. eng.
91. Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol. 2001 Nov;1(2):135-45. PubMed PMID: 11905821. eng.
92. Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. Science. 1996 Apr;272(5258):50-3. PubMed PMID: 8600536. eng.
93. Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. Nat Immunol. 2001 Oct;2(10):947-50. PubMed PMID: 11547333. eng.
94. Garcia KC, Teyton L, Wilson IA. Structural basis of T cell recognition. Annu Rev Immunol. 1999;17:369-97. PubMed PMID: 10358763. eng.
95. West AP, Koblansky AA, Ghosh S. Recognition and signaling by toll-like receptors. Annu Rev Cell Dev Biol. 2006;22:409-37. PubMed PMID: 16822173. eng.
96. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004 Jul;4(7):499-511. PubMed PMID: 15229469. eng.
97. Slack JL, Schooley K, Bonnert TP, Mitcham JL, Qwarnstrom EE, Sims JE, et al. Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. J Biol Chem. 2000 Feb;275(7):4670-8. PubMed PMID: 10671496. eng.
98. Banerjee A, Gerondakis S. Coordinating TLR-activated signaling pathways in cells of the immune system. Immunol Cell Biol. 2007 2007 Aug-Sep;85(6):420-4. PubMed PMID: 17637696. eng.
99. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity. 1999 Oct;11(4):443-51. PubMed PMID: 10549626. eng.
100. Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, et al. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science. 1999 Jul;285(5428):736-9. PubMed PMID: 10426996. eng.
101. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001 Oct;413(6857):732-8. PubMed PMID: 11607032. eng.
102. Hoshino K, Kaisho T, Iwabe T, Takeuchi O, Akira S. Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation. Int Immunol. 2002 Oct;14(10):1225-31. PubMed PMID: 12356687. eng.
103. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998 Dec;282(5396):2085-8. PubMed PMID: 9851930. eng.
104. Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. Nat Rev Immunol. 2003 Feb;3(2):169-76. PubMed PMID: 12563300. eng.

105. Ohashi K, Burkart V, Flohé S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J Immunol. 2000 Jan;164(2):558-61. PubMed PMID: 10623794. eng.
106. Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, et al. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. J Exp Med. 2002 Jan;195(1):99-111. PubMed PMID: 11781369. Pubmed Central PMCID: PMC2196009. eng.
107. Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, et al. The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem. 2001 Mar;276(13):10229-33. PubMed PMID: 11150311. eng.
108. Matzinger P. The danger model: a renewed sense of self. Science. 2002 Apr;296(5566):301-5. PubMed PMID: 11951032. eng.
109. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature. 2001 Apr;410(6832):1099-103. PubMed PMID: 11323673. eng.
110. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science. 2004 Mar;303(5663):1529-31. PubMed PMID: 14976261. eng.
111. Toshchakov V, Jones BW, Perera PY, Thomas K, Cody MJ, Zhang S, et al. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat Immunol. 2002 Apr;3(4):392-8. PubMed PMID: 11896392. eng.
112. DelaRosa O, Lombardo E. Modulation of adult mesenchymal stem cells activity by toll-like receptors: implications on therapeutic potential. Mediators Inflamm. 2010;2010:865601. PubMed PMID: 20628526. Pubmed Central PMCID: PMC2902124. eng.
113. Björkbacka H, Kunjathoor VV, Moore KJ, Koehn S, Ordija CM, Lee MA, et al. Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. Nat Med. 2004 Apr;10(4):416-21. PubMed PMID: 15034566. eng.
114. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 2004 Jul;118(2):229-41. PubMed PMID: 15260992. eng.
115. Hasan UA, Trinchieri G, Vlach J. Toll-like receptor signaling stimulates cell cycle entry and progression in fibroblasts. J Biol Chem. 2005 May;280(21):20620-7. PubMed PMID: 15788393. eng.
116. Hwa Cho H, Bae YC, Jung JS. Role of toll-like receptors on human adipose-derived stromal cells. Stem Cells. 2006 Dec;24(12):2744-52. PubMed PMID: 16902195. eng.
117. Pevsner-Fischer M, Morad V, Cohen-Sfady M, Rousso-Noori L, Zanin-Zhorov A, Cohen S, et al. Toll-like receptors and their ligands control mesenchymal stem cell functions. Blood. 2007 Feb;109(4):1422-32. PubMed PMID: 17038530. eng.
118. Waterman RS, Tomchuck SL, Henkle SL, Betancourt AM. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. PLoS One. 2010;5(4):e10088. PubMed PMID: 20436665. Pubmed Central PMCID: PMC2859930. eng.
119. Tomchuck SL, Zwezdaryk KJ, Coffelt SB, Waterman RS, Danka ES, Scandurro AB. Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. Stem Cells. 2008 Jan;26(1):99-107. PubMed PMID: 17916800. Pubmed Central PMCID: PMC2757778. eng.
120. Wang ZJ, Zhang FM, Wang LS, Yao YW, Zhao Q, Gao X. Lipopolysaccharides can protect mesenchymal stem cells (MSCs) from oxidative stress-induced apoptosis and enhance proliferation of MSCs via Toll-like receptor(TLR)-4 and PI3K/Akt. Cell Biol Int. 2009 Jun;33(6):665-74. PubMed PMID: 19376254. eng.
121. Mo IF, Yip KH, Chan WK, Law HK, Lau YL, Chan GC. Prolonged exposure to bacterial toxins downregulated expression of toll-like receptors in mesenchymal stromal cell-derived osteoprogenitors. BMC Cell Biol. 2008;9:52. PubMed PMID: 18799018. Pubmed Central PMCID: PMC2567970. eng.
122. Yao Y, Zhang F, Wang L, Zhang G, Wang Z, Chen J, et al. Lipopolysaccharide preconditioning enhances the efficacy of mesenchymal stem cells transplantation in a rat model of acute myocardial infarction. J Biomed Sci. 2009;16:74. PubMed PMID: 19691857. Pubmed Central PMCID: PMC2739513. eng.
123. Liotta F, Angeli R, Cosmi L, Filì L, Manuelli C, Frosali F, et al. Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell

modulatory activity by impairing Notch signaling. Stem Cells. 2008 Jan;26(1):279-89. PubMed PMID: 17962701. eng.
124. Caroff M, Karibian D. Structure of bacterial lipopolysaccharides. Carbohydr Res. 2003 Nov;338(23):2431-47. PubMed PMID: 14670707. eng.
125. Muroi M, Tanamoto K. The polysaccharide portion plays an indispensable role in Salmonella lipopolysaccharide-induced activation of NF-kappaB through human toll-like receptor 4. Infect Immun. 2002 Nov;70(11):6043-7. PubMed PMID: 12379680. Pubmed Central PMCID: PMC130318. eng.
126. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. Annu Rev Biochem. 2002;71:635-700. PubMed PMID: 12045108. Pubmed Central PMCID: PMC2569852. eng.
127. Darkow R, Groth T, Albrecht W, Lützow K, Paul D. Functionalized nanoparticles for endotoxin binding in aqueous solutions. Biomaterials. 1999 Jul;20(14):1277-83. PubMed PMID: 10403045. eng.
128. Gutsmann T, Schromm AB, Brandenburg K. The physicochemistry of endotoxins in relation to bioactivity. Int J Med Microbiol. 2007 Sep;297(5):341-52. PubMed PMID: 17452014. eng.
129. Caroff M, Karibian D, Cavaillon JM, Haeffner-Cavaillon N. Structural and functional analyses of bacterial lipopolysaccharides. Microbes Infect. 2002 Jul;4(9):915-26. PubMed PMID: 12106784. eng.
130. Joiner KA, Schmetz MA, Goldman RC, Leive L, Frank MM. Mechanism of bacterial resistance to complement-mediated killing: inserted C5b-9 correlates with killing for Escherichia coli O111B4 varying in O-antigen capsule and O-polysaccharide coverage of lipid A core oligosaccharide. Infect Immun. 1984 Jul;45(1):113-7. PubMed PMID: 6203836. Pubmed Central PMCID: PMC263282. eng.
131. Gorbet MB, Sefton MV. Endotoxin: the uninvited guest. Biomaterials. 2005 Dec;26(34):6811-7. PubMed PMID: 16019062. eng.
132. Miyake K. Endotoxin recognition molecules, Toll-like receptor 4-MD-2. Semin Immunol. 2004 Feb;16(1):11-6. PubMed PMID: 14751758. eng.
133. Nalepka JL, Greenfield EM. Detection of bacterial endotoxin in human tissues. Biotechniques. 2004 Sep;37(3):413-7. PubMed PMID: 15470896. eng.
134. Glauser MP, Zanetti G, Baumgartner JD, Cohen J. Septic shock: pathogenesis. Lancet. 1991 Sep;338(8769):732-6. PubMed PMID: 1679876. eng.
135. Opal SM. The host response to endotoxin, antilipopolysaccharide strategies, and the management of severe sepsis. Int J Med Microbiol. 2007 Sep;297(5):365-77. PubMed PMID: 17452016. eng.
136. Horn DL, Opal SM, Lomastro E. Antibiotics, cytokines, and endotoxin: a complex and evolving relationship in gram-negative sepsis. Scand J Infect Dis Suppl. 1996;101:9-13. PubMed PMID: 9060045. eng.
137. Opal SM, Palardy JE, Marra MN, Fisher CJ, McKelligon BM, Scott RW. Relative concentrations of endotoxin-binding proteins in body fluids during infection. Lancet. 1994 Aug;344(8920):429-31. PubMed PMID: 7520106. eng.
138. Greenfield EM, Bi Y, Ragab AA, Goldberg VM, Nalepka JL, Seabold JM. Does endotoxin contribute to aseptic loosening of orthopedic implants? J Biomed Mater Res B Appl Biomater. 2005 Jan;72(1):179-85. PubMed PMID: 15449253. eng.
139. Sundfeldt M, Carlsson LV, Johansson CB, Thomsen P, Gretzer C. Aseptic loosening, not only a question of wear: a review of different theories. Acta Orthop. 2006 Apr;77(2):177-97. PubMed PMID: 16752278. eng.
140. Nelson CL, McLaren AC, McLaren SG, Johnson JW, Smeltzer MS. Is aseptic loosening truly aseptic? Clin Orthop Relat Res. 2005 Aug(437):25-30. PubMed PMID: 16056022. eng.
141. Nalepka JL, Lee MJ, Kraay MJ, Marcus RE, Goldberg VM, Chen X, et al. Lipopolysaccharide found in aseptic loosening of patients with inflammatory arthritis. Clin Orthop Relat Res. 2006 Oct;451:229-35. PubMed PMID: 16735873. eng.
142. Engesaeter LB, Lie SA, Espehaug B, Furnes O, Vollset SE, Havelin LI. Antibiotic prophylaxis in total hip arthroplasty: effects of antibiotic prophylaxis systemically and in bone cement on the revision rate of 22,170 primary hip replacements followed 0-14 years in the Norwegian Arthroplasty Register. Acta Orthop Scand. 2003 Dec;74(6):644-51. PubMed PMID: 14763692. eng.
143. Bi Y, Seabold JM, Kaar SG, Ragab AA, Goldberg VM, Anderson JM, et al. Adherent endotoxin on orthopedic wear particles stimulates cytokine production and osteoclast differentiation. J Bone Miner Res. 2001 Nov;16(11):2082-91. PubMed PMID: 11697805. eng.
144. Santavirta S, Konttinen YT, Bergroth V, Eskola A, Tallroth K, Lindholm TS. Aggressive granulomatous lesions associated with hip arthroplasty. Immunopathological studies. J Bone Joint Surg Am. 1990 Feb;72(2):252-8. PubMed PMID: 1968067. eng.

145. Lassus J, Waris V, Xu JW, Li TF, Hao J, Nietosvaara Y, et al. Increased interleukin-8 (IL-8) expression is related to aseptic loosening of total hip replacement. Arch Orthop Trauma Surg. 2000;120(5-6):328-32. PubMed PMID: 10853906. eng.
146. Takagi M, Tamaki Y, Hasegawa H, Takakubo Y, Konttinen L, Tiainen VM, et al. Toll-like receptors in the interface membrane around loosening total hip replacement implants. J Biomed Mater Res A. 2007 Jun;81(4):1017-26. PubMed PMID: 17415764. eng.
147. Tatro JM, Taki N, Islam AS, Goldberg VM, Rimnac CM, Doerschuk CM, et al. The balance between endotoxin accumulation and clearance during particle-induced osteolysis in murine calvaria. J Orthop Res. 2007 Mar;25(3):361-9. PubMed PMID: 17106883. eng.
148. Cho DR, Shanbhag AS, Hong CY, Baran GR, Goldring SR. The role of adsorbed endotoxin in particle-induced stimulation of cytokine release. J Orthop Res. 2002 Jul;20(4):704-13. PubMed PMID: 12168658. eng.
149. Munford RS. Detoxifying endotoxin: time, place and person. J Endotoxin Res. 2005;11(2):69-84. PubMed PMID: 15949133. eng.
150. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. N Engl J Med. 2003 Jan;348(2):138-50. PubMed PMID: 12519925. eng.
151. Hamann L, Alexander C, Stamme C, Zähringer U, Schumann RR. Acute-phase concentrations of lipopolysaccharide (LPS)-binding protein inhibit innate immune cell activation by different LPS chemotypes via different mechanisms. Infect Immun. 2005 Jan;73(1):193-200. PubMed PMID: 15618154. Pubmed Central PMCID: PMC538978. eng.
152. Corradin SB, Mauël J, Gallay P, Heumann D, Ulevitch RJ, Tobias PS. Enhancement of murine macrophage binding of and response to bacterial lipopolysaccharide (LPS) by LPS-binding protein. J Leukoc Biol. 1992 Oct;52(4):363-8. PubMed PMID: 1402386. eng.
153. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science. 1990 Sep;249(4975):1431-3. PubMed PMID: 1698311. eng.
154. Wang D, Pabst KM, Aida Y, Pabst MJ. Lipopolysaccharide-inactivating activity of neutrophils is due to lactoferrin. J Leukoc Biol. 1995 Jun;57(6):865-74. PubMed PMID: 7790769. eng.
155. Wang X, Quinn PJ. Lipopolysaccharide: Biosynthetic pathway and structure modification. Prog Lipid Res. 2010 Apr;49(2):97-107. PubMed PMID: 19815028. eng.
156. Llewelyn M, Cohen J. New insights into the pathogenesis and therapy of sepsis and septic shock. Curr Clin Top Infect Dis. 2001;21:148-71. PubMed PMID: 11572150. eng.
157. Rietschel ET, Brade H, Holst O, Brade L, Müller-Loennies S, Mamat U, et al. Bacterial endotoxin: Chemical constitution, biological recognition, host response, and immunological detoxification. Curr Top Microbiol Immunol. 1996;216:39-81. PubMed PMID: 8791735. eng.
158. Kawaguchi H, Hizuta A, Tanaka N, Orita K. Role of endotoxin in wound healing impairment. Res Commun Mol Pathol Pharmacol. 1995 Sep;89(3):317-27. PubMed PMID: 8680800. eng.
159. Udagawa N, Sato N, Yang S, Nakamura M, Yamashita T, Nakamura H, et al. Signal transduction of lipopolysaccharide-induced osteoclast differentiation. Periodontol 2000. 2007;43:56-64. PubMed PMID: 17214835. eng.
160. Benahmed M, Heymann D, Pilet P, Bienvenu J, Daculsi G. LPS increases biomaterial degradation by human monocytes in vitro. J Biomed Mater Res. 1997 Jan;34(1):115-9. PubMed PMID: 8978660. eng.
161. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature. 2003 May;423(6937):337-42. PubMed PMID: 12748652. eng.
162. Itoh K, Udagawa N, Kobayashi K, Suda K, Li X, Takami M, et al. Lipopolysaccharide promotes the survival of osteoclasts via Toll-like receptor 4, but cytokine production of osteoclasts in response to lipopolysaccharide is different from that of macrophages. J Immunol. 2003 Apr;170(7):3688-95. PubMed PMID: 12646634. eng.
163. Gruber R. Cell biology of osteoimmunology. Wien Med Wochenschr. 2010 Sep;160(17-18):438-45. PubMed PMID: 20714814. eng.
164. Maitz MF, Teichmann J, Sperling C, Werner C. Surface endotoxin contamination and hemocompatibility evaluation of materials. J Biomed Mater Res B Appl Biomater. 2009 Jul;90(1):18-25. PubMed PMID: 18973273. eng.
165. Ragab AA, Van De Motter R, Lavish SA, Goldberg VM, Ninomiya JT, Carlin CR, et al. Measurement and removal of adherent endotoxin from titanium particles and implant surfaces. J Orthop Res. 1999 Nov;17(6):803-9. PubMed PMID: 10632445. eng.

166. Nakagawa Y, Murai T, Hasegawa C, Hirata M, Tsuchiya T, Yagami T, et al. Endotoxin contamination in wound dressings made of natural biomaterials. J Biomed Mater Res B Appl Biomater. 2003 Jul;66(1):347-55. PubMed PMID: 12808594. eng.
167. Hasiwa M, Kullmann K, von Aulock S, Klein C, Hartung T. An in vitro pyrogen safety test for immune-stimulating components on surfaces. Biomaterials. 2007 Mar;28(7):1367-75. PubMed PMID: 17157907. eng.
168. Bryans TD, Braithwaite C, Broad J, Cooper JF, Darnell KR, Hitchins VM, et al. Bacterial endotoxin testing: a report on the methods, background, data, and regulatory history of extraction recovery efficiency. Biomed Instrum Technol. 2004 2004 Jan-Feb;38(1):73-8. PubMed PMID: 15008051. eng.
169. Raetz CR. Bacterial endotoxins: extraordinary lipids that activate eucaryotic signal transduction. J Bacteriol. 1993 Sep;175(18):5745-53. PubMed PMID: 8376321. Pubmed Central PMCID: PMC206651. eng.
170. Elias JA, Homer RJ, Hamid Q, Lee CG. Chitinases and chitinase-like proteins in T(H)2 inflammation and asthma. J Allergy Clin Immunol. 2005 Sep;116(3):497-500. PubMed PMID: 16159614. eng.
171. Henrissat B, Davies G. Structural and sequence-based classification of glycoside hydrolases. Curr Opin Struct Biol. 1997 Oct;7(5):637-44. PubMed PMID: 9345621. eng.
172. Boot RG, Blommaart EF, Swart E, Ghauharali-van der Vlugt K, Bijl N, Moe C, et al. Identification of a novel acidic mammalian chitinase distinct from chitotriosidase. J Biol Chem. 2001 Mar;276(9):6770-8. PubMed PMID: 11085997. eng.
173. Renkema GH, Boot RG, Muijsers AO, Donker-Koopman WE, Aerts JM. Purification and characterization of human chitotriosidase, a novel member of the chitinase family of proteins. J Biol Chem. 1995 Feb;270(5):2198-202. PubMed PMID: 7836450. eng.
174. Millis AJ, Hoyle M, Kent L. In vitro expression of a 38,000 dalton heparin-binding glycoprotein by morphologically differentiated smooth muscle cells. J Cell Physiol. 1986 Jun;127(3):366-72. PubMed PMID: 3086326. eng.
175. Hu B, Trinh K, Figueira WF, Price PA. Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family. J Biol Chem. 1996 Aug;271(32):19415-20. PubMed PMID: 8702629. eng.
176. Buhi WC. Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. Reproduction. 2002 Mar;123(3):355-62. PubMed PMID: 11882012. eng.
177. Kzhyshkowska J, Mamidi S, Gratchev A, Kremmer E, Schmuttermaier C, Krusell L, et al. Novel stabilin-1 interacting chitinase-like protein (SI-CLP) is up-regulated in alternatively activated macrophages and secreted via lysosomal pathway. Blood. 2006 Apr;107(8):3221-8. PubMed PMID: 16357325. eng.
178. van Aalten DM, Komander D, Synstad B, Gåseidnes S, Peter MG, Eijsink VG. Structural insights into the catalytic mechanism of a family 18 exo-chitinase. Proc Natl Acad Sci U S A. 2001 Jul;98(16):8979-84. PubMed PMID: 11481469. Pubmed Central PMCID: PMC55359. eng.
179. Bussink AP, Speijer D, Aerts JM, Boot RG. Evolution of mammalian chitinase(-like) members of family 18 glycosyl hydrolases. Genetics. 2007 Oct;177(2):959-70. PubMed PMID: 17720922. Pubmed Central PMCID: PMC2034658. eng.
180. Fusetti F, Pijning T, Kalk KH, Bos E, Dijkstra BW. Crystal structure and carbohydrate-binding properties of the human cartilage glycoprotein-39. J Biol Chem. 2003 Sep;278(39):37753-60. PubMed PMID: 12851408. eng.
181. Bleau G, Massicotte F, Merlen Y, Boisvert C. Mammalian chitinase-like proteins. EXS. 1999;87:211-21. PubMed PMID: 10906962. eng.
182. Lee CG, Da Silva CA, Dela Cruz CS, Ahangari F, Ma B, Kang MJ, et al. Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. Annu Rev Physiol. 2011 Mar;73:479-501. PubMed PMID: 21054166. eng.
183. Lee CG. Chitin, chitinases and chitinase-like proteins in allergic inflammation and tissue remodeling. Yonsei Med J. 2009 Feb;50(1):22-30. PubMed PMID: 19259344. Pubmed Central PMCID: PMC2649864. eng.
184. Kawada M, Seno H, Kanda K, Nakanishi Y, Akitake R, Komekado H, et al. Chitinase 3-like 1 promotes macrophage recruitment and angiogenesis in colorectal cancer. Oncogene. 2011 Nov. PubMed PMID: 22056877. Pubmed Central PMCID: PMC3290745. ENG.

185. Johansen JS. Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodelling, fibroses and cancer. Dan Med Bull. 2006 May;53(2):172-209. PubMed PMID: 17087877. eng.
186. Zhu Z, Zheng T, Homer RJ, Kim YK, Chen NY, Cohn L, et al. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. Science. 2004 Jun;304(5677):1678-82. PubMed PMID: 15192232. eng.
187. van Eijk M, van Roomen CP, Renkema GH, Bussink AP, Andrews L, Blommaart EF, et al. Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. Int Immunol. 2005 Nov;17(11):1505-12. PubMed PMID: 16214810. eng.
188. Hakala BE, White C, Recklies AD. Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. J Biol Chem. 1993 Dec;268(34):25803-10. PubMed PMID: 8245017. eng.
189. Krause SW, Rehli M, Kreutz M, Schwarzfischer L, Paulauskis JD, Andreesen R. Differential screening identifies genetic markers of monocyte to macrophage maturation. J Leukoc Biol. 1996 Oct;60(4):540-5. PubMed PMID: 8864140. eng.
190. Houston DR, Recklies AD, Krupa JC, van Aalten DM. Structure and ligand-induced conformational change of the 39-kDa glycoprotein from human articular chondrocytes. J Biol Chem. 2003 Aug;278(32):30206-12. PubMed PMID: 12775711. eng.
191. Recklies AD, Ling H, White C, Bernier SM. Inflammatory cytokines induce production of CHI3L1 by articular chondrocytes. J Biol Chem. 2005 Dec;280(50):41213-21. PubMed PMID: 16234240. eng.
192. Ling H, Recklies AD. The chitinase 3-like protein human cartilage glycoprotein 39 inhibits cellular responses to the inflammatory cytokines interleukin-1 and tumour necrosis factor-alpha. Biochem J. 2004 Jun;380(Pt 3):651-9. PubMed PMID: 15015934. Pubmed Central PMCID: PMC1224214. eng.
193. Johansen JS, Christoffersen P, Møller S, Price PA, Henriksen JH, Garbarsch C, et al. Serum YKL-40 is increased in patients with hepatic fibrosis. J Hepatol. 2000 Jun;32(6):911-20. PubMed PMID: 10898311. eng.
194. Johansen JS, Jensen BV, Roslind A, Nielsen D, Price PA. Serum YKL-40, a new prognostic biomarker in cancer patients? Cancer Epidemiol Biomarkers Prev. 2006 Feb;15(2):194-202. PubMed PMID: 16492905. eng.
195. Jacques C, Recklies AD, Levy A, Berenbaum F. HC-gp39 contributes to chondrocyte differentiation by inducing SOX9 and type II collagen expressions. Osteoarthritis Cartilage. 2007 Feb;15(2):138-46. PubMed PMID: 16949314. eng.
196. Rehli M, Niller HH, Ammon C, Langmann S, Schwarzfischer L, Andreesen R, et al. Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation. J Biol Chem. 2003 Nov;278(45):44058-67. PubMed PMID: 12933821. eng.
197. Junker N, Johansen JS, Andersen CB, Kristjansen PE. Expression of YKL-40 by peritumoral macrophages in human small cell lung cancer. Lung Cancer. 2005 May;48(2):223-31. PubMed PMID: 15829322. eng.
198. De Ceuninck F, Gaufillier S, Bonnaud A, Sabatini M, Lesur C, Pastoureau P. YKL-40 (cartilage gp-39) induces proliferative events in cultured chondrocytes and synoviocytes and increases glycosaminoglycan synthesis in chondrocytes. Biochem Biophys Res Commun. 2001 Jul;285(4):926-31. PubMed PMID: 11467840. eng.
199. Recklies AD, White C, Ling H. The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways. Biochem J. 2002 Jul;365(Pt 1):119-26. PubMed PMID: 12071845. Pubmed Central PMCID: PMC1222662. eng.
200. Malinda KM, Ponce L, Kleinman HK, Shackelton LM, Millis AJ. Gp38k, a protein synthesized by vascular smooth muscle cells, stimulates directional migration of human umbilical vein endothelial cells. Exp Cell Res. 1999 Jul;250(1):168-73. PubMed PMID: 10388530. eng.
201. Nishikawa KC, Millis AJ. gp38k (CHI3L1) is a novel adhesion and migration factor for vascular cells. Exp Cell Res. 2003 Jul;287(1):79-87. PubMed PMID: 12799184. eng.
202. Johansen JS, Høyer PE, Larsen LA, Price PA, Møllgård K. YKL-40 protein expression in the early developing human musculoskeletal system. J Histochem Cytochem. 2007 Dec;55(12):1213-28. PubMed PMID: 17712177. eng.
203. Brøchner CB, Johansen JS, Larsen LA, Bak M, Mikkelsen HB, Byskov AG, et al. YKL-40 is differentially expressed in human embryonic stem cells and in cell progeny of the three germ layers. J

Histochem Cytochem. 2012 Mar;60(3):188-204. PubMed PMID: 22140133. Pubmed Central PMCID: PMC3351129. eng.
204. Kurita K. Chitin and chitosan: functional biopolymers from marine crustaceans. Mar Biotechnol (NY). 2006 2006 May-Jun;8(3):203-26. PubMed PMID: 16532368. eng.
205. Cederkvist FH, Parmer PP, Vårum KM, Eijsink VGH, Sorlie M. Inhibition of a family 18 chitinase by chitooligosaccharides. Carbohydrate Polymers. 2008;74:41-9.
206. Khor E, Lim LY. Implantable applications of chitin and chitosan. Biomaterials. 2003 Jun;24(13):2339-49. PubMed PMID: 12699672. eng.
207. Jayakumar R, Prabaharan M, Sudheesh Kumar PT, Nair SV, Tamura H. Biomaterials based on chitin and chitosan in wound dressing applications. Biotechnol Adv. 2011 2011 May-Jun;29(3):322-37. PubMed PMID: 21262336. eng.
208. Rinaudo M. Chitin and chitosan: Properties and applications. Prog Polym Sci. 2006;31:603-32.
209. Aam BB, Heggset EB, Norberg AL, Sørlie M, Vårum KM, Eijsink VG. Production of chitooligosaccharides and their potential applications in medicine. Mar Drugs. 2010;8(5):1482-517. PubMed PMID: 20559485. Pubmed Central PMCID: PMC2885077. eng.
210. Minke R, Blackwell J. The structure of alpha-chitin. J Mol Biol. 1978 Apr;120(2):167-81. PubMed PMID: 642008. eng.
211. Gardner KH, Blackwell J. Refinement of the structure of beta-chitin. Biopolymers. 1975 Aug;14(8):1581-95. PubMed PMID: 1156656. eng.
212. Kim S-K, Jung W-K. Effects of chitin, chitosan and their derivatives on human hemostasis. In: Kim S-K, editor. Chitin, chitosan, oligosaccharides and their derivatives: Biological activities and applications. Boca Raton, FL: CRC Press; 2011. p. 251-62.
213. Minagawa T, Okamura Y, Shigemasa Y, Minami S, Okamoto Y. Effects of molecular weight and deacetylation degree of chitin/chitosan on wound healing. Carbohydr Polym. 2007;67:640-4.
214. Lee CG, Da Silva CA, Lee JY, Hartl D, Elias JA. Chitin regulation of immune responses: an old molecule with new roles. Curr Opin Immunol. 2008 Dec;20(6):684-9. PubMed PMID: 18938241. Pubmed Central PMCID: PMC2605627. eng.
215. Da Silva CA, Chalouni C, Williams A, Hartl D, Lee CG, Elias JA. Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. J Immunol. 2009 Mar;182(6):3573-82. PubMed PMID: 19265136. eng.
216. Suzuki K, Okawa Y, Hashimoto K, Suzuki S, Suzuki M. Protecting effect of chitin and chitosan on experimentally induced murine candidiasis. Microbiol Immunol. 1984;28(8):903-12. PubMed PMID: 6094990. eng.
217. Nishimura K, Nishimura S, Nishi N, Saiki I, Tokura S, Azuma I. Immunological activity of chitin and its derivatives. Vaccine. 1984 Mar;2(1):93-9. PubMed PMID: 6397928. eng.
218. Sandford PA. Advances in Chitin Science. Trondheim: NTNU; 2003. p. 35-42.
219. Muzzarelli RA. Human enzymatic activities related to the therapeutic administration of chitin derivatives. Cell Mol Life Sci. 1997 Feb;53(2):131-40. PubMed PMID: 9118001. eng.
220. Krajewska B. Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. Enzyme Microbiol Technol. 2004;35:126-39.
221. Datta PK, Basu PS, Datta TK. Isolation and characterization of Vicia faba lectin affinity purified on chitin column. Prep Biochem. 1984;14(4):373-87. PubMed PMID: 6514678. eng.
222. Songkroah C, Nakbanpote W, Thiravetyan P. Recovery of silver-thiosulfate complexes with chitin. Process Biochem. 2004;39:1553-9.
223. Zhang J, Xia W, Liu P, Cheng Q, Tahirou T, Gu W, et al. Chitosan modification and pharmaceutical/biomedical applications. Mar Drugs. 2010;8:1962-87.
224. Kim IY, Seo SJ, Moon HS, Yoo MK, Park IY, Kim BC, et al. Chitosan and its derivatives for tissue engineering applications. Biotechnol Adv. 2008 2008 Jan-Feb;26(1):1-21. PubMed PMID: 17884325. eng.
225. Ermak IM, Davydova VN, Gorbach VI, Berdyshev EL, Kuznetsova TA, Ivanushko IA, et al. Modification of biological activity of lipopolysaccharide in the complex with chitosan. Bull Exp Biol Med. 2004 Apr;137(4):379-81. PubMed PMID: 15452608. eng.
226. Lamb Machado R, de Arruda EJ, Costapinto Santana C, Alves Bueno SM. Evaluation of a chitosan membrane for removal of endotoxin from human IgG solutions. Process Biochemistry: Elsevier Ltd.; 2006. p. 2252-7.
227. Harish Prashanth KV, Tharanathan RN. Chitin/chitosan: modifications and their unlimited application potential - an overview. Trends Food Sci Technol. 2007;18:117-31.

228. Dutta J, Dutta PK. Antimicrobial activity of chitin, chitosan and their oligosaccharides. In: Kim S-K, editor. Chitin, Chitosan, Oligosaccharides and their Derivatives: Biological Activities and Applications. Boca Raton, FL: CRC Press; 2011. p. 195-214.
229. Chung YC, Chen CY. Antibacterial characteristics and activity of acid-soluble chitosan. Bioresour Technol. 2008 May;99(8):2806-14. PubMed PMID: 17697776. eng.
230. Peluso G, Petillo O, Ranieri M, Santin M, Ambrosio L, Calabró D, et al. Chitosan-mediated stimulation of macrophage function. Biomaterials. 1994 Dec;15(15):1215-20. PubMed PMID: 7703317. eng.
231. Mori T, Okumura M, Matsuura M, Ueno K, Tokura S, Okamoto Y, et al. Effects of chitin and its derivatives on the proliferation and cytokine production of fibroblasts in vitro. Biomaterials. 1997 Jul;18(13):947-51. PubMed PMID: 9199765. eng.
232. Ueno H, Yamada H, Tanaka I, Kaba N, Matsuura M, Okumura M, et al. Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs. Biomaterials. 1999 Aug;20(15):1407-14. PubMed PMID: 10454012. eng.
233. Ueno H, Mori T, Fujinaga T. Topical formulations and wound healing applications of chitosan. Adv Drug Deliv Rev. 2001 Nov;52(2):105-15. PubMed PMID: 11718934. eng.
234. Mi FL, Shyu SS, Wu YB, Lee ST, Shyong JY, Huang RN. Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing. Biomaterials. 2001 Jan;22(2):165-73. PubMed PMID: 11101160. eng.
235. Paul W, Sharma CP. Chitin and alginates wound dressings: a short review. Trends Biomater Artif Organs. 2004;18:18-23.
236. Hamilton V, Yuan Y, Rigney DA, Puckett AD, Ong JL, Yang Y, et al. Characterization of chitosan films and effects on fibroblast cell attachment and proliferation. J Mater Sci Mater Med. 2006 Dec;17(12):1373-81. PubMed PMID: 17143770. eng.
237. Mansouri S, Lavigne P, Corsi K, Benderdour M, Beaumont E, Fernandes JC. Chitosan-DNA nanoparticles as non-viral vectors in gene therapy: Strategies to improve transfection efficacy. Eur J Pharmaceutics Biopharmaceutics. 2004;57:1.
238. Baldrick P. The safety of chitosan as a pharmaceutical excipient. Regul Toxicol Pharmacol. 2010 Apr;56(3):290-9. PubMed PMID: 19788905. eng.
239. Bumgardner JD, Wiser R, Elder SH, Jouett R, Yang Y, Ong JL. Contact angle, protein adsorption and osteoblast precursor cell attachment to chitosan coatings bonded to titanium. J Biomater Sci Polym Ed. 2003;14(12):1401-9. PubMed PMID: 14870943. eng.
240. Ge Z, Baguenard S, Lim LY, Wee A, Khor E. Hydroxyapatite-chitin materials as potential tissue engineered bone substitutes. Biomaterials. 2004 Mar;25(6):1049-58. PubMed PMID: 14615170. eng.
241. Lin SB, Lin YC, Chen HH. Low molecular weight chitosan prepared with the aid of cellulase, lysozyme and chitinase: Characterisation and antibacterial activity. Food Chem. 2009;116(1):47-53.
242. Ilyina AV, Tikhanov VE, Albulov AI, Varlmov VP. Enzymic preparation of acid-free water-soluble chitosan. Process Biochem. 2000;35:563-8.
243. Harish Prashanth KV, Tharanathan RN. Depolymerized products of chitosan as potent inhibitors of tumor-induced angiogenesis. Biochim Biophys Acta. 2005 Feb;1722(1):22-9. PubMed PMID: 15716061. eng.
244. Qin C, Du Y, Xiao L, Li Z, Gao X. Enzymic preparation of water-soluble chitosan and their antitumor activity. Int J Biol Macromol. 2002 Dec;31(1-3):111-7. PubMed PMID: 12559434. eng.
245. Ribeiro MP, Espiga A, Silva D, Baptista P, Henriques J, Ferreira C, et al. Development of a new chitosan hydrogel for wound dressing. Wound Repair Regen. 2009 2009 Nov-Dec;17(6):817-24. PubMed PMID: 19903303. eng.
246. Köping-Höggård M, Mel'nikova YS, Vårum KM, Lindman B, Artursson P. Relationship between the physical shape and the efficiency of oligomeric chitosan as a gene delivery system in vitro and in vivo. J Gene Med. 2003 Feb;5(2):130-41. PubMed PMID: 12539151. eng.
247. Lin CW, Chen LJ, Lee PL, Lee CI, Lin JC, Chiu JJ. The inhibition of TNF-alpha-induced E-selectin expression in endothelial cells via the JNK/NF-kappaB pathways by highly N-acetylated chitooligosaccharides. Biomaterials. 2007 Mar;28(7):1355-66. PubMed PMID: 17126899. eng.
248. Xiong C, Wu H, Wei P, Pan M, Tuo Y, Kusakabe I, et al. Potent angiogenic inhibition effects of deacetylated chitohexaose separated from chitooligosaccharides and its mechanism of action in vitro. Carbohydr Res. 2009 Oct;344(15):1975-83. PubMed PMID: 19631932. eng.
249. Jung WK, Moon SH, Kim SK. Effect of chitooligosaccharides on calcium bioavailability and bone strength in ovariectomized rats. Life Sci. 2006 Jan;78(9):970-6. PubMed PMID: 16137703. eng.

250. Kim SK, Park PJ, Yang HP, Han SS. Subacute toxicity of chitosan oligosaccharide in Sprague-Dawley rats. Arzneimittelforschung. 2001 Sep;51(9):769-74. PubMed PMID: 11642011. eng.
251. Suzuki K, Mikami T, Okawa Y, Tokoro A, Suzuki S, Suzuki M. Antitumor effect of hexa-N-acetylchitohexaose and chitohexaose. Carbohydr Res. 1986 Aug;151:403-8. PubMed PMID: 3768901. eng.
252. Wei X, Wang Y, Xiao J, Xia W. Separation of chitooligosaccharides and the potent effects on gene expression of cell surface receptor CR3. Int J Biol Macromol. 2009 Nov;45(4):432-6. PubMed PMID: 19635497. eng.
253. Tokoro A, Tatewaki N, Suzuki K, Mikami T, Suzuki S, Suzuki M. Growth-inhibitory effect of hexa-N-acetylchitohexaose and chitohexaose against Meth-A solid tumor. Chem Pharm Bull (Tokyo). 1988 Feb;36(2):784-90. PubMed PMID: 3409386. eng.
254. Okamoto Y, Inoue A, Miyatake K, Ogihara K, Shigemasa Y, Minami S. Effects of chitin/chitosan and their oligomers/monomers on migrations of macrophages. Macromol Biosci. 2003;3:587-90.
255. Moon JS, Kim HK, Koo HC, Joo YS, Nam HM, Park YH, et al. The antibacterial and immunostimulative effect of chitosan-oligosaccharides against infection by Staphylococcus aureus isolated from bovine mastitis. Appl Microbiol Biotechnol. 2007 Jul;75(5):989-98. PubMed PMID: 17361430. eng.
256. Yoon HJ, Moon ME, Park HS, Im SY, Kim YH. Chitosan oligosaccharide (COS) inhibits LPS-induced inflammatory effects in RAW 264.7 macrophage cells. Biochem Biophys Res Commun. 2007 Jul;358(3):954-9. PubMed PMID: 17512902. eng.
257. Wu GJ, Tsai GJ. Chitooligosaccharides in combination with interferon-gamma increase nitric oxide production via nuclear factor-kappaB activation in murine RAW264.7 macrophages. Food Chem Toxicol. 2007 Feb;45(2):250-8. PubMed PMID: 17107743. eng.
258. Qiao Y, Bai XF, Du YG. Chitosan oligosaccharides protect mice from LPS challenge by attenuation of inflammation and oxidative stress. Int Immunopharmacol. 2011 Jan;11(1):121-7. PubMed PMID: 21059391. eng.
259. Park P, Je J, Kim S. Free radical scavenging activities of differently deacetylated chitosans using an ESR spectrometer. Carbohydr Polymers. 2004;55:17-22.
260. Liu HT, Li WM, Li XY, Xu QS, Liu QS, Bai XF, et al. Chitosan oligosaccharides inhibit the expression of interleukin-6 in lipopolysaccharide-induced human umbilical vein endothelial cells through p38 and ERK1/2 protein kinases. Basic Clin Pharmacol Toxicol. 2010 May;106(5):362-71. PubMed PMID: 19929981. eng.
261. Klokkevold PR, Vandemark L, Kenney EB, Bernard GW. Osteogenesis enhanced by chitosan (poly-N-acetyl glucosaminoglycan) in vitro. J Periodontol. 1996 Nov;67(11):1170-5. PubMed PMID: 8959566. eng.
262. Kim SK, Park PJ, Jung WK, Byun HG, Mendis E, Cho YI. Inhibitory activity of phosphorylated chitooligosaccharides on the formation of calcium phosphate. Carbohydr Polym. 2005;60:483-7.
263. Anderson JW, Nicolosi RJ, Borzelleca JF. Glucosamine effects in humans: a review of effects on glucose metabolism, side effects, safety considerations and efficacy. Food Chem Toxicol. 2005 Feb;43(2):187-201. PubMed PMID: 15621331. eng.
264. Kirkham SG, Samarasinghe RK. Review article: Glucosamine. J Orthop Surg (Hong Kong). 2009 Apr;17(1):72-6. PubMed PMID: 19398798. eng.
265. Setnikar I, Giacchetti C, Zanolo G. Pharmacokinetics of glucosamine in the dog and in man. Arzneimittelforschung. 1986 Apr;36(4):729-35. PubMed PMID: 3718596. eng.
266. Setnikar I, Giachetti C, Zanolo G. Absorption, distribution and excretion of radioactivity after a single intravenous or oral administration of [14C] glucosamine to the rat. Pharmatherapeutica. 1984;3(8):538-50. PubMed PMID: 6422479. eng.
267. Derfoul A, Miyoshi AD, Freeman DE, Tuan RS. Glucosamine promotes chondrogenic phenotype in both chondrocytes and mesenchymal stem cells and inhibits MMP-13 expression and matrix degradation. Osteoarthritis Cartilage. 2007 Jun;15(6):646-55. PubMed PMID: 17337215. eng.
268. Ganno T, Yamada S, Ohara N, Matsunaga T, Yanagiguchi K, Ikeda T, et al. Early gene expression analyzed by cDNA microarray and real-time PCR in osteoblasts cultured with chitosan monomer. J Biomed Mater Res A. 2007 Jul;82(1):188-94. PubMed PMID: 17269148. eng.
269. McAlindon TE, LaValley MP, Gulin JP, Felson DT. Glucosamine and chondroitin for treatment of osteoarthritis: a systematic quality assessment and meta-analysis. JAMA. 2000 Mar;283(11):1469-75. PubMed PMID: 10732937. eng.

270. Varghese S, Theprungsirikul P, Sahani S, Hwang N, Yarema KJ, Elisseeff JH. Glucosamine modulates chondrocyte proliferation, matrix synthesis, and gene expression. Osteoarthritis Cartilage. 2007 Jan;15(1):59-68. PubMed PMID: 16849037. eng.
271. Gouze JN, Gouze E, Popp MP, Bush ML, Dacanay EA, Kay JD, et al. Exogenous glucosamine globally protects chondrocytes from the arthritogenic effects of IL-1beta. Arthritis Res Ther. 2006;8(6):R173. PubMed PMID: 17109745. Pubmed Central PMCID: PMC1794517. eng.
272. Dodge GR, Jimenez SA. Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes. Osteoarthritis Cartilage. 2003 Jun;11(6):424-32. PubMed PMID: 12801482. eng.
273. Largo R, Alvarez-Soria MA, Díez-Ortego I, Calvo E, Sánchez-Pernaute O, Egido J, et al. Glucosamine inhibits IL-1beta-induced NFkappaB activation in human osteoarthritic chondrocytes. Osteoarthritis Cartilage. 2003 Apr;11(4):290-8. PubMed PMID: 12681956. eng.
274. Richy F, Bruyere O, Ethgen O, Cucherat M, Henrotin Y, Reginster JY. Structural and symptomatic efficacy of glucosamine and chondroitin in knee osteoarthritis: a comprehensive meta-analysis. Arch Intern Med. 2003 Jul;163(13):1514-22. PubMed PMID: 12860572. eng.
275. Chan PS, Caron JP, Rosa GJ, Orth MW. Glucosamine and chondroitin sulfate regulate gene expression and synthesis of nitric oxide and prostaglandin E(2) in articular cartilage explants. Osteoarthritis Cartilage. 2005 May;13(5):387-94. PubMed PMID: 15882562. eng.
276. Ma L, Rudert WA, Harnaha J, Wright M, Machen J, Lakomy R, et al. Immunosuppressive effects of glucosamine. J Biol Chem. 2002 Oct;277(42):39343-9. PubMed PMID: 12176986. eng.
277. Hua J, Sakamoto K, Nagaoka I. Inhibitory actions of glucosamine, a therapeutic agent for osteoarthritis, on the functions of neutrophils. J Leukoc Biol. 2002 Apr;71(4):632-40. PubMed PMID: 11927650. eng.
278. Huang CH, Tseng WY, Yao CC, Jeng JH, Young TH, Chen YJ. Glucosamine promotes osteogenic differentiation of dental pulp stem cells through modulating the level of the transforming growth factor-beta type I receptor. J Cell Physiol. 2010 Oct;225(1):140-51. PubMed PMID: 20458730. eng.
279. Ivanovska N, Dimitrova P. Bone resorption and remodeling in murine collagenase-induced osteoarthritis after administration of glucosamine. Arthritis Res Ther. 2011 Mar;13(2):R44. PubMed PMID: 21410959. ENG.
280. Matsunaga T, Yanagiguchi K, Yamada S, Ohara N, Ikeda T, Hayashi Y. Chitosan monomer promotes tissue regeneration on dental pulp wounds. J Biomed Mater Res A. 2006 Mar;76(4):711-20. PubMed PMID: 16315192. eng.
281. Fleming JE, Cornell CN, Muschler GF. Bone cells and matrices in orthopedic tissue engineering. Orthop Clin North Am. 2000 Jul;31(3):357-74. PubMed PMID: 10882463. eng.
282. Wheeler DL, Enneking WF. Allograft bone decreases in strength in vivo over time. Clin Orthop Relat Res. 2005 Jun(435):36-42. PubMed PMID: 15930919. eng.
283. Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials. 2006 Jun;27(18):3413-31. PubMed PMID: 16504284. eng.
284. Hench LL, Polak JM. Third-generation biomedical materials. Science. 2002 Feb;295(5557):1014-7. PubMed PMID: 11834817. eng.
285. Esposito M, Coulthard P, Thomsen P, Worthington HV. The role of implant surface modifications, shape and material on the success of osseointegrated dental implants. A Cochrane systematic review. Eur J Prosthodont Restor Dent. 2005 Mar;13(1):15-31. PubMed PMID: 15819145. eng.
286. Puleo DA, Nanci A. Understanding and controlling the bone-implant interface. Biomaterials. 1999 Dec;20(23-24):2311-21. PubMed PMID: 10614937. eng.
287. Szmukler-Moncler S, Salama H, Reingewirtz Y, Dubruille JH. Timing of loading and effect of micromotion on bone-dental implant interface: review of experimental literature. J Biomed Mater Res. 1998;43(2):192-203. PubMed PMID: 9619438. eng.
288. García AJ, Vega MD, Boettiger D. Modulation of cell proliferation and differentiation through substrate-dependent changes in fibronectin conformation. Mol Biol Cell. 1999 Mar;10(3):785-98. PubMed PMID: 10069818. Pubmed Central PMCID: PMC25202. eng.
289. Ito Y, Kajihara M, Imanishi Y. Materials for enhancing cell adhesion by immobilization of cell-adhesive peptide. J Biomed Mater Res. 1991 Nov;25(11):1325-37. PubMed PMID: 1797805. eng.
290. Boyan BD, Hummert TW, Dean DD, Schwartz Z. Role of material surfaces in regulating bone and cartilage cell response. Biomaterials. 1996 Jan;17(2):137-46. PubMed PMID: 8624390. eng.

291. Yao J, Cs-Szabó G, Jacobs JJ, Kuettner KE, Glant TT. Suppression of osteoblast function by titanium particles. J Bone Joint Surg Am. 1997 Jan;79(1):107-12. PubMed PMID: 9010191. eng.
292. Vermes C, Chandrasekaran R, Jacobs JJ, Galante JO, Roebuck KA, Glant TT. The effects of particulate wear debris, cytokines, and growth factors on the functions of MG-63 osteoblasts. J Bone Joint Surg Am. 2001 Feb;83-A(2):201-11. PubMed PMID: 11216681. eng.
293. LeBaron RG, Athanasiou KA. Extracellular matrix cell adhesion peptides: functional applications in orthopedic materials. Tissue Eng. 2000 Apr;6(2):85-103. PubMed PMID: 10941205. eng.
294. Stephansson SN, Byers BA, García AJ. Enhanced expression of the osteoblastic phenotype on substrates that modulate fibronectin conformation and integrin receptor binding. Biomaterials. 2002 Jun;23(12):2527-34. PubMed PMID: 12033600. eng.
295. Moursi AM, Globus RK, Damsky CH. Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation in vitro. J Cell Sci. 1997 Sep;110 (Pt 18):2187-96. PubMed PMID: 9378768. eng.
296. Conti M, Donati G, Cianciolo G, Stefoni S, Samorì B. Force spectroscopy study of the adhesion of plasma proteins to the surface of a dialysis membrane: role of the nanoscale surface hydrophobicity and topography. J Biomed Mater Res. 2002 Sep;61(3):370-9. PubMed PMID: 12115462. eng.
297. Meadows PY, Walker GC. Force microscopy studies of fibronectin adsorption and subsequent cellular adhesion to substrates with well-defined surface chemistries. Langmuir. 2005 Apr;21(9):4096-107. PubMed PMID: 15835980. eng.
298. Klebe RJ, Bentley KL, Schoen RC. Adhesive substrates for fibronectin. J Cell Physiol. 1981 Dec;109(3):481-8. PubMed PMID: 7320060. eng.
299. Schwarz MA, Juliano RL. Surface activation of the cell adhesion fragment of fibronectin. Exp Cell Res. 1984 Aug;153(2):550-5. PubMed PMID: 6734759. eng.
300. Siebers MC, ter Brugge PJ, Walboomers XF, Jansen JA. Integrins as linker proteins between osteoblasts and bone replacing materials. A critical review. Biomaterials. 2005 Jan;26(2):137-46. PubMed PMID: 15207460. eng.
301. Anselme K, Linez P, Bigerelle M, Le Maguer D, Le Maguer A, Hardouin P, et al. The relative influence of the topography and chemistry of TiAl6V4 surfaces on osteoblastic cell behaviour. Biomaterials. 2000 Aug;21(15):1567-77. PubMed PMID: 10885729. eng.
302. Anselme K, Bigerelle M, Noel B, Dufresne E, Judas D, Iost A, et al. Qualitative and quantitative study of human osteoblast adhesion on materials with various surface roughnesses. J Biomed Mater Res. 2000 Feb;49(2):155-66. PubMed PMID: 10571901. eng.
303. Kieswetter K, Schwartz Z, Hummert TW, Cochran DL, Simpson J, Dean DD, et al. Surface roughness modulates the local production of growth factors and cytokines by osteoblast-like MG-63 cells. J Biomed Mater Res. 1996 Sep;32(1):55-63. PubMed PMID: 8864873. eng.
304. Rupp F, Scheideler L, Olshanska N, de Wild M, Wieland M, Geis-Gerstorfer J. Enhancing surface free energy and hydrophilicity through chemical modification of microstructured titanium implant surfaces. J Biomed Mater Res A. 2006 Feb;76(2):323-34. PubMed PMID: 16270344. eng.
305. Lee JH, Khang G, Lee JW, Lee HB. Interaction of Different Types of Cells on Polymer Surfaces with Wettability Gradient. J Colloid Interface Sci. 1998 Sep;205(2):323-30. PubMed PMID: 9735195. ENG.
306. Hamamoto N, Hamamoto Y, Nakajima T, Ozawa H. Histological, histocytochemical and ultrastructural study on the effects of surface charge on bone formation in the rabbit mandible. Arch Oral Biol. 1995 Feb;40(2):97-106. PubMed PMID: 7540834. eng.
307. Krukowski M, Shively RA, Osdoby P, Eppley BL. Stimulation of craniofacial and intramedullary bone formation by negatively charged beads. J Oral Maxillofac Surg. 1990 May;48(5):468-75. PubMed PMID: 1691778. eng.
308. Davies JE, Causton B, Bovell Y, Davy K, Sturt CS. The migration of osteoblasts over substrata of discrete surface charge. Biomaterials. 1986 May;7(3):231-3. PubMed PMID: 3719043. eng.
309. Shelton RM, Rasmussen AC, Davies JE. Protein adsorption at the interface between charged polymer substrata and migrating osteoblasts. Biomaterials. 1988 Jan;9(1):24-9. PubMed PMID: 3349119. eng.
310. Keselowsky BG, Collard DM, García AJ. Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. J Biomed Mater Res A. 2003 Aug;66(2):247-59. PubMed PMID: 12888994. eng.

311. Webb K, Hlady V, Tresco PA. Relationships among cell attachment, spreading, cytoskeletal organization, and migration rate for anchorage-dependent cells on model surfaces. J Biomed Mater Res. 2000 Mar;49(3):362-8. PubMed PMID: 10602069. Pubmed Central PMCID: PMC3262179. eng.
312. Healy KE, Thomas CH, Rezania A, Kim JE, McKeown PJ, Lom B, et al. Kinetics of bone cell organization and mineralization on materials with patterned surface chemistry. Biomaterials. 1996 Jan;17(2):195-208. PubMed PMID: 8624396. eng.
313. Keselowsky BG, Collard DM, García AJ. Integrin binding specificity regulates biomaterial surface chemistry effects on cell differentiation. Proc Natl Acad Sci U S A. 2005 Apr;102(17):5953-7. PubMed PMID: 15827122. Pubmed Central PMCID: PMC1087905. eng.
314. Faucheux N, Schweiss R, Lützow K, Werner C, Groth T. Self-assembled monolayers with different terminating groups as model substrates for cell adhesion studies. Biomaterials. 2004 Jun;25(14):2721-30. PubMed PMID: 14962551. eng.
315. Davies JE. In vitro modeling of the bone/implant interface. Anat Rec. 1996 Jun;245(2):426-45. PubMed PMID: 8769677. eng.
316. Coelho PG, Granjeiro JM, Romanos GE, Suzuki M, Silva NR, Cardaropoli G, et al. Basic research methods and current trends of dental implant surfaces. J Biomed Mater Res B Appl Biomater. 2009 Feb;88(2):579-96. PubMed PMID: 18973274. eng.
317. Haycock JW. 3D cell culture: a review of current approaches and techniques. Methods Mol Biol. 2011;695:1-15. PubMed PMID: 21042962. eng.
318. Groth T, Falk P, Miethke RR. Cytotoxicity of biomaterials-basic mechanisms and in-vitro test methods - a review. Altern Lab Anim. 1995;23:790-9.
319. Guarino V, Causa F, Ambrosio L. Bioactive scaffolds for bone and ligament tissue. Expert Rev Med Devices. 2007 May;4(3):405-18. PubMed PMID: 17488233. eng.
320. Long M, Rack HJ. Titanium alloys in total joint replacement--a materials science perspective. Biomaterials. 1998 Sep;19(18):1621-39. PubMed PMID: 9839998. eng.
321. Elias CN, Lima JHC, Valiev R, Meyers MA. Biomedical applications of titanium and its alloys. Biological Materials Science. 2008:46-9.
322. Niinomi M, Nakai M, Hieda J. Development of new metallic alloys for biomedical applications. Acta Biomater. 2012 Jul. PubMed PMID: 22765961. ENG.
323. Sporer SM, Paprosky WG. Biologic fixation and bone ingrowth. Orthop Clin North Am. 2005 Jan;36(1):105-11, vii. PubMed PMID: 15542129. eng.
324. Goodacre CJ, Kan JY, Rungcharassaeng K. Clinical complications of osseointegrated implants. J Prosthet Dent. 1999 May;81(5):537-52. PubMed PMID: 10220658. eng.
325. Bagno A, Di Bello C. Surface treatments and roughness properties of Ti-based biomaterials. J Mater Sci Mater Med. 2004 Sep;15(9):935-49. PubMed PMID: 15448401. eng.
326. Schwartz Z, Nasazky E, Boyan BD. Surface microtopography regulates osteointegration: the role of implant surface microtopography in osteointegration. Alpha Omegan. 2005 Jul;98(2):9-19. PubMed PMID: 16122142. eng.
327. Cooper LF, Masuda T, Yliheikkilä PK, Felton DA. Generalizations regarding the process and phenomenon of osseointegration. Part II. In vitro studies. Int J Oral Maxillofac Implants. 1998 1998 Mar-Apr;13(2):163-74. PubMed PMID: 9581401. eng.
328. Niinomi M. Mechanical biocompatibilities of titanium alloys for biomedical applications. J Mech Behav Biomed Mater. 2008 Jan;1(1):30-42. PubMed PMID: 19627769. eng.
329. Li C, Zhu Z. Dynamic Young's modulus of open-porosity titanium measured by the electromagnetic acoustic resonance method. J Porous Mater. 2006;13:21-6.
330. Kim HW, Lee EJ, Jun IK, Kim HE. On the feasibility of phosphate glass and hydroxyapatite engineered coating on titanium. J Biomed Mater Res A. 2005 Dec;75(3):656-67. PubMed PMID: 16108050. eng.
331. Barber TA, Gamble LJ, Castner DG, Healy KE. In vitro characterization of peptide-modified p(AAm-co-EG/AAc) IPN-coated titanium implants. J Orthop Res. 2006 Jul;24(7):1366-76. PubMed PMID: 16732610. eng.
332. Kim KH, Kwon TY, Kim SY, Kang IK, Kim S, Yang Y, et al. Preparation and characterization of anodized titanium surfaces and their effect on osteoblast responses. J Oral Implantol. 2006;32(1):8-13. PubMed PMID: 16526576. eng.
333. Zhang C, Leng Y, Chen J. In vitro mechanical integrity of hydroxyapatite coatings on Ti-6Al-4V implants under shear loading. J Biomed Mater Res. 2001 Sep;56(3):342-50. PubMed PMID: 11372051. eng.

334. Ong JL, Chan DCN. Hydroxyapatite and their use as coatings in dental implants: a review. Crit Rev Biomed Eng. 1999;28:667-707.
335. Tomihata K, Ikada Y. In vitro and in vivo degradation of films of chitin and its deacetylated derivatives. Biomaterials. 1997 Apr;18(7):567-75. PubMed PMID: 9105597. eng.
336. Chatelet C, Damour O, Domard A. Influence of the degree of acetylation on some biological properties of chitosan films. Biomaterials. 2001 Feb;22(3):261-8. PubMed PMID: 11197501. eng.
337. Amaral IF, Cordeiro AL, Sampaio P, Barbosa MA. Attachment, spreading and short-term proliferation of human osteoblastic cells cultured on chitosan films with different degrees of acetylation. J Biomater Sci Polym Ed. 2007;18(4):469-85. PubMed PMID: 17540120. eng.
338. Uygun BE, Bou-Akl T, Albanna M, Matthew HW. Membrane thickness is an important variable in membrane scaffolds: Influence of chitosan membrane structure on the behavior of cells. Acta Biomater. 2010 Jun;6(6):2126-31. PubMed PMID: 19925888. Pubmed Central PMCID: PMC2862882. eng.
339. Feng F, Liu Y, Zhao B, Hu Ka. In Vitro Biomineralization of Glutaraldehyde Crosslinked Chitosan Films. Journal of Wuhan University of Technology - Mater.Sci.Ed.2005. p. 20-3.
340. Lim LY, Khor E, Koo O. Gamma irradiation of chitosan. J Biomed Mater Res. 1998;43(3):282-90. PubMed PMID: 9730066. eng.
341. Sannan T, Kurita K, Iwakura Y. Studies on chitin, 2. Effect of deacetylation on solubility. Die Makromolekulare Chemie1976. p. 3589-600.
342. Rao SB, Sharma CP. Sterilization of chitosan: implications. J Biomater Appl. 1995 Oct;10(2):136-43. PubMed PMID: 8618207. eng.
343. Lim LY, Khor E, Ling CE. Effects of dry heat and saturated steam on the physical properties of chitosan. J Biomed Mater Res. 1999;48(2):111-6. PubMed PMID: 10331902. eng.
344. Hsu S, Whu SW, Tsai CL, Wu YH, Chen HW, Hsieh KH. Chitosan as scaffold materials: effects of molecular weight and degree of deacetylation. J Polym Res. 2004;11:141-7.
345. Fakhry A, Schneider GB, Zaharias R, Senel S. Chitosan supports the initial attachment and spreading of osteoblasts preferentially over fibroblasts. Biomaterials. 2004 May;25(11):2075-9. PubMed PMID: 14741622. eng.
346. Amaral IF, Lamghari M, Sousa SR, Sampaio P, Barbosa MA. Rat bone marrow stromal cell osteogenic differentiation and fibronectin adsorption on chitosan membranes: the effect of the degree of acetylation. J Biomed Mater Res A. 2005 Nov;75(2):387-97. PubMed PMID: 16092111. eng.
347. Abarrategi A, García-Cantalejo J, Moreno-Vicente C, Civantos A, Ramos V, Casado JV, et al. Gene expression profile on chitosan/rhBMP-2 films: A novel osteoinductive coating for implantable materials. Acta Biomater. 2009 Sep;5(7):2633-46. PubMed PMID: 19342322. eng.
348. Bumgardner JD, Chesnutt BM, Yuan Y, Yang Y, Appleford M, Oh S, et al. The integration of chitosan-coated titanium in bone: an in vivo study in rabbits. Implant Dent. 2007 Mar;16(1):66-79. PubMed PMID: 17356373. eng.
349. Thierry B, Winnik FM, Merhi Y, Silver J, Tabrizian M. Bioactive coatings of endovascular stents based on polyelectrolyte multilayers. Biomacromolecules. 2003 2003 Nov-Dec;4(6):1564-71. PubMed PMID: 14606881. eng.
350. Bumgardner JD, Wiser R, Gerard PD, Bergin P, Chestnutt B, Marin M, et al. Chitosan: potential use as a bioactive coating for orthopaedic and craniofacial/dental implants. J Biomater Sci Polym Ed. 2003;14(5):423-38. PubMed PMID: 12807145. eng.
351. Martin HJ, Schulz KH, Bumgardner JD, Walters KB. XPS study on the use of 3-aminopropyltriethoxysilane to bond chitosan to a titanium surface. Langmuir. 2007 Jun;23(12):6645-51. PubMed PMID: 17488131. eng.
352. Martin HJ, Schulz KH, Bumgardner JD, Schneider JA. Enhanced bonding of chitosan to implant quality titanium via four treatment combinations. Thin Solid Films. 2008;516:6277-86.
353. Boccaccini AR, Keim S, Ma R, Li Y, Zhitomirsky I. Electrophoretic deposition of biomaterials. J R Soc Interface. 2010 Oct;7 Suppl 5:S581-613. PubMed PMID: 20504802. Pubmed Central PMCID: PMC2952181. eng.
354. Mehdipour M, Afshar A. A stduy of the electrophoretic deposition of bioactive glass-chitosan composite coating. Ceramics International. 2012;38:471-6.
355. Cai K, Rechtenbach A, Hao J, Bossert J, Jandt KD. Polysaccharide-protein surface modification of titanium via a layer-by-layer technique: characterization and cell behaviour aspects. Biomaterials. 2005 Oct;26(30):5960-71. PubMed PMID: 15913761. eng.

356. Cai K, Hu Y, Jandt KD, Wang Y. Surface modification of titanium thin film with chitosan via electrostatic self-assembly technique and its influence on osteoblast growth behavior. J Mater Sci Mater Med. 2008 Feb;19(2):499-506. PubMed PMID: 17619966. eng.
357. Freshney RI. Culture of animal cells : a manual of basic technique. 5th ed. Hoboken, N.J.: Wiley-Liss; 2005. xxvi, 642 p. p.
358. Dohle E, Fuchs S, Kolbe M, Hofmann A, Schmidt H, Kirkpatrick CJ. Sonic hedgehog promotes angiogenesis and osteogenesis in a coculture system consisting of primary osteoblasts and outgrowth endothelial cells. Tissue Eng Part A. 2010 Apr;16(4):1235-7. PubMed PMID: 19886747. Pubmed Central PMCID: PMC2875976. eng.
359. Cohen DE, Melton D. Turning straw into gold: directing cell fate for regenerative medicine. Nat Rev Genet. 2011 Apr;12(4):243-52. PubMed PMID: 21386864. eng.
360. Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol. 1983 Jan;96(1):191-8. PubMed PMID: 6826647. Pubmed Central PMCID: PMC2112252. eng.
361. Franceschi RT, Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J Bone Miner Res. 1992 Feb;7(2):235-46. PubMed PMID: 1373931. eng.
362. Franceschi RT, Iyer BS, Cui Y. Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. J Bone Miner Res. 1994 Jun;9(6):843-54. PubMed PMID: 8079660. eng.
363. Choi JY, Lee BH, Song KB, Park RW, Kim IS, Sohn KY, et al. Expression patterns of bone-related proteins during osteoblastic differentiation in MC3T3-E1 cells. J Cell Biochem. 1996 Jun;61(4):609-18. PubMed PMID: 8806085. eng.
364. Holt PG. Infections and the development of allergy. Toxicol Lett. 1996 Aug;86(2-3):205-10. PubMed PMID: 8711774. eng.
365. Disthabanchong S, Radinahamed P, Stitchantrakul W, Hongeng S, Rajatanavin R. Chronic metabolic acidosis alters osteoblast differentiation from human mesenchymal stem cells. Kidney Int. 2007 Feb;71(3):201-9. PubMed PMID: 17183249. eng.
366. Sammons J, Ahmed N, El-Sheemy M, Hassan HT. The role of BMP-6, IL-6, and BMP-4 in mesenchymal stem cell-dependent bone development: effects on osteoblastic differentiation induced by parathyroid hormone and vitamin D(3). Stem Cells Dev. 2004 Jun;13(3):273-80. PubMed PMID: 15186723. eng.
367. Kikuchi T, Matsuguchi T, Tsuboi N, Mitani A, Tanaka S, Matsuoka M, et al. Gene expression of osteoclast differentiation factor is induced by lipopolysaccharide in mouse osteoblasts via Toll-like receptors. J Immunol. 2001 Mar;166(5):3574-9. PubMed PMID: 11207318. eng.
368. Goodman SB, Gómez Barrena E, Takagi M, Konttinen YT. Biocompatibility of total joint replacements: A review. J Biomed Mater Res A. 2009 Aug;90(2):603-18. PubMed PMID: 18508337. eng.
369. Di Martino A, Sittinger M, Risbud MV. Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. Biomaterials. 2005 Oct;26(30):5983-90. PubMed PMID: 15894370. eng.
370. Naberezhnykh GA, Gorbach VI, Likhatskaya GN, Davidova VN, Solov'eva TF. Interaction of chitosans and their N-acylated derivatives with lipopolysaccharide of gram-negative bacteria. Biochemistry (Mosc). 2008 Apr;73(4):432-41. PubMed PMID: 18457573. eng.
371. Radon K. The two sides of the "endotoxin coin". Occup Environ Med. 2006 Jan;63(1):73-8, 10. PubMed PMID: 16361410. Pubmed Central PMCID: PMC2078037. eng.
372. Pirog KA, Stabinsky Y, Goldman R. PeproTech Cytokine Index. In: Inc. P, editor. 3 ed. Rocky Hill, New Jersey, USA: PeproTech Inc.; 2010. p. 131;238.
373. Ness TL, Carpenter KJ, Ewing JL, Gerard CJ, Hogaboam CM, Kunkel SL. CCR1 and CC chemokine ligand 5 interactions exacerbate innate immune responses during sepsis. J Immunol. 2004 Dec;173(11):6938-48. PubMed PMID: 15557190. eng.
374. Sweeney SM, Guy CA, Fields GB, San Antonio JD. Defining the domains of type I collagen involved in heparin- binding and endothelial tube formation. Proc Natl Acad Sci U S A. 1998 Jun;95(13):7275-80. PubMed PMID: 9636139. Pubmed Central PMCID: PMC22588. eng.
375. Davydova VN, Yermak IM, Gorbach VI, Krasikova IN, Solov'eva TF. Interaction of bacterial endotoxins with chitosan. Effect of endotoxin structure, chitosan molecular mass, and ionic strength of the solution on the formation of the complex. Biochemistry (Mosc). 2000 Sep;65(9):1082-90. PubMed PMID: 11042502. eng.

376. Lombardo E, DelaRosa O, Mancheño-Corvo P, Menta R, Ramírez C, Büscher D. Toll-like receptor-mediated signaling in human adipose-derived stem cells: implications for immunogenicity and immunosuppressive potential. Tissue Eng Part A. 2009 Jul;15(7):1579-89. PubMed PMID: 19061425. eng.
377. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev. 1999 Jun;20(3):345-57. PubMed PMID: 10368775. eng.
378. Shahidi F, Abuzaytoun R. Chitin, chitosan, and co-products: chemistry, production, applications, and health effects. Adv Food Nutr Res. 2005;49:93-135. PubMed PMID: 15797344. eng.
379. Prasitsilp M, Jenwithisuk R, Kongsuwan K, Damrongchai N, Watts P. Cellular responses to chitosan in vitro: the importance of deacetylation. J Mater Sci Mater Med. 2000 Dec;11(12):773-8. PubMed PMID: 15348059. eng.
380. Zheng Z, Zhang L, Kong L, Wang A, Gong Y, Zhang X. The behaviour of MC3T3-E1 cells on chitosan/poly-L-lysine composite films: Effect of nanotopography, surface chemistry and wettability. J Biomed Mater Res. 2009;89A:453-65.
381. Rhazi M, Desbrières J, Tolaimate A, Alagui A, Vottero P. Investigation of different natural sources of chitin: influence of the source and deactylation process on the physicochemical characteristics of chitosan. Polymer International. 2000;49:337-44.
382. Steele JG, McFarland C, Dalton BA, Johnson G, Evans MD, Howlett CR, et al. Attachment of human bone cells to tissue culture polystyrene and to unmodified polystyrene: the effect of surface chemistry upon initial cell attachment. J Biomater Sci Polym Ed. 1993;5(3):245-57. PubMed PMID: 7512377. eng.
383. Silva RM, Silva GA, Coutinho OP, Mano JF, Reis RL. Preparation and characterisation in simulated body conditions of glutaraldehyde crosslinked chitosan membranes. J Mater Sci Mater Med. 2004 Oct;15(10):1105-12. PubMed PMID: 15516871. eng.
384. Marquis ME, Lord E, Bergeron E, Drevelle O, Park H, Cabana F, et al. Bone cells-biomaterials interactions. Front Biosci. 2009;14:1023-67. PubMed PMID: 19273115. eng.
385. Junker R, Dimakis A, Thoneick M, Jansen JA. Effects of implant surface coatings and composition on bone integration: a systematic review. Clin Oral Implants Res. 2009 Sep;20 Suppl 4:185-206. PubMed PMID: 19663965. eng.
386. Wenling C, Duohui J, Jiamou L, Yandao G, Nanming Z, Xiufang Z. Effects of the degree of deacetylation on the physicochemical properties and Schwann cell affinity of chitosan films. J Biomater Appl. 2005 Oct;20(2):157-77. PubMed PMID: 16183675. eng.
387. Ma Z, Mao Z, Gao C. Surface modification and property analysis of biomedical polymers used for tissue engineering. Colloids Surf B Biointerfaces. 2007 Nov;60(2):137-57. PubMed PMID: 17683921. eng.
388. Yi H, Wu LQ, Bentley WE, Ghodssi R, Rubloff GW, Culver JN, et al. Biofabrication with chitosan. Biomacromolecules. 2005 2005 Nov-Dec;6(6):2881-94. PubMed PMID: 16283704. eng.
389. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol. 2007 Apr;7(4):292-304. PubMed PMID: 17380158. eng.
390. Bauer TW. Particles and periimplant bone resorption. Clin Orthop Relat Res. 2002 Dec(405):138-43. PubMed PMID: 12461365. eng.
391. Jirik FR, Podor TJ, Hirano T, Kishimoto T, Loskutoff DJ, Carson DA, et al. Bacterial lipopolysaccharide and inflammatory mediators augment IL-6 secretion by human endothelial cells. J Immunol. 1989 Jan;142(1):144-7. PubMed PMID: 2783321. eng.
392. Chen CC, Rosenbloom CL, Anderson DC, Manning AM. Selective inhibition of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 expression by inhibitors of I kappa B-alpha phosphorylation. J Immunol. 1995 Oct;155(7):3538-45. PubMed PMID: 7561050. eng.
393. Goldblum SE, Ding X, Brann TW, Campbell-Washington J. Bacterial lipopolysaccharide induces actin reorganization, intercellular gap formation, and endothelial barrier dysfunction in pulmonary vascular endothelial cells: concurrent F-actin depolymerization and new actin synthesis. J Cell Physiol. 1993 Oct;157(1):13-23. PubMed PMID: 8408232. eng.
394. Harlan JM, Harker LA, Reidy MA, Gajdusek CM, Schwartz SM, Striker GE. Lipopolysaccharide-mediated bovine endothelial cell injury in vitro. Lab Invest. 1983 Mar;48(3):269-74. PubMed PMID: 6827807. eng.
395. Wang W, Deng M, Liu X, Ai W, Tang Q, Hu J. TLR4 activation induces nontolerant inflammatory response in endothelial cells. Inflammation. 2011 Dec;34(6):509-18. PubMed PMID: 20878353. eng.

396. Corni I, Ryan MP, Boccaccini AR. Electrophoretic deposition: from traditional ceramics to nanotechnology. Journal of the European Ceramic Society. 2008;28:1353-67.
397. Yuan Y, Chesnutt BM, Wright L, Haggard WO, Bumgardner JD. Mechanical property, degradation rate, and bone cell growth of chitosan coated titanium influenced by degree of deacetylation of chitosan. J Biomed Mater Res B Appl Biomater. 2008 Jul;86(1):245-52. PubMed PMID: 18161778. eng.

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Paper I

"Science never solves a problem without creating ten more"

George Bernard Shaw, political activist (1856-1950)

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Glucosamine increases the expression of YKL-40 and osteogenic marker genes in hMSC during osteogenic differentiation

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Abstract: Human mesenchymal stem cells (hMSC) can be expanded *in vitro* and differentiated towards osteogenic, chondrogenic or adipogenic lineages, making them an attractive source for tissue engineering and regenerative medicine. Chitinase-like-proteins (CLPs) belong to the family 18 glycosyl hydrolases and are believed to play a role in inflammation and tissue remodelling. The aim of this study was to determine the effect of the aminosugar glucosamine on the expression of the CLP YKL-40 during osteogenic differentiation of hMSC. Glucosamine did not affect multipotency of hMSC nor proliferation rate of undifferentiated hMSC. YKL-40 was expressed during both expansion of undifferentiated hMSC and during osteogenic differentiation. A slight but non-significant increase in YKL-40 expression was observed with glucosamine, accompanied by a pH-dependent delay in mineralization. However, glucosamine induced higher expression of osteogenic marker genes.

Keywords: YKL-40, mesenchymal stem cells, osteogenic differentiation, chitinase-like-protein

Introduction

Stem cells hold a great promise as a tool in basic research and development of drugs and new treatments in regenerative medicine. Human multipotent mesenchymal stromal cells (hMSC) is a commonly used term for a heterogeneous population of cells that can be derived from human bone marrow, adipose tissue, peripheral blood or umbilical cord blood[1,2]. These cells can differentiate into cells of the mesenchymal lineage, e.g. to osteoblasts, chondrocytes, fibroblasts and adipocytes, as well as, stromal cells of the bone marrow[3].

In connection to the regeneration of the osteogenic matrix, chitinases and chitinase-like-proteins (CLP) have been suggested to have a role in defence mechanisms against chitin-containing particles or organisms and to participate in tissue remodelling and inflammation[4,5]. Whether triggering of the immune response and the potential role in tissue remodelling is directly affected by the chitinase-like-proteins or dependent on the downstream induction of cytokines and growth factors has not been investigated[5]. There have been indications that the chitinase-like-proteins are expressed during tissue damage or regeneration in an attempt to counter the negative effect of inflammatory cytokines[6]. In humans, three chitinase-like-proteins are known, YKL-39, YKL-40 and oviductin, as well as the active chitinases acidic mammalian chitinase (AMCase) and chitotriosidase[7]. The active chitinases belong to the family 18 glycosyl hydrolases, the same family as the CLPs. CLPs have lost the ability to degrade chitin due to an amino acid substitution in the binding cleft[7,8], but the ability to bind chitin and chitooligosaccharides is still retained[9]. Binding of the ligand induces a large conformational change in YKL-40 which indicates a potential signalling role of the enzyme[10]. YKL-40 is expressed by articular chondrocytes, synoviocytes, osteoblasts and differentiated macrophages and is up-regulated in inflammatory diseases like rheumatoid arthritis and osteoarthritis[6,11]. YKL-40 expression has been suggested to be regulated by the transcription factor NF-κBeta[6]. Also, it has been proposed that YKL-40 might prevent damage to the extra-cellular matrix during inflammation by reducing the deleterious effect of pro-inflammatory cytokines[12].

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Due to chitinases, chitin, the second most abundant compound in nature, does not accumulate in the environment[13]. Chitin, a structural component in the cell wall of crustaceans and fungi, can be used for the production of glucosamine, chitooligosaccharides and chitosan, which have wide ranging uses in biotechology, agriculture and food science[13]. Glucosamine is an aminosugar that naturally occurs in the body and has been claimed to have beneficial effects on osteoarthritis as it constitutes one of the subunits in glycosaminoglycans found in the extra-cellular matrix of cartilage[14]. It is unknown how glucosamine might work to ameliorate arthritis, but a connection to nitric oxide production has been suggested[14,15]. The effect of glucosamine on bone resorption and remodelling has been evaluated in a collagenase-induced osteoarthritis model in mice[16]. It was shown that excessive bone formation and bone remodelling were reduced and bone resorption was decreased[16]. The effect of glucosamine on the CLPs and its role during osteogenesis of hMSC has not been investigated.

In this study, we show that the chitinase-like-protein YKL-40 is expressed in hMSC and is maintained during osteogenic differentiation of these cells. The effect of glucosamine on the expression of YKL-40 and osteogenic differentiation was analysed.

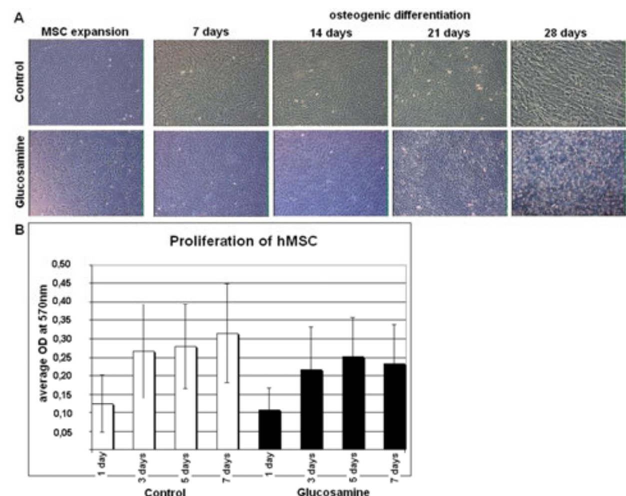

Figure 1. Effect of glucosamine on hMSC phenotype and proliferation. (A) Phenotype consistency in the expansion and osteogenic differentiation of hMSC (representative pictures); (B) Proliferation of hMSC grown in control media and media supplemented with 200 μg/mL Glucosamine. Bars illustrate proliferation on days 1, 3, 5 and 7 (n = 42 measurements).

Results and Discussion

hMSC Morphology and Proliferation. Short-term expansion of cells (single passage) in control media and glucosamine supplemented media did not lead to changes in the expression of cell surface markers and the cells comply with the characteristics of bone-marrow derived mesenchymal stromal cells (supplementary Figure 1)[17]. Cell morphology was typical of hMSC-small spindle-shaped cells attached to the surface of tissue culture plastic (Fig. 1A). Glucosamine did not affect cell attachment or morphology during expansion. During osteogenic differentiation, cell morphology changed to a more cuboidal and flattened shape. In later stages of differentiation, osteoblastic cells deposited calcium hydroxyapatite crystals and cell layers pulled together. No difference in phenotype was observed in the presence of glucosamine, except for the lack of calcium deposition.

Proliferation was similar during short-term expansion of hMSC in control media and glucosamine media, with a decrease in proliferation on days 5 and 7 (Fig. 1B).

Furthermore, the cells could be successfully differentiated towards the osteogenic, chondrogenic and adipogenic lineage, proving multi-lineage potential (supplementary Fig. 2).

Expression of the Chitinase-Like-Proteins (CLPs) in hMSC. CLPs have putative roles in defence mechanisms against chitin-containing particles and participate in tissue remodelling and inflammation[4,5]. Crystallography studies revealed that the enzyme can bind chitin and chitooligosaccharides with high affinity[9,10]. We therefore examined, whether the aminosugar glucosamine could affect the expression of the CLPs during short-term expansion of hMSC.

hMSC expressed the chitinase-like protein YKL-40 at day 3 and day 7 during expansion (supplementary Fig. 3). No statistical difference was found between control samples (0.85 ± 0.85 fold increase over 7 days) and glucosamine treated samples (3.24 ± 2.17 fold increase over 7 days) in the expression of YKL-40. The active chitinases, chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase) were not detected (data not shown).

During osteogenic differentiation, YKL-40 was expressed both in control (1.82 ± 0.53 fold increase over 28 days) and glucosamine treated samples (3.52 ± 0.63; $p = 0.0828$ fold increase over 28 days) (Fig. 2A). A trend in which glucosamine increased YKL-40 expression during osteogenic differentiation was observed, but statistical significance ($p < 0.05$) was not met.

Osteoblast Gene Expression. The following osteogenic marker genes: alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN) and runt-related transcription factor 2 (RUNX-2) were expressed both in control and glucosamine treated samples during osteogenic differentiation (Fig. 2A). A statistically significant increase in the presence of glucosamine was observed for ALP (0.63 ± 0.24 fold increase in control vs. 1.84 ± 0.13 fold increase in glucosamine; $p = 0.0011$), OCN (1.41 ± 0.41 fold increase in control vs. 2.85 ± 0.18 fold increase in glucosamine; $p = 0.0087$) and RUNX-2 (1.06 ± 0.31 fold increase in control vs. 2.39 ± 0.17 fold increase in glucosamine; $p = 0.0037$) gene expression. The difference in OPN expression (0.20 ± 0.41 fold increase in control vs. 1.56 ± 0.62 fold increase in glucosamine; $p = 0.097$) was not statistically significant but a trend was observed that suggested glucosamine could increase the expression of OPN.

To visualize the correlation between the expression of YKL-40 and the expression of the osteogenic marker genes, a heatmap was generated (Fig. 2B)[18]. Clustering divided the sample population into three distinct groups: (1) control samples from day 7 to day 25 of osteogenic differentiation; (2) glucosamine treated samples from day 7 to day 21; and (3) late osteogenesis including control samples at day 28 and glucosamine treated samples from day 25 to day 28. In the first

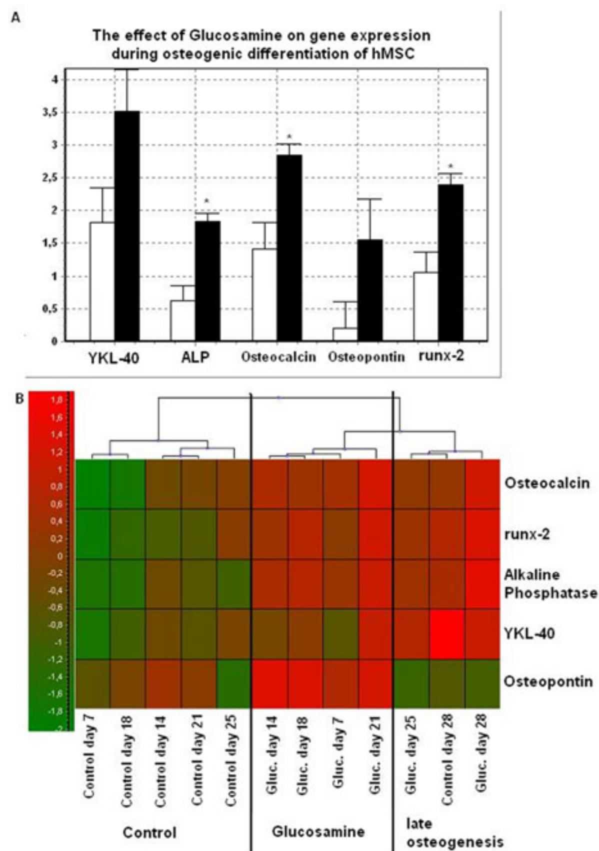

Figure 2. Effect of glucosamine on gene expression during osteogenic differentiation of hMSC. (A) Average expression of YKL-40, Alkaline Phosphatase (ALP), Osteocalcin (OCN), Osteopontin (OPN) and runt-related transcription factor 2 (RUNX-2) in control (white bars) and glucosamine (black bars) supplemented cultures over a period of 28 days of osteogenic differentiation. Bars describe average fold change with standard error. n = 2; * = $p < 0.05$; (B) Heatmap and hierarchical clustering of gene expression data auto-scaled to genes. Red colour depicts high expression and green colour depicts low expression (n = 2).

group overall expression of genes was low, except for the expression of OPN. The second group, i.e. early glucosamine treated samples, showed high expression of all osteogenic genes but only intermediate expression of YKL-40. Late osteogenesis, the third group, was characterized by peak expression of YKL-40 as well as OCN, RUNX-2 and ALP, but lower expression of OPN.

The increased expression of YKL-40 was correlated to the increased expression of the osteogenic marker genes, with the exception of OPN, which was highest in early glucosamine treated samples (Fig. 2B). Correlation was determined by calculating the Spearman correlation coefficient (r^2), which was shown to be 77.64% for YKL-40/RUNX-2, 90.45% for ALP/RUNX-2 and 78.87% for OCN/RUNX-2. Visualization by means of a heatmap suggested furthermore that glucosamine might modify osteogenic gene expression to match late osteogenesis compared to control samples. How and why glucosamine is affecting the expression of YKL-40 during long-term cultures was not investigated.

Our data suggest that YKL-40, in combination with increased expression of ALP, RUNX-2 and OCN, could play a role during late osteogenic differentiation of hMSC and this process could be modified by glucosamine, a subunit of the natural substrate for chitinases. YKL-40 is known to take part in tissue remodelling and regeneration but also protects the tissue from negative effects of pro-inflammatory cytokines[12]. This indicates that up-regulation of YKL-40 in developing and regenerating tissues might benefit the remodelling process.

Mineralization. Control cells increased the amount of mineralization deposited over a period of 28 days, as shown in von Kossa staining (Fig. 3A). Glucosamine treatment resulted in an overall impairment of mineral deposition with the first deposits visible after 21 days. Alizarin Red Staining showed the typical increase of calcium deposition expected in control cultures, whereas glucosamine supplemented cells did not show any sign of calcification (Fig. 3B). Photometric evaluation of Alizarin Red Staining visualized the lack of calcification in the presence of glucosamine, while control cells mineralized from day 21 onward (data not shown).

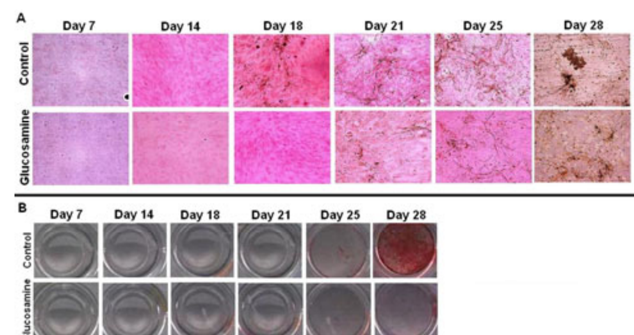

Figure 3. Characterization of mineralization processes during osteogenic differentiation of hMSC. (A) von Kossa Staining of hMSC differentiated for 28 days towards the osteogenic lineage. Dark brown colour is indicative of mineralization. (B) Alizarin Red Staining of hMSC differentiated for 28 days towards the osteogenic lineage. Dark red colour is indicative of calcification. Pictures are representative from 9 experiments.

A likely explanation for the lack of mineralization in glucosamine supplemented cultures could be acidosis of the cell culture media. All glucosamine stock solutions were prepared in PBS, but when the final concentration of glucosamine was added to the cell culture media, a pH change of the media was observed. Acidosis has been shown to delay mineralization in hMSC-derived osteoblasts and to increase OCN and RUNX-2 expression as well as decrease ALP, osterix (OSX) and OPN gene expression[19]. However, acidosis, as shown by Disthabanchong et al., does not explain the effect of glucosamine on gene expression observed in the present study, apart from the delay in mineralization in hMSC[19]. ALP mRNA expression and activity have been shown as well by other groups to be increased in the presence of glucosamine, when pH was stabilized at 7.4[20]. Furthermore, when media pH was adjusted using sodium bicarbonate solution in our study,

delay in mineralization was resolved (data not shown).

Effect of Glucosamine on Cytokine Expression. Whether the influence of YKL-40 on the activation of the immune system and the tissue remodelling process is due to the proteins themselves or the secondary induction of growth factors and cytokines is not known[5]. Furthermore, increased levels of YKL-40 are believed to protect the tissue from the negative effects of pro-inflammatory cytokines[6]. Therefore, we determined the secretion of several pro- and anti-inflammatory cytokines and growth factors during the osteogenic differentiation of hMSC.

No secretion of IL-1β, IL-12p (40), EGF and RANTES was observed in control cells or glucosamine supplemented cells. The anti-inflammatory cytokines IL-4 (3.9 ± 2.4 pg/mL in control vs. 3.5 ± 2.0 pg/mL in glucosamine), IL-10 (8.0 ± 3.5 pg/mL in control vs. 7.3 ± 1.1 pg/mL in glucosamine) and TNF-α (16.5 ± 6.9 pg/mL in control vs. 15.1 ± 6.4 pg/mL in glucosamine) were secreted in low amounts over 28 days of osteogenic differentiation (Fig. 4A). The growth factor VEGF (174.5 ± 133.1 pg/mL in control vs. 390.7 ± 27.7 pg/mL in glucosamine) showed intermediate secretion and the inflammatory cytokines IL-6 (1725.6 ± 307.3 pg/mL in control vs. 1321.9 ± 538.2 pg/mL in glucosamine) and IL-8 (344.6 ± 133.1 pg/mL in control vs. 390.7 ± 165.4 pg/mL in glucosamine) showed high secretion (Fig. 4B). The differences in secretion levels between control samples and glucosamine treated samples were not statistically significant with any of the cytokines determined.

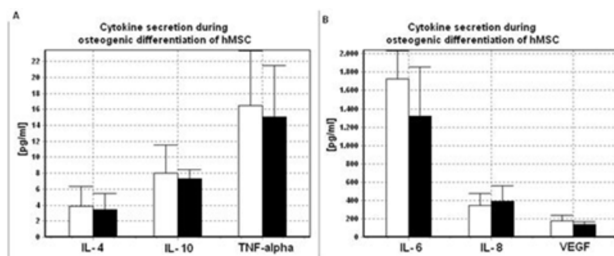

Figure 4. Average cytokine secretion during osteogenic differentiation of hMSC in control media or glucosamine (200 μg/mL) supplemented media. (A) Anti-inflammatory cytokines IL-4, IL-10 and TNF-α. (B) Inflammatory cytokines IL-6, IL-8 and growth-factor VEGF (average expression over 28 days); error bars describe standard error; n = 2.

Experimental Section

Cell Culture. Human, bone-marrow-derived, mesenchymal stromal cells (hMSC) (Lonza, Basel, Switzerland) were cultured in Standard Mesenchymal Stem Cell Basal media (Lonza) supplemented with L-Glutamine, Gentamicin Sulfate/ Amphotericin, and hMSC growth supplement following manufacturer's instructions. For osteogenic differentiation, Differentiation Basal Medium Osteogenic (Lonza) supplemented with dexamethasone, ascorbate, L-Glutamine, Streptamycine/Penicilline, β-Glycerophosphate and hMSC growth supplement was used. MTT proliferation assays were performed in DMEM/F12 media (Gibco, Carlsbad, California, USA) supplemented with Penicillin/Streptomycin (Invitrogen) and 10% hMSC-approved fetal calf serum (Stem Cell, Vancouver, BC, Canada). To determine the effect of glucosamine on hMSC, standard media and osteogenic differentiation media were supplemented with 200 μg/mL D-glucosamine hydrochloride-salt (YSK—Yaizu Suisankagaku Industry, Shizuoka, Japan). Glucosamine stock solutions were prepared by dissolving glucosamine in sterile PBS and filtered through 0.45 μm and 0.22 μm filters.

To prove multi-lineage differentiation potential, unstimulated cells were differentiated towards the osteogenic, chondrogenic and adipogenic lineage after appropriate expansion. All experiments were done at minimum in triplicate and hMSC from passage 3–5 were used in all experiments.

MTT Proliferation Assays. hMSC were expanded in a 96-well-plate and proliferation determined with the ATCC MTT proliferation kit (ATCC Bioproducts, Boras, Sweden) following manufacturer's instructions.

RNA Isolation and cDNA Transcription. RNA isolation was performed using Qiagen BioRobot workstation (Quiagen, Hilden, Germany) and the EZ-1 RNA Cell Mini Kit (Qiagen), following manufacturer's instructions. High-Capacitiy cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, California, USA) was used in all experiments.

Gene Expression. To quantify gene expression of selected genes, qPCR was performed in a StepOne Real Time PCR System (Applied Biosystems). 10 μL aliquots of Taqman master mix and 1 μL of Taqman assay (Applied Biosystems) were added to 9 μL of 1:10 diluted sample cDNA for each reaction.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. Taqman assays included YKL-40 (Hs00609691_m1), acidic mammalian chitinase (AMCase) (Hs00757767_m1), chitotriosidase (CHIT1) (Hs00185753_m1), osteocalcin (OCN) (Hs00609452_g1), osteopontin (*OPN*) (Hs00167093_m1), alkaline phosphatase (ALP) (Hs01029141_g1) and runt-related transcription factor 2 (RUNX-2) (Hs00231692_m1), all from Applied Biosystems.

Data were analysed using GenEX 5.3.2.13 software (MultiD Analyses, Gothenburg, Sweden). Data pre-processing was based on averaged qPCR repeats, normalized to the reference gene GAPDH and relative quantities were calculated relative to the earliest control sample (3 days expansion during short-term expansion and 7 days osteogenic differentiation of control samples for osteogenesis experiments). Graphs were created from averaged fold changes over 28 days relative to the respective control sample.

Luminex 10Plex Human Cytokine Assay. Luminex 10Plex Human Cytokine Assay (Panomics, Fremont, California, USA) was performed using media supernatants from stimulated and unstimulated cells during osteogenic differentiation. The following cytokines were analysed: Interleukin-1β (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12 (p40), tumor necrosis factor-α (TNF-α), RANTES, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) (detection limit of cytokines: 1.3 pg/mL).

Alizarin Red Staining and Quantitation. Cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde. Next, cells were washed with distilled H_2O (dH_2O) and stained with a 2% Alizarin Red Solution (Sigma Aldrich Inc., St. Louis, MO, USA) in dH_2O at pH 4.1 for 20 min. Cells were then washed with dH_2O and pictures were taken in a scanner (ScanJet ADF, Hewlett Packard).

For quantification, dried and stained wells were hydrated over-night in dH_2O and incubated with 10% cetyl-pyridinium chloride (Sigma) in dH_2O for 15 min on a shaker. Cells were spun down and supernatant aliquots were analysed in a MultiSkan spectrometer (Thermo Scientific) at 562 nm.

Von Kossa *S*taining. Cells were washed with PBS and fixed in 4% paraformaldehyde. Standard protocols were used to perform von Kossa staining and pictures were taken in a fluorescent microscope (Olympus BX51) using Cell A Imaging Software (Olympus, Center Valley, PA, USA).

Statistical Analysis. Data are presented as means and standard deviations, with the exception of gene expression data and cytokine secretion, where standard error was used. Statistical analysis was performed using GenEx 5.3.2.13 software. To evaluate treatment effect, t-test was used and a heatmap based on hierarchical clustering was constructed from autoscaled, pre-processed relative fold changes. Correlation of gene expression data visualized in the heatmap set-up was evaluated by Spearman correlation; $p < 0.05$ was considered statistically significant.

Conclusion

In this study, we showed the expression of the chitinase-like-protein YKL-40 in hMSC, both during expansion of undifferentiated cells and after osteogenic differentiation. Glucosamine did neither affect the maintenance of hMSC pluripotency nor proliferation. The active chitinases, chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase), were not detected. Glucosamine increased the expression of YKL-40 during osteogenic differentiation, but did not affect expression during short-term expansion. Glucosamine was furthermore associated with an increase in the expression of osteogenic marker genes during osteogenic differentiation of hMSC.

Electronic Supplementary Material

Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s13659-012-0017-0 and is accessible for authorized users.

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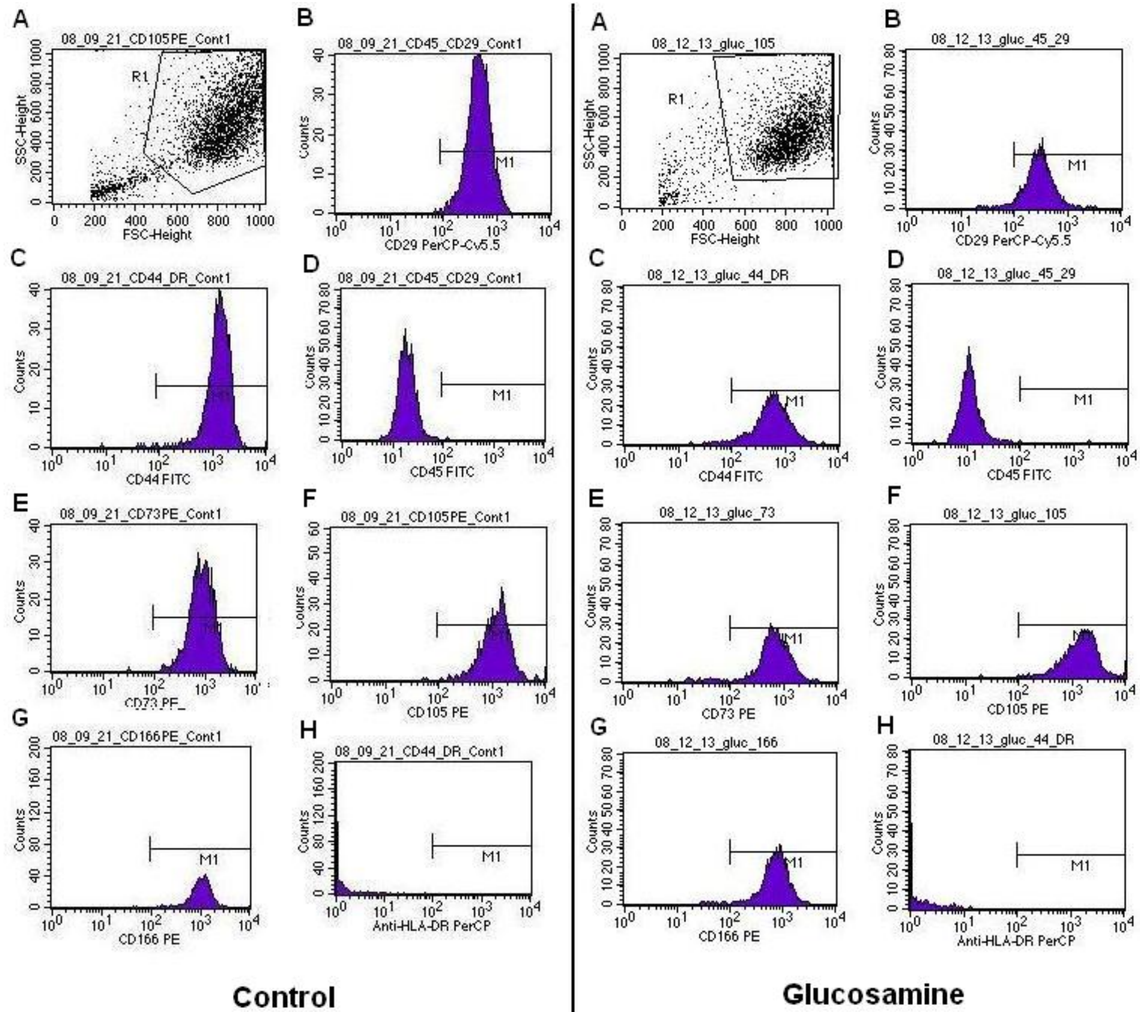
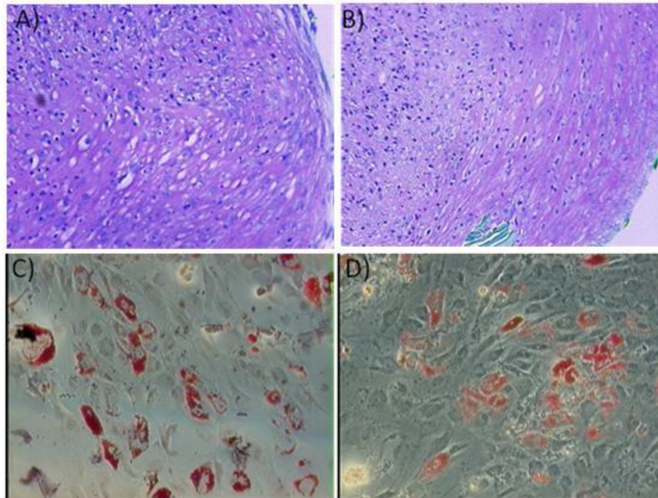
References

[1] Sakaguchi, Y.; Sekiya, I.; Yagishita, K.; Muneta, T. *Arthritis Rheum.* **2005**, *52*, 2521–2529.
[2] Zuk, P. A.; Zhu, M.; Mizuno, H.; Huang, J.; Futrell, J. W.; Katz, A. J.; Benhaim, P.; Lorenz, H. P.; Hedrick, M. H. *Tissue Eng.* **2001**, *7*, 211–228.
[3] Jiang, Y.; Jahagirdar, B. N.; Reinhardt, R. L.; Schwartz, R. E.; Keene, C. D.; Ortiz-Gonzalez, X. R.; Reyes, M.; Lenvik, T.; Lund, T.: Blackstad, M.; Du, J.; Aldrich, S.; Lisberg, A.; Low, W. C.; Largaespada, D. A.; Verfaillie, C. M. *Nature* **2002**, *418*, 41–49.
[4] Bleau, G.; Massicotte, F.; Merlen, Y.; Boisvert, C. *EXS* **1999**, *87*, 211–221.
[5] Lee, C. G. *Yonsei Med. J.* **2009**, *50*, 22–30.
[6] Recklies, A. D.; Ling. H.; White, C.; Bernier, S. M. *J. Biol. Chem.* **2005**, *280*, 41213–41221.
[7] Bussink, A. P.; Speijer, D.; Aerts, J. M.; Boot, R. G. *Genetics* **2007**, *177*, 959–970.
[8] Renkema, G. H.; Boot, R. G.; Au, F. L.; Donker-Koopman, W. E.; Strijland, A.; Muijsers, A. O.; Hrebicek, M.; Aerts, J. M. *Eur. J. Biochem.* **1998**, *251*, 504–509.
[9] Holt, P. G. *Toxicol. Lett.* **1996**, *86*, 205–210.
[10] Houston, D. R.; Recklies, A. D.; Krupa, J. C.; van Aalten, D. M. *J. Biol. Chem.* **2003**, *278*, 30206–30212.
[11] Hakala, B. E.; White, C.; Recklies, A. D. *J. Biol. Chem.* **1993**, *268*, 25803–25810.
[12] Ling, H.; Recklies, A. D. *Biochem. J.* **2004**, *380*, 651–659.
[13] Sandford, P. A. *Advances in Chitin Science*; NTNU: Trondheim, 2003; pp. 35–42.
[14] Kirkham, S. G.; Samarasinghe, R. K. *J. Orthop. Surg. (Hong Kong)* **2009**, *17*, 72–76.
[15] Meininger, C. J.; Kelly, K. A.; Li, H.; Haynes, T. E.; Wu, G. *Biochem. Biophys. Res. Commun.* **2000**, *279*, 234–239.
[16] Ivanovska, N.; Dimitrova, P. *Arthritis Res. Ther.* **2011**, *13*, R44.
[17] Dominici, M.; Le Blanc, K.; Mueller, I.; Slaper-Cortenbach, I.; Marini, F.; Krause, D.; Deans, R.; Keating, A.; Prockop, D.; Horwitz, E. *Cytotherapy* **2006**, *8*, 315–317.
[18] Bergkvist, A.; Rusnakova, V.; Sindelka, R.; Garda, J. M.; Sjögreen, B.; Lindh, D.; Forootan, A.; Kubista, M. *Methods* **2010**, *50*, 323–335.
[19] Disthabanchong, S.; Radinahamed, P.; Stitchantrakul, W.; Hongeng, S.; Rajatanavin, R. *Kidney Int.* **2007**, *71*, 201–209.
[20] Ganno, T.; Yamada, S.; Ohara, N.; Matsunaga, T.; Yanagiguchi, K.; Ikeda, T.; Ishizaki, H.; Hayashi, Y. *J. Biomed. Mater. Res. A* **2007**, *82*, 188–194.

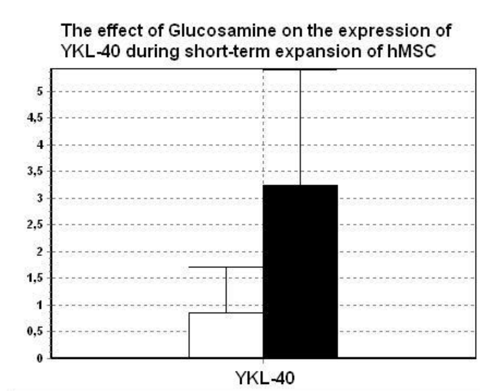
Supplemental Figures



Supplementary Figure 1. Surface marker antigen expression during short-term expansion of hMSC in control media and media supplemented with 200 µg/ml glucosamine. (A) dot plot with size (x-axis) and granularity (y-axis) of analysed cells. Histogram plots of (B) expression of CD29, (C) expression of CD44, (D) expression of CD45, (E) expression of CD73, (F) expression of CD105, (G) expression of CD166 and (H) expression of HLA-DR; for each graph 5000 events were obtained; n=2.



Supplementary Figure 2. Characterization of chondrogenic and adipogenic differentiation of hMSC with and without glucosamine. (A-B) Toluidine blue staining of hMSC differentiated for 28 days towards the chondrogenic lineage with glucosamine (A) and without glucosamine (B). Purple color and arrangement of cells are indicative of chondrogenic differentiation. (C-D) Oil red O staining of hMSC differentiated for 14 days towards the adipogenic lineage with glucosamine (C) and without glucosamine (D). Red color is indicative of lipid deposition which confirms adipogenic differentiation. Images are representative from 3 experiments.



Supplementary Figure 3. Effect of 200 µg/ml glucosamine on the expression of the chitinase-like-protein *YKL-40* during short-term expansion of undifferentiated hMSC. Expression was averaged from C_t values at days 3 and 7 for control cultures (white bars) and glucosamine supplemented cultures (black bars). Bars describe average fold change with standard error (n=2).

Paper II

2

"The great tragedy of science -
the slaying of a beautiful hypothesis by an ugly fact."

Thomas Henry Huxley, English biologist (1825-1895)

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Chitosan and Chitin Hexamers affect expansion and differentiation of mesenchymal stem cells differently

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Osteogenesis

ABSTRACT

Chitooligosaccharides are of interest as potential drugs due to their bioactivity and water solubility. We compared the effect of acetylated and deacetylated chitooligomers (Hexamers) on short-term expansion (7 days) and osteogenic differentiation of bone-marrow derived, human mesenchymal stem cells in terms of gene expression, cytokine secretion and quality of osteogenic differentiation. We show that chitooligomers affect hMSC gene expression and cytokine secretion, but not mineralization. The effect of chitooligomers was shown to be dependent on the acetylation degree, with significantly stronger effects when cells are stimulated with chitin-derived Hexamers (N-Acetyl Chitohexaose) than with Chitosan Hexamers (Chitohexaose).

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1. Introduction

Chitinases and chitinase-like-proteins (CLPs) belong to the family 18 glycosylhydrolases [1,2]. The latter lack enzymatic activity due to an amino acid substitution in the binding cleft, but can bind chitin and chitooligosaccharides with high affinity [1,2]. YKL-40, one of the three CLPs known in man, is expressed by articular chondrocytes, differentiated macrophages, synoviocytes and osteoblasts [3,4]. Binding of the putative ligand induces a conformational change, indicating a potential signaling role for the protein, but the definite *in vivo* function is still controversial [5]. The expression of YKL-40 has been suggested to affect remodeling of the extra-cellular matrix and protect the tissue from the degenerative effect of pro-inflammatory cytokines [3]. Furthermore, there is an indication that CLPs are able to sense the presence of invading microorganisms through recognition of chitin containing particles and subsequently activate immune responses [6,7]. Whether these actions are mitigated by the proteins themselves or by secondary induction of cytokines and growth factors is not known [7]. Based on the changes in YKL-40 expression in patients with inflammation, the protein is expected to play a role in the body's acute phase response system [8,9].

Chitin, which is the second most abundant compound in nature, does not accumulate in the environment due to the activity of chitinases [10]. The structural component in the cell wall of fungi and crustaceans can be used to derive chitosan, chitooligosaccharides (ChOS) and glucosamine, which are widely used in biotechnology and food science [10]. Since dissolution of chitin and chitosan in aqueous solutions without organic acids is difficult, the interest in ChOS as potential drugs for asthma, as vectors in gene therapy and as wound dressings has been increasing [11–14]. Additionally, ChOS have been shown to exhibit appealing properties including antitumor activity, inhibition of angiogenesis and immune-stimulatory effects [11,15,16]. The potential effect of ChOS depends on the degree of acetylation, the size of the oligomers (pentamers, hexamers or higher) and the molecular weight [17,18]. Particularly the hexamer and heptamer oligomers both acetylated and deacetylated, display highest levels of biological activity [15,18,19].

Human multipotent mesenchymal stem cells (hMSC) are a heterogeneous population of cells that can be isolated from the bone marrow, adipose tissue, peripheral blood and umbilical cord blood [20–23]. These cells can be differentiated into cells of the mesenchymal lineage including osteoblasts, chondrocytes, stromal cells and adipocytes [24]. Stem cells hold a great promise as a tool

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in basic research, drug development and in developing new treatments in regenerative medicine [25].

In this study, we showed the effect of Chitosan Hexamers (Chitohexaose) and Chitin Hexamers (N-Acetyl Chitohexaose) on expansion (7 days) and osteogenic differentiation potential of human, bone-marrow derived, mesenchymal stem cells. We evaluated gene expression, secretion of selected cytokines and quality of osteogenic differentiation. The results demonstrate that chitooligomers can affect hMSC gene expression and cytokine secretion, but do not affect mineralization and that the degree of influence depends on the deacetylation of the oligomers. Chitin Hexamers were shown to exhibit significantly stronger effects on hMSC than do Chitosan Hexamers.

2. Methods and materials

2.1. Cell culture

Human, bone marrow-derived, mesenchymal stem cells (hMSC) (Lonza, Basel, Switzerland) were cultured in DMEM/F12 media (Gibco, CA, USA) supplemented with Penicillin/Streptomycin (Invitrogen, CA, USA) and 10% MSC-approved fetal calf serum (Stem Cell, Vancouver, Canada). Osteogenic differentiation was induced in Differentiation Basal Medium Osteogenic (Lonza) supplemented with dexamethasone, ascorbate, L-Glutamine, Streptomycin/Penicllin, β-Glycerophosphate and MSC growth supplement. Two independent donors were used for control and Chitosan Hexamer stimulated cells, one donor was used for stimulation with Chitin Hexamers.

For stimulation, hMSC culture media was supplemented during the culture period with 200 μg/ml Chitosan Hexamers (Chitohexaose 6 HCl, chemical formula: $C_{36}H_{68}N_6O_{25}$–6HCl, molecular weight: 1203.73 g/mol, 96% purity per HPLC, 100% degree of deacetylation; cat. no. OC09273, Carbosynth Limited, UK) or 200 μg/ml Chitin Hexamers (hexa-N-acetyl-Chitohexaose, chemical formula: $C_{48}H_{80}N_6O_3$, molecular weight: 1237.1 g/mol, 95% purity per HPLC, 0% degree of deacetylation; cat. no. 56/11-0050, IsoSep AB, Sweden). To ensure the absence of endotoxins, Chitosan Hexamers and Chitin Hexamers were run through Detoxi-Gel Endotoxin Removing Columns (Thermo Scientific, USA) with immobilized Polymyxin B before use in cell culture systems.

Proliferation and viability were assessed with MTT proliferation assay (ATCC Bioproducts, Boras, Sweden) following manufacturer's instructions.

2.2. Analysis of gene expression

RNA isolation was performed using Quiagen BioRobot workstation (Quiagen, Germany) and the EZ-1 RNA Cell Mini Kit (Quiagen) following manufacturer's instructions. Samples from osteogenic differentiation experiments were homogenized in a FastPrep 24 instrument (MP Biomedicals, USA) using Lysing Matrix D tubes (MP Biomedicals) containing 1.4 mm ceramic spheres before RNA isolation.

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used for reverse transcription of RNA with a master mix containing: 2.0 μl RT buffer, 0.8 μl 25× dNTP (100 mM), 2.0 μl 10× Random Primers RT, 1.0 μl Multiscribe Reverse Transcriptase, 1.0 μl RNase Inhibitor and 3.2 μl Nuclease free H_2O per sample. Cycle conditions were as following: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 s and then 4 °C. Samples were stored at −20 °C before qPCR analysis.

Gene expression of selected genes was quantified using the 7500 Real Time PCR System (Applied Biosystems). 10 μl ready-made Taqman master mix (Applied Biosystems), 1 μl Taqman assay (Applied Biosystems) and 9 μl of 1:10 diluted sample cDNA were prepared per sample. Samples were analyzed at least in duplicate for each of the donors. *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) was used as internal reference gene. Taqman assays used during this study were: *YKL-40* (Hs00609691_m1), *TLR3* (Hs00152933_m1), *TLR4* (Hs00152939_m1), *COL1A2* (collagen I; Hs01028970_m1), *ALP* (Alkaline Phosphatase; Hs01029141_g1) and *RUNX-2* (runt-related transcription factor 2; Hs00231692_m1).

Data analysis of qPCR results was performed using GenEX 5.3.2.13 software (MultiD, Sweden). Missing data was handled with RT-PCR replicates and outliers determined and deleted using Grubb's test. qPCR repeats were averaged and normalized to the reference gene *GAPDH* for hMSC data. Osteogenic differentiation results from qPCR were globally normalized with a cut off at $C_t = 34$.

2.3. Analysis of osteogenic differentiation

For specific staining of calcification during osteogenic differentiation, media was removed and cell layers washed three times with 1 ml PBS before fixation in 4% para-formaldehyde for 15 min at 22 °C. Fixation agent was removed and cell layers washed three times for 5 min with distilled H_2O (dH_2O) on a shaker and then stained with a 2% Alizarin Red Solution (Sigma Aldrich Inc.) in dH_2O at pH 4.1 for 20 min on a shaker (pH was adjusted with 0.5% ammoniumhydroxide). Cell layers were then washed four times for 5 min with dH_2O on a shaker and then dried upside down. Pictures were taken in an inverted microscope (Leica DM IRB) with Infinity Capture 5.0.2 software.

2.4. Cytokine immunoassay

Secretion of eight cytokines into media supernatants was detected using a custom designed Procarta 8Plex Human Cytokine assay (Panomics/Affymetrix, USA) with a detection limit of 1 pg/ml. Supernatant aliquots were stored at −80 °C and analyzed in duplicates. Analysis was done on a Luminex instrument that was calibrated and cleaned as recommend by the manufacturer. Concentration of cytokines was derived from a standard curve and corrected for the blank (basic cell culture media). The assay was performed according to manufacturer's instructions.

2.5. YKL-40 ELISA

Levels of YKL-40 in the media supernatants of cells were determined using a sandwich enzyme immunoassay ELISA (Quidel, USA) with a detection limit of 5.4 ng/ml. Supernatant aliquots were stored at −80 °C and analyzed in duplicates. ELISA was performed according to manufacturer's instructions and concentration of YKL-40 determined from a standard curve as ng/ml.

2.6. Statistical analysis

Data are presented as means plus/minus standard errors. Statistical analysis was performed using Prism 5.01 software (GraphPad Software Inc., USA) and for qPCR analysis using GenEX 5.3.2.13 software. One-way ANOVA was used to evaluate the effect of stimulation during osteogenesis and two-way ANOVA for analysis of time-dependent effect of stimulation with chitosan oligomers during expansion. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. hMSC morphology and proliferation

Cell morphology was typical of hMSC with small, spindle-shaped cells attached to the surface of tissue culture plastic.

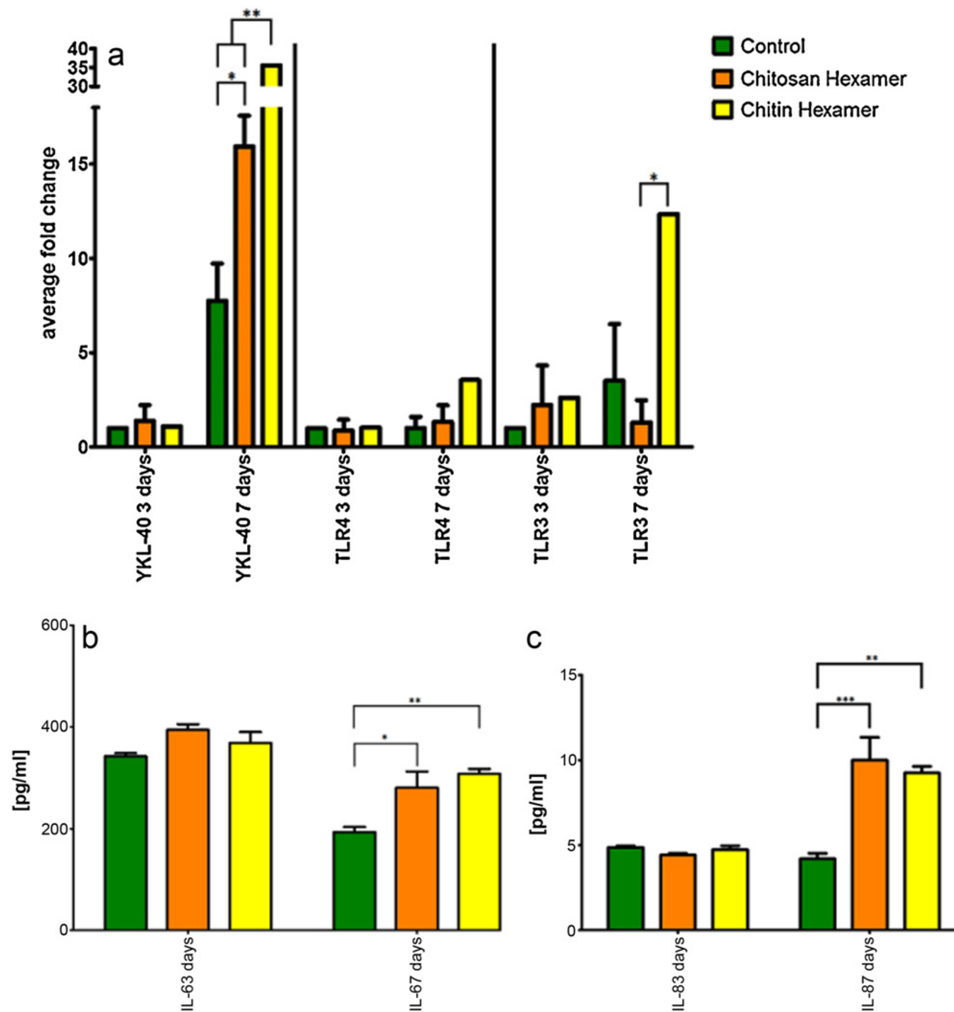

Fig. 1. Short-term effect of hMSC stimulation with 200 μg/ml Chitosan Hexamers or 200 μg/ml Chitin Hexamers. (a) Expression of *YKL-40*, *TLR 3* and *TLR 4* after 3 days and 7 days of expansion. Error bars are standard errors ($n = 2$ measurements/donor) ($^{*}p < 0.05$; $^{**}p < 0.01$). (b) and (c) Cytokine secretion of IL-6 and IL-8 after 3 days and 7 days in expansion. Error bars are standard errors ($n = 2$ measurements/donor) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

Stimulation with Chitosan Hexamers or Chitin Hexamers did not affect cell attachment or morphology during 1 passage expansion (7 days) (data not shown). During osteogenic differentiation, cell morphology changed to a cuboidal and flattened shape. In later stages of the differentiation process, osteoblastic cells deposited calcium hydroxyapatite crystals and cell layers pulled together, most likely due to collagen I secretion. Morphology and mineralization during differentiation were also not affected by the presence of Chitosan Hexamers and Chitin Hexamers.

An important issue in the clinical application of hMSC is the restricted ability for *in vitro* expansion [26]. Proliferation and viability can be affected by substances present in the cell culture media and change the usual latent exponential growth curve observed for this cell type [26]. Proliferation was similar in control cells and stimulated cultures, with a slight decrease in proliferation after 5 days of expansion (Supplementary Fig. 1). No statistical difference was found in proliferation and viability of hMSC over a period of 7 days in the presence of Chitosan Hexamers and Chitin Hexamers.

Chitooligomers did not affect proliferation, morphology or cell attachment of hMSC, therefore the bioactivity observed in subsequently performed experiments is expected to be due to the effect of the chitooligomers and not based on the alteration of hMSC basic characteristics.

3.2. Gene expression during short-term expansion

One of the first events in sensing substances that pose potential harm to the host is based on the activation of Toll-like receptors (TLR), which play an important role in innate immunity [27]. Binding of a ligand to this group of receptors activates signaling cascades that lead to the induction of cytokines and chemokines [27]. We determined the expression of *TLR 4*, sensor of endotoxins and gram-negative pathogens, and *TLR 3*, which is believed to play a role in facilitating stress responses in hMSC [28]. The expression of *TLR 4* did not change between 3 day and 7 day periods of culture and was likewise not affected by the presence of the chitooligomers (Fig. 1a). An increase in expression of *TLR 3* was observed for Chitin Hexamers after 7 days of stimulation ($p < 0.05$), whereas Chitosan Hexamers did not affect the expression of this gene. TLRs have been linked to epithelial homeostasis by inducing proliferation and tissue repair after injury, indicating a potential tissue remodeling role [29].

We determined the effect of chitooligomers on the expression of *YKL-40* and found that after 7 days both Chitosan Hexamers and Chitin Hexamers strongly induce the expression of this gene (Fig. 1a). The effect of Chitin Hexamers is stronger than the increase observed in the presence of Chitosan Hexamers ($p < 0.01$). In control cells, the expression of *YKL-40* increases over time. YKL-40 is

indicated in the functions of the innate immune system and the increase in expression might therefore be related to the expression of *TLR 3*, a first-line defense receptor in the innate immune system. TLRs and YKL-40 share some signaling molecules including the transcription factor NF-κBeta and crystallography studies revealed that the protein can bind chitin and chitooligosaccharides with high affinity [3,30].

3.3. Cytokine secretion during expansion

The activation of TLRs induces a signaling cascade that can result in the secretion of cytokines and chemokines such as IL-6, IL-8, TNF-α and IFN-γ [31]. These cytokines can create an inflammatory environment that attracts cells of the adaptive immune system [32]. hMSC are known to secrete factors that can alter immune responses and several studies have evaluated the ability of MSC to inhibit dendritic cell maturation, suppress T- and B-cell activation and inhibit the action of antigen-presenting cells [32,33]. Since *TLR 3* is significantly up-regulated in the presence of Chitin Hexamers we determined the secretion of 8 human cytokines in the cell culture media during short-term expansion. No secretion of IL-1β, IL-10, RANTES or TNF-α was observed in control cells or stimulated cultures. Base line levels of IL-4 and IL-12p(40) were detected in all samples (data not shown). The secretion of IL-6 was increased in the presence of Chitosan Hexamers ($p < 0.05$) and Chitin Hexamers ($p < 0.01$) as compared to the control samples (Fig. 1b). No effect was observed after 3 days in culture, but an overall decrease of IL-6 secretion over time was noted. Similarly, the secretion of IL-8 was significantly higher in Chitosan Hexamer ($p < 0.001$) and Chitin Hexamer ($p < 0.01$) stimulated cultures, whereas the secretion in control samples did not change over time (Fig. 1c). The increase in secretion of IL-6 and IL-8 can be seen as an important step in host defense to regulate inflammatory responses and can directly affect bone metabolism [34]. Furthermore, pro-inflammatory cytokines are thought to decrease wound healing through the increase in cellular activation and chemotaxis [35]. The ability of Hexamer oligomers to induce secretion of IL-8 was shown before in rat fibroblasts *in vitro* and was assumed to be connected to the wound healing process by attracting polymorphonuclear cells [36]. Other studies on the effect of chitooligosaccharides on the secretion of cytokines have shown the down-regulation of IL-6 and TNF-α in murine macrophages after challenge with lipopolysaccharide and suggested that chitooligosaccharides might prevent inflammatory processes through inhibition of NF-κBeta [37]. The difference to our study is in so far as murine macrophages express CD14 receptors, which are known to bind chitooligosaccharides and can therefore compete with the binding of lipopolysaccharide, reducing the induction of cytokines [38]. hMSC do not express CD14 per definition and the presence of chitooligosaccharides after stimulation with lipopolysaccharide does not decrease the secretion of pro-inflammatory cytokines (unpublished observations).

3.4. Osteogenic marker gene expression

The ability of hMSC to differentiate along the osteogenic lineage makes them an attractive tool in tissue engineering [39]. The level of differentiation can be monitored by the expression of selected osteogenic marker genes, such as Alkaline Phosphatase (*ALP*), runt-related transcription factor 2 (*RUNX-2*), osteocalcin (*OCN*) and osteopontin (*OPN*) [40,41]. Not only will the expression of the marker genes be increased, the process of differentiation will be accompanied by the deposition of calcium hydroxyapatite and other morphological changes [40,41]. The expression of the osteogenic marker genes *RUNX-2* ($p < 0.01$) and collagen I (*COL1A2*) ($p < 0.05$) was only affected by the presence of Chitin Hexamers, but not after stimulation with Chitosan Hexamers as compared to the control group (Fig. 2a). The difference in expression levels between the chitooligomers was significant in the case of *RUNX-2* ($p < 0.05$), but not *COL1A2*. The expression of *ALP* was not significantly different between the stimulated groups and the control samples. Runx-2 is also known as the master-regulator of osteogenic differentiation and can be detected throughout the process of osteogenic and chondrogenic differentiation [42,43]. This transcription factor is essential for the expression of down-stream genes important for osteogenesis, *i.e. OPN*, *OCN* and *COL1A2* [44,45].

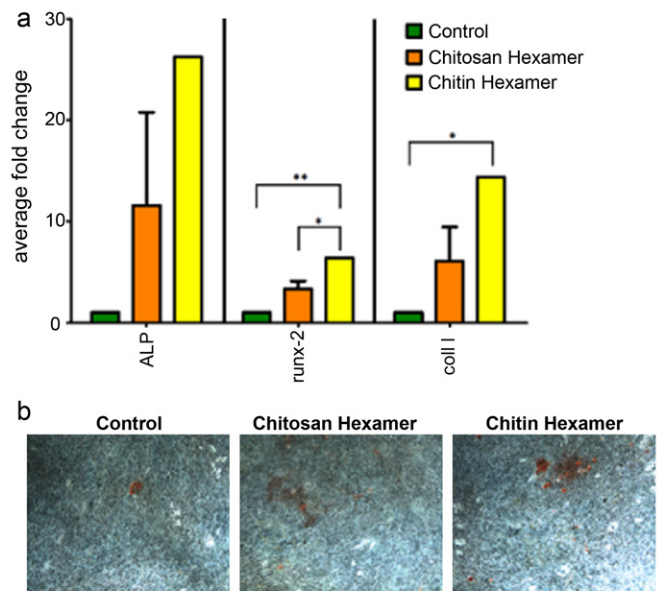

Fig. 2. Quality of osteogenic differentiation in hMSC. (a) Expression of Alkaline Phosphatase (*ALP*), runt-related transcription factor 2 (*RUNX-2*) and collagen I (*COL1A2*) after 21 days of osteogenic differentiation. Error bars are standard errors ($n = 2$ measurements/donor) (*$p < 0.05$; **$p < 0.01$) and (b) Alizarin Red Staining of calcification at 21 days of osteogenesis. Red staining is specific for calcium deposition (a representative picture of two donors).

3.5. Mineralization

The deposition of calcium hydroxyapatite crystals in the course of osteogenic differentiation can be visualized by Alizarin Red Staining. In control cultures, the typical increase of calcium deposition can be observed over a period of 21 days (Fig. 2b). The amount of mineralization and changes in osteoblast morphology was very similar in the presence of Chitosan Hexamers and Chitin Hexamers. Despite the increase in osteogenic marker genes, *RUNX-2* and *COL1A2*, the process of mineralization was not affected. There is a possibility that the effect on calcium deposition was not obvious after 21 days in culture, but may become prominent after longer differentiation periods.

3.6. YKL-40 and TLR 3 gene expression during osteogenic differentiation

The expression of *YKL-40* was similar in all samples and was accompanied by equal amounts of secreted YKL-40 protein in the different groups (Fig. 3a and b). The effect of chitooligomers on *YKL-40* gene expression could be short-lived, since increased gene expression can be observed during the expansion of hMSC for 7 days but not after osteogenic differentiation for 21 days. There have been indications that the interaction of YKL-40 with its possible ligand chitooligosaccharides might not be a pre-requisite for activation and that in some cases a lack of potential activation through interaction could be observed [46].

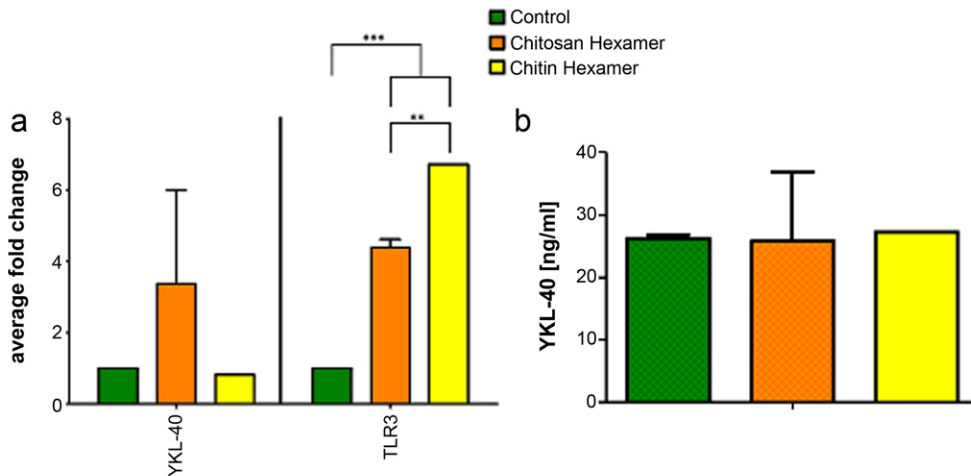

Fig. 3. Long-term effect of hMSC stimulation with 200 μg/ml Chitosan Hexamers or 200 μg/ml Chitin Hexamers. (a) Expression of *YKL-40* and *TLR 3*. Error bars are standard errors ($n = 2$ measurements/donor) and (b) YKL-40 protein secretion during osteogenic differentiation of hMSC. Error bars are standard errors ($n = 2$ measurements/donor).

In contrast to this, the effect of Chitosan Hexamers and Chitin Hexamers on the expression of *TLR 3* is prolonged (Fig. 3a). Both chitooligomers induced significantly greater expression of *TLR 3* compared to the control samples ($p < 0.001$), but again the difference between the two chitooligomers was clear. The expression of *TLR 3* was additionally significantly higher in Chitin Hexamer ($p < 0.01$) stimulated cells than in the presence of Chitosan Hexamers. This might indicate a prolonged stress response in hMSC induced by the chitooligomers.

3.7. Effect on cytokine expression during differentiation

The effect of chitooligomers on cytokine secretion during osteogenic differentiation was very similar to the observations made during short-term expansion of hMSC. No secretion of IL-1β, IL-10 or RANTES could be detected in any of the samples. Base line levels of IL-4, IL-12p(40) and TNF-α were observed, but there was no significant difference between the groups. The secretion of both IL-6 and IL-8 was increased in the presence of Chitosan Hexamers ($p < 0.001$ for IL-6 and $p < 0.01$ for IL8 secretion) and Chitin Hexamers ($p < 0.001$ for IL-6 and IL-8 secretion) (Fig. 4). Nevertheless, the increase in cytokine secretion between the two oligomers was very similar.

The main event connecting innate immune responses to bone biology in this study is the induction of pro-inflammatory cytokines like IL-6. This cytokine was tightly linked to osteoclast-induced bone resorption and plays an important role in endotoxin-mediated responses of hMSC [34,47]. *In vitro*, the increased secretion of IL-6 and IL-8 can promote osteogenic differentiation and mineralization, but *in vivo* or in co-culture with pre-osteoclasts, increased rates of bone-turnover will be induced.

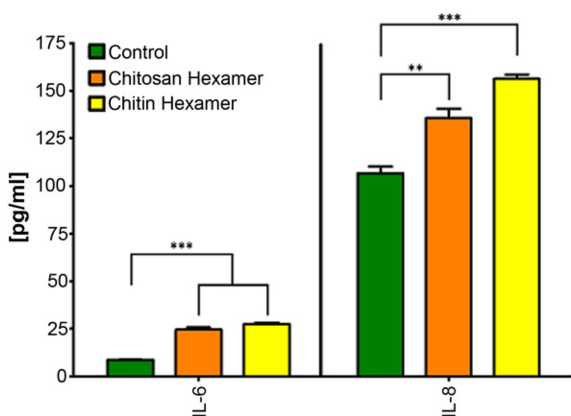

Fig. 4. Effect of hMSC stimulation with 200 μg/ml Chitosan Hexamers or 200 μg/ml Chitin Hexamers on cytokine secretion during osteogenesis. Secretion of IL-6 and IL-8 at 20 days of osteogenic differentiation. Error bars are standard errors ($n = 2$ measurements/donor) (**$p < 0.01$; ***$p < 0.001$).

4. Conclusion

Acetylated and deacetylated Hexamer oligomers derived from chitosan are of interest as potential anti-tumor drugs, immune-stimulating agents and inhibitors of tumor-related angiogenesis [11,16]. In this study, we compared the effect of acetylated and deacetylated Hexamers, derived from chitin on the short-term expansion (7 days) and osteogenic differentiation of bone-marrow derived, human mesenchymal stem cells. Chitooligomers can affect gene expression (*YKL-40* during expansion, *TLR 3*, *RUNX-2* and *COL1A2*) and cytokine secretion of IL-6 and IL-8, but not mineralization during osteogenic differentiation. The effect of chitooligomers was shown to depend on the degree of acetylation, with significantly stronger effects after stimulation with chitin-derived Hexamers (N-Acetyl Chitohexaose) than with chitosan-derived Hexamers (Chitohexaose). Based on these findings, we believe that the appropriate use of either acetylated or deacetylated chitooligomers has strong impact on the outcome of cell-based assays and strong considerations should be placed upon the decision for either chitooligomer considering the desired effect *in vitro* and *in vivo*.

Disclosure

The authors declare no competing financial interest.

Contributors

R.L., P.H.P. and O.E.S. contributed to the conception, design, analysis and interpretation of data, drafted the manuscript, critically read it, and approved the final version to be published. F.T., C.-H.N., J.M.E. and J.G. critically read the manuscript and approved the final version to be published.

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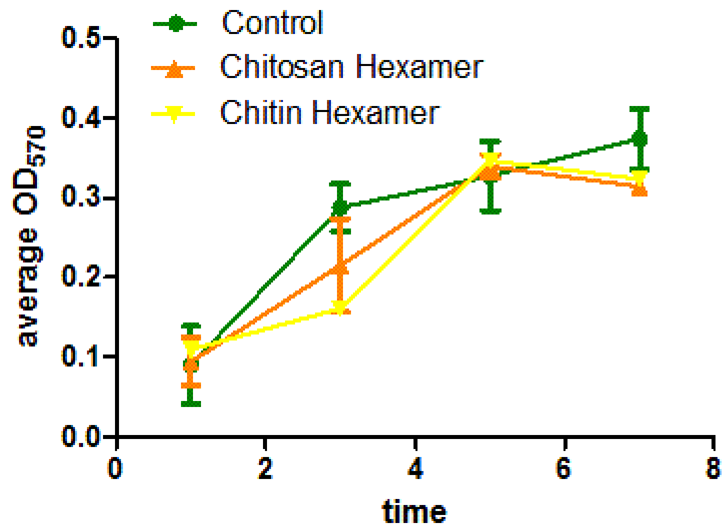
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2012.07.005.

References

[1] A.P. Bussink, D. Speijer, J.M. Aerts, R.G. Boot, Genetics 177 (2007) 959–970.
[2] G.H. Renkema, R.G. Boot, F.L. Au, W.E. Donker-Koopman, A. Strijland, A.O. Muijsers, M. Hrebicek, J.M. Aerts, European Journal of Biochemistry 251 (1998) 504–509.
[3] A.D. Recklies, H. Ling, C. White, S.M. Bernier, Journal of Biological Chemistry 280 (2005) 41213–41221.
[4] B.E. Hakala, C. White, A.D. Recklies, Journal of Biological Chemistry 268 (1993) 25803–25810.
[5] D.R. Houston, A.D. Recklies, J.C. Krupa, D.M. van Aalten, Journal of Biological Chemistry 278 (2003) 30206–30212.
[6] G. Bleau, F. Massicotte, Y. Merlen, C. Boisvert, EXS 87 (1999) 211–221.
[7] C.G. Lee, Yonsei Medical Journal 50 (2009) 22–30.
[8] G. Kronborg, C. Ostergaard, N. Weis, H. Nielsen, N. Obel, S.S. Pedersen, P.A. Price, J.S. Johansen, Scandinavian Journal of Infectious Diseases 34 (2002) 323–326.
[9] C. Nordenbaek, J.S. Johansen, P. Junker, N. Borregaard, O. Sørensen, P.A. Price, Journal of Infectious Diseases 180 (1999) 1722–1726.
[10] P.A. Sandford, Advances in Chitin Science 6 (2003) 35–42.
[11] K.V. Harish Prashanth, R.N. Tharanathan, Biochimica et Biophysica Acta 1722 (2005) 22–29.
[12] J.A. Elias, R.J. Homer, Q. Hamid, C.G. Lee, Journal of Allergy and Clinical Immunology 116 (2005) 497–500.
[13] M.P. Ribeiro, A. Espiga, D. Silva, P. Baptista, J. Henriques, C. Ferreira, J.C. Silva, J.P. Borges, E. Pires, P. Chaves, I.J. Correia, Wound Repair and Regeneration 17 (2009) 817–824.
[14] M. Köping-Höggård, Y.S. Mel'nikova, K.M. Vårum, B. Lindman, P. Artursson, The Journal of Gene Medicine 5 (2003) 130–141.
[15] K. Suzuki, T. Mikami, Y. Okawa, A. Tokoro, S. Suzuki, M. Suzuki, Carbohydrate Research 151 (1986) 403–408.
[16] X. Wei, Y. Wang, J. Xiao, W. Xia, International Journal of Biological Macromolecules 45 (2009) 432–436.
[17] C.W. Lin, L.J. Chen, P.L. Lee, C.I. Leel, J.C. Lin, J.J. Chiu, Biomaterials 28 (2007) 1355–1366.
[18] C. Xiong, H. Wu, P. Wei, M. Pan, Y. Tuo, I. Kusakabe, Y. Du, Carbohydrate Research 344 (2009) 1975–1983.
[19] C. Qin, Y. Du, L. Xiao, Z. Li, X. Gao, International Journal of Biological Macromolecules 31 (2002) 111–117.
[20] A. Erices, P. Conget, J.J. Minguell, British Journal of Haematology 109 (2000) 235–242.
[21] Y. Sakaguchi, I. Sekiya, K. Yagishita, T. Muneta, Arthritis and Rheumatism 52 (2005) 2521–2529.
[22] P.A. Zuk, M. Zhu, H. Mizuno, J. Huang, J.W. Futrell, A.J. Katz, P. Benhaim, H.P. Lorenz, M.H. Hedrick, Tissue Engineering 7 (2001) 211–228.
[23] N.J. Zvaifler, L. Marinova-Mutafchieva, G. Adams, C.J. Edwards, J. Moss, J.A. Burger, R.N. Maini, Arthritis Research 2 (2000) 477–488.
[24] Y. Jiang, B.N. Jahagirdar, R.L. Reinhardt, R.E. Schwartz, C.D. Keene, X.R. Ortiz-Gonzalez, M. Reyes, T. Lenvik, T. Lund, M. Blackstad, J. Du, S. Aldrich, A. Lisberg, W.C. Low, D.A. Largaespada, C.M. Verfaillie, Nature 418 (2002) 41–49.
[25] D.E. Cohen, D. Melton, Nature Reviews Genetics 12 (2011) 243–252.
[26] M.E. Bernardo, F. Locatelli, W.E. Fibbe, Annals of the New York Academy of Sciences 1176 (2009) 101–117.
[27] S. Akira, K. Takeda, Nature Reviews Immunology 4 (2004) 499–511.
[28] S.L. Tomchuck, K.J. Zwezdaryk, S.B. Coffelt, R.S. Waterman, E.S. Danka, A.B. Scandurro, Stem Cells 26 (2008) 99–107.
[29] F. Liotta, R. Angeli, L. Cosmi, L. Filì, C. Manuelli, F. Frosali, B. Mazzinghi, L. Maggi, A. Pasini, V. Lisi, V. Santarlasci, L. Consoloni, M.L. Angelotti, P. Romagnani, P. Parronchi, M. Krampera, E. Maggi, S. Romagnani, F. Annunziato, Stem Cells 26 (2008) 279–289.
[30] P.G. Holt, Toxicology Letters 86 (1996) 205–210.
[31] C. Pasare, R. Medzhitov, Microbes and Infection 6 (2004) 1382–1387.
[32] A. Roeder, C.J. Kirschning, R.A. Rupec, M. Schaller, H.C. Korting, Trends in Microbiology 12 (2004) 44–49.
[33] R. Medzhitov, Nature Reviews Immunology 1 (2001) 135–145.
[34] J. Sammons, N. Ahmed, M. El-Sheemy, H.T. Hassan, Stem Cells and Development 13 (2004) 273–280.
[35] W.G. Brodbeck, Y. Nakayama, T. Matsuda, E. Colton, N.P. Ziats, J.M. Anderson, Cytokine 18 (2002) 311–319.
[36] T. Mori, M. Okumura, M. Matsuura, K. Ueno, S. Tokura, Y. Okamoto, S. Minami, T. Fujinaga, Biomaterials 18 (1997) 947–951.
[37] H.T. Liu, W.M. Li, X.Y. Li, Q.S. Xu, Q.S. Liu, X.F. Bai, C. Yu, Y.G. Du, Basic & Clinical Pharmacology & Toxicology 106 (2010) 362–371.
[38] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop, E. Horwitz, Cytotherapy 8 (2006) 315–317.
[39] R. Quarto, M. Mastrogiacomo, R. Cancedda, S.M. Kutepov, V. Mukhachev, A. Lavroukov, E. Kon, M. Marcacci, New England Journal of Medicine 344 (2001) 385–386.
[40] J.Y. Choi, B.H. Lee, K.B. Song, R.W. Park, I.S. Kim, K.Y. Sohn, J.S. Jo, H.M. Ryoo, Journal of Cellular Biochemistry 61 (1996) 609–618.
[41] J. Caetano-Lopes, H. Canhão, J.E. Fonseca, Acta Reumatológica Portuguesa 32 (2007) 103–110.
[42] K. Nakashima, X. Zhou, G. Kunkel, Z. Zhang, J.M. Deng, R.R. Behringer, B. de Crombrugghe, Cell 108 (2002) 17–29.
[43] S. Harada, G.A. Rodan, Nature 423 (2003) 349–355.
[44] T. Komori, Journal of Cellular Biochemistry 99 (2006) 1233–1239.
[45] E.J. Mackie, International Journal of Biochemistry and Cell Biology 35 (2003) 1301–1305.
[46] A.D. Recklies, C. White, H. Ling, Biochemical Journal 365 (2002) 119–126.
[47] T. Kikuchi, T. Matsuguchi, N. Tsuboi, A. Mitani, S. Tanaka, M. Matsuoka, G. Yamamoto, T. Hishikawa, T. Noguchi, Y. Yoshikai, Journal of Immunology 166 (2001) 3574–3579.

Supplementary Figure Legends



Supplementary Figure 1. Proliferation of hMSC during short-term expansion. Proliferation was determined using MTT proliferation assay at days 1, 3, 5 and 7. Error bars are standard errors (n=15).

Paper III

3

"There is one thing even more vital to science than intelligent methods;
And that is the sincere desire to find out the truth, whatever it may be."

Charles Sanders Peirce, Mathematician (1839-1914)

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Endotoxins affect bioactivity of chitosan derivatives in cultures of bone marrow-derived human mesenchymal stem cells

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ABSTRACT

Biomaterials research has been expanding over the last decade, in part to provide improved medical devices for the treatment of orthopedic tissue injuries. In the quest to provide the best performance combined with low cost for medical implants, an increasing number of non-chemists have entered the field of biomaterials research without the profound knowledge of chemistry needed to understand the complex interaction mechanisms and characteristics of natural substances. Likewise, non-biologists often lack understanding when it comes to the presence of the contaminating biota frequently found in natural substances. This lack of knowledge by researchers in the field, combined with sensitive in vitro cell-based assays, can lead to inaccurate evaluation of biomaterials. Hence, there should be both an active effort to assemble multi-disciplinary teams and a genuine concern for the possible effects of contamination on in vitro assays. Here, we show that the presence of bacterial endotoxins in chitosan derivatives can result in false-positive results, profoundly altering product performance in in vitro assays. False-positive results through uncritical use of natural substances in vitro can be avoided by proper endotoxin testing and careful evaluation of cytokine secretion patterns.

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1. Introduction

Clinical treatment of orthopedic tissue injuries often involves the use of metal implants coated with bioactive materials to improve osseointegration and performance at the bone–biomaterial interface [1]. One of the materials used for this purpose is chitosan, the partly deacetylated form of chitin [2]. Flexible molding abilities, fungistatic and bacteriostatic properties, and recent reports on the positive influence of chitosan on osteogenesis in vitro and in vivo make this polymer a promising candidate for use in regenerative medicine [3–5]. The cationic charge of the polymer, introduced by the removal of the N-acetyl group in the deacetylation process, accounts for its interaction with negatively charged cytokines and growth factors [4]. However, the positive charge also predisposes chitosan to interaction with harmful endotoxins derived from Gram-negative bacteria, based on the formation of hydrogen bonds and electrostatic interactions [6,7]. In fact, chitosan so strongly binds to endotoxins that it was used for the removal of endotoxins from medical preparations before its introduction into biomaterials research and use in clinical applications [8].

Endotoxins are located in the outer cell membrane of Gram-negative bacteria and are released upon cell death, growth and division. Endotoxins and lipopolysaccharide (LPS) are often used as synonyms, even though LPS is only the toxicity-inducing component in endotoxins [9]; here, they are used interchangeably. Endotoxins signal the presence of bacteria to eukaryotic cells. The building blocks of endotoxins include a core oligosaccharide, an O-antigen and a lipid A component, which is the main factor contributing to the induction of immune responses in the host [10]. One of the first events in sensing Gram-negative pathogens and endotoxins is based on the activation of Toll-like receptor 4 (TLR 4) and its interaction with co-modulators, such as MD2 and CD14 [11]. The TLR group consists of receptors that play an important role in innate immunity, and binding of their ligands activates signaling cascades that lead to the secretion of various cytokines and chemokines [11].

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Chitinase-like proteins, e.g. YKL-40 and YKL-39, have putative roles in defense mechanisms against chitin-containing particles, indicating the presence of invading microorganisms, and participate in tissue remodeling and inflammation [12]. Whether activation of immune responses and tissue remodeling are affected by the proteins themselves or by secondary induction of cytokines and growth factors remains unknown [13]. Up-regulation of *YKL-40* in inflammatory and degenerative diseases, such as rheumatoid arthritis, osteoarthritis and certain cancers, suggests that the YKL-40 protein acts in the prevention of damage to the extracellular matrix by reducing the effect of proinflammatory cytokines [14]. Not only is the induction of *YKL-40* dependent on the transcription factor nuclear factor kappa Beta (NF-κB), the main transcriptional pathway activated by the Toll-like receptors, but crystallography studies have also revealed that the enzyme binds chitin and chitooligosaccharides (ChOS) with high affinity [15,16].

Endotoxins are known for their effect on cellular functions, including the stimulation of cytokine secretion, activation of monocytes and macrophages, and an increase in bone turnover in vivo [17,18]. In high concentrations, LPS can cause septic shock and acute renal failure in humans [17]. Therefore, in clinical practice strict regulations are in place to decrease the risk of endotoxin contamination in medical preparations. However, during the in vitro evaluation of biomaterials for clinical applications, endotoxin testing is often neglected. This negligence may ultimately result in erroneous interpretation of bioactivity, posing the risk for undesired health complications during subsequent clinical trials.

Here, we show that the presence of bacterial endotoxins (10 ng ml^{-1}) in chitosan derivatives can result in false-positive results, profoundly altering bioactivity in in vitro assays. False-positive results through uncritical use of natural substances in vitro can be avoided by proper endotoxin testing and careful evaluation of cytokine secretion patterns. We intend to raise awareness in the field of biomaterials research to the threat of endotoxin contamination in natural substances, and the resulting discrepancies between in vitro and in vivo studies.

2. Materials and methods

2.1. Production of ChOS

Chitin Flakes (Primex, Iceland) were deacetylated in 50 wt.% aqueous NaOH at 60 °C for 40 min at 25 rpm. The suspension was washed in flowing cold water at 6 °C for 10–12 min in double bag cheesecloth. The resulting chitosan was mixed with water and the pH adjusted to 3.8 with 30% HCl. Degradation of chitosan was performed using chitinase from *Penicillium* species (750 chitinolytic units per g) for 22 h at 25 °C at 50 rpm. ChOS were then separated by ultrafiltration with a Helicon SS50 spiral-wound ultrafiltration membrane (PTGC, 10 kDa cut-off, Millipore, USA) using tangential flow in a Millipore PUF-200-FG pilot module. The filtrate was desalted and subjected to a 1 kDa cut-off Helicon SS50 membrane using the same module. The volume of filtrate was kept constant by the addition of deionized water until oligosaccharides with a degree of polymerization (DP) of 1–3 comprised less than 10% of the total composition of ChOS, as determined by high-performance liquid chromatography (HPLC; HP-SEC with a TSK-oligo column, TosoHaas, Japan).

Before use in cell culture systems, all ChOS were subjected to endotoxin cleaning using Detoxi-Gel Endotoxin Removing Gel (Thermo Scientific, USA). Successful removal of endotoxins was assessed with the PyroGene Recombinant Factor C Endotoxin Detection System (Lonza, USA). For comparison, sample batches were also analyzed by Lonza (Verviers, Belgium) using the same protocol.

2.2. Characterization of ChOS

HP-SEC in a Beckman Gold System with a TSK-oligo column and 5 mM ammonium hydroxide (pH 10.0) as eluent at a flow rate of 0.5 ml min^{-1} was used to determine the distribution and quantity of different degree of polymerization oligomers. Twenty microliters of 10 mg ml^{-1} ChOS solution was injected and analyzed with an ultraviolet detector at 205 nm. Ethanol served as an internal reference for the inclusion volume and Beckman Gold analysis software was used for peak analysis.

The degree of deacetylation was analyzed using ^{1}H-nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) analysis. ^{1}H-NMR samples were measured in a Bruker AVANCE 400 spectrometer (Bruker Biospin GmbH, Germany) at 400.14 MHz at 298°K. Measurements were performed without water suppression in either D_2O or D_2O/DCl (deuterium chloride) as solvent. The sample concentration was 20–25 mg ml^{-1} and the N-acetyl peak was used as an internal reference. The degree of acetylation was calculated using the combined integrals of the proton peaks. IR measurements were performed in an AVTAR 370 FTIR instrument (Thermo Nicolet Corporation, USA). For this, 2–5 mg of samples was thoroughly mixed with KBr and then pressed into pellets with a Specac compressor (Specac Inc. USA).

2.3. Cell culture

Cell culture experiments were carried out with human bone marrow-derived mesenchymal stem cells (Lonza, Switzerland) in DMEM/F12 medium (Gibco, USA) supplemented with penicillin/streptomycin (Invitrogen, USA) and 10% MSC-approved fetal calf serum (Stem Cell, Canada) at 37 °C, 5% CO_2 and 95% humidity. To induce osteogenic differentiation, the basal expansion medium was switched to Differentiation Basal Medium Osteogenic (Lonza) supplemented with dexamethasone, ascorbate, L-glutamine, streptomycin/penicillin, β-glycerophosphate and MSC growth supplement, and 4000 cells cm^{-2} were seeded on vacuum gas plasma-treated tissue culture plastic. Three independent donors were used to determine the effect of 10 ng ml^{-1} LPS (Sigma Aldrich Inc., USA), 400 μg ml^{-1} ChOS (Genis ehf, Iceland) and 10 ng ml^{-1} LPS in combination with 400 μg ml^{-1} ChOS. Proliferation and viability was assessed using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay (ATCC Bioproducts, Sweden) following the manufacturer's instructions.

2.4. Analysis of gene expression

RNA isolation was performed using a Quiagen BioRobot workstation (Quiagen, Germany) and the EZ-1 RNA Cell Mini Kit (Quiagen). Samples from osteogenic differentiation experiments were homogenized in a FastPrep 24-instrument (MP Biomedicals, USA) using Lysing Matrix D tubes (MP Biomedicals) containing 1.4 mm ceramic spheres before RNA isolation. RNA was transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and gene expression of selected genes was quantified in a 7500 Real Time PCR System (Applied Biosystems) and analyzed using GenEx 5.3.2.13 software (MultiD, Sweden). Samples were analyzed at least in duplicate for each of the three donors. *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) was used as the internal reference gene. Taqman assays used during this study included: *YKL-40* (Hs00609691_m1), *TLR2* (Hs00152932_m1), *TLR3* (Hs00152933_m1), *TLR4* (Hs00152939_m1), *COL1A2* (collagen type I; Hs01028970_m1), *ALP* (Alkaline phosphatase; Hs01029141_g1), *OPN* (osteopontin; Hs00167093_m1) and *RUNX-2* (runt-related transcription factor 2; Hs00231692_m1).

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2.5. Validation of osteogenic differentiation

Osteogenic differentiation was evaluated by Alizarin Red staining and subsequent quantitation using 10% cetylpyridinium chloride (Sigma Aldrich Inc.), and validated by von Kossa staining, following standard protocols [19]. Alkaline phosphatase activity was determined in cell lysates by adding p-nitrophenyl phosphate solution (Sigma Aldrich Inc.) and measuring the optical density at 405 nm in a MultiSkan spectrometer. Alkaline phosphatase activity was calculated as nmol of p-nitrophenol per min following the general Beer–Lambert law.

2.6. Polystyrene beads cytokine assay

Secretion of eight cytokines into cell culture medium supernatants was determined using a custom-designed Luminex 8Plex Human Cytokine assay (Panomics, USA). The cytokines determined were IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p(40), RANTES and TNF-α with a limit of detection of 1 pg ml^{-1} in medium supernatant.

2.7. YKL-40 enzyme-linked immunosorbent assay

YKL-40 protein concentration in medium supernatants was analyzed with MicroVue YKL-40 enzyme immunoassay (Quidel, USA). The limit of detection was 5.4 ng ml^{-1} and the assay was performed according to the manufacturer‘s instructions.

2.8. Statistical analysis

Data are presented as mean ± standard error. Statistical analysis was performed using Prism 5.01 software (GraphPad Software Inc., USA) and for quantitative real-time polymerase chain reaction (qPCR) analysis using GenEx 5.3.2.13 software. One-, two- and three-way analyses of variance (ANOVAs) were used to evaluate the effect of endotoxin contamination in ChOS biomaterials. For experiments other than qPCR, one-way ANOVA was used to evaluate the effect of endotoxin contamination. Student's *t*-test was used for direct comparison between two groups; $p < 0.05$ was considered statistically significant. Three independent donors were used in all experiments.

3. Results

3.1. Characterization of ChOS

ChOS preparations were analyzed for degree of acetylation, ash content, solubility and appearance, and are summarized in Table 1. The degree of acetylation was calculated using the combined integrals of the proton peaks (Supplementary Fig. 1). The integral of the protons H-2 (GluNAc), H-3, H-4, H-5, H-6 and H-6′, belonging to the sugar backbone of ChOS, were found to be at δ 3.3–3.9 ppm and H-2 (GluN) was found at δ 2.7 ppm (for D_2O/DCl as solvent at δ 3.2 ppm). The integrals of these protons were compared to the integral for the N-acetyl peak at δ 2.08 ppm. Detailed results regarding characterization of ChOS using ^{1}H-NMR and FTIR are summarized in Supplementary Table 1 and Supplementary Fig. 2.

Before use, ChOS were characterized regarding the distribution of the DP using HPLC (Fig. 1). The effect of endotoxin removal on the structure of the ChOS was determined with ^{1}H-NMR and revealed that the endotoxin cleansing procedure did not affect the ChOS structure, the degree of acetylation or the DP (Fig. 1, Supplementary Figs. 1 and 2, and Supplementary Table 1). The NMR and IR investigations showed that there was no change in ChOS materials subsequent to endotoxin removal (Supplementary Fig. 1 and Supplementary Table 1). All characteristic NMR and IR peaks were identical and the degree of acetylation was unaltered. Furthermore, the absence of changes in the intensity of the H-1 α peak, signaling the reducing end, and the HPLC elution pattern (Supplementary Fig. 2) also confirmed that the DP had not been affected by the endotoxin removal step.

Table 1
Characterization of ChOS.

Characterization of ChOS	
F_A (degree of acetylation)	0.40$_{\text{(determined by }^1\text{H-NMR)}}$
Ash content	8.3%
Solubility in H_2O	100%
Appearance	White powder (spray-dried)
Escherichia coli	Absent
Coliform bacteria	Absent
Salmonella spp.	Absent
Endotoxin	0.38 ± 0.13 EU mg^{-1}

3.2. Short-term effect of LPS and ChOS on human mesenchymal stem cells (hMSC)

We estimated proliferation rate and viability with an MTT proliferation assay and determined that 10 ng ml^{-1} LPS over a 7 day period did not affect the proliferation potential of hMSC (Supplementary Fig. 3).

During the analysis of cytokine secretion patterns, the presence of LPS is apparent after 3 days in culture (Fig. 2a and b), manifesting in a significant increase ($p < 0.05$) in the secretion of the inflammatory cytokines IL-6 and IL-8, as expected. RANTES and IL-12p(40), two cytokines not usually secreted under standard culturing conditions in hMSC, could also be detected with LPS present.

However, during the standard evaluation of proliferation, morphology and expression levels of genes previously shown to be sensitive to endotoxins, the effect of 10 ng ml^{-1} LPS could not be detected during the 7 day evaluation period of expansion (Fig. 2c and d). An increase in the expression of *TLR3* was observed between days 3 and 7 during the expansion of hMSC, but neither LPS nor ChOS affected its gene expression (Fig. 2c). The expression of *TLR4* did not change between 3 and 7 day periods of culture, and was likewise not significantly affected by the presence of LPS (Fig. 2c). The expression of *YKL-40* in the presence or absence of LPS and ChOS did not vary significantly, but a time-dependent increase was observed (Fig. 2d).

3.3. Quality of osteogenic differentiation in hMSC

The osteogenic differentiation potential of hMSC makes them an attractive tool to treat osteogenesis-related injuries, with or without the use of scaffolds and implants [20]. We therefore examined the expression of osteogenic marker genes in the presence of ChOS and LPS.

ChOS alone increased the expression of *COL1A2* ($p < 0.01$), whereas the presence of LPS completely abrogated this effect. No difference in the expression of *ALP* and *RUNX-2* was detected with ChOS. The expression of the osteogenic marker genes *ALP* ($p < 0.001$) and *RUNX-2* ($p < 0.01$) was significantly up-regulated with LPS.

Furthermore, we observed an increase in ALP activity ($p < 0.001$) and the deposition of calcium hydroxyapatite crystals in the presence of LPS or LPS in combination with ChOS, though no increase was detected when cells were differentiated with ChOS alone (Fig. 3b and c; Supplementary Fig. 4).

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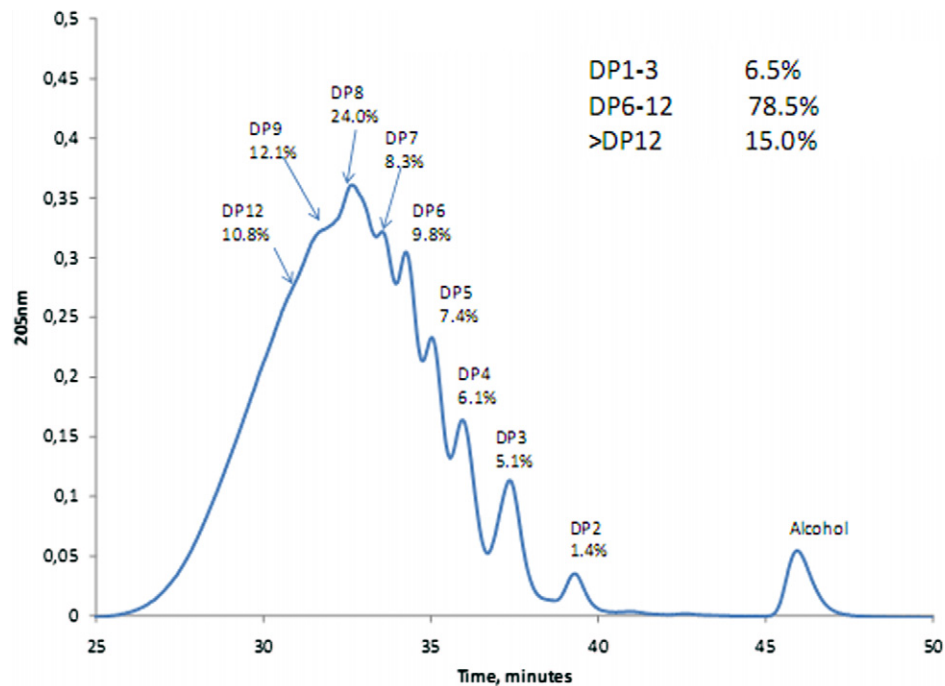

Fig. 1. HPLC characterization of ChOS. Distribution and quantity of different degree of polymerization oligomers was determined with HPLC using a TSK-Oligo column.

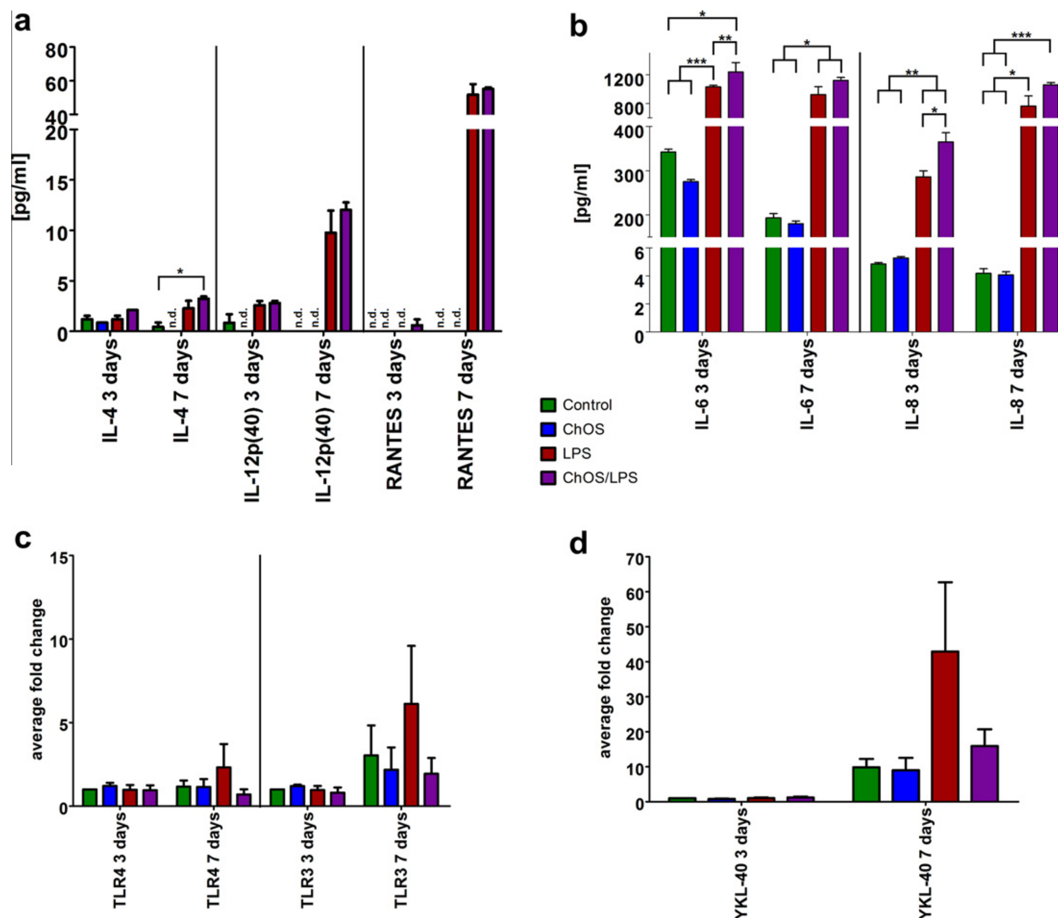

Fig. 2. Short-term effect of LPS and ChOS on hMSC. (a and b) Cytokine secretion of IL-4, IL-12p(40), RANTES, IL-6 and IL-8 after 3 and 7 days in expansion. A significant increase in IL-6 and IL-8 secretion was observed in the presence of endotoxins, as well as the induction of RANTES and IL-12p(40) secretion. Error bars are standard errors ($n = 2$) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). (c and d) Expression of *YKL-40*, *TLR4* and *TLR 3* after 3 and 7 days of expansion. A time-dependent increase in expression can be observed for *YKL-40* independent of the presence of endotoxins. Error bars are standard errors ($n = 6$).

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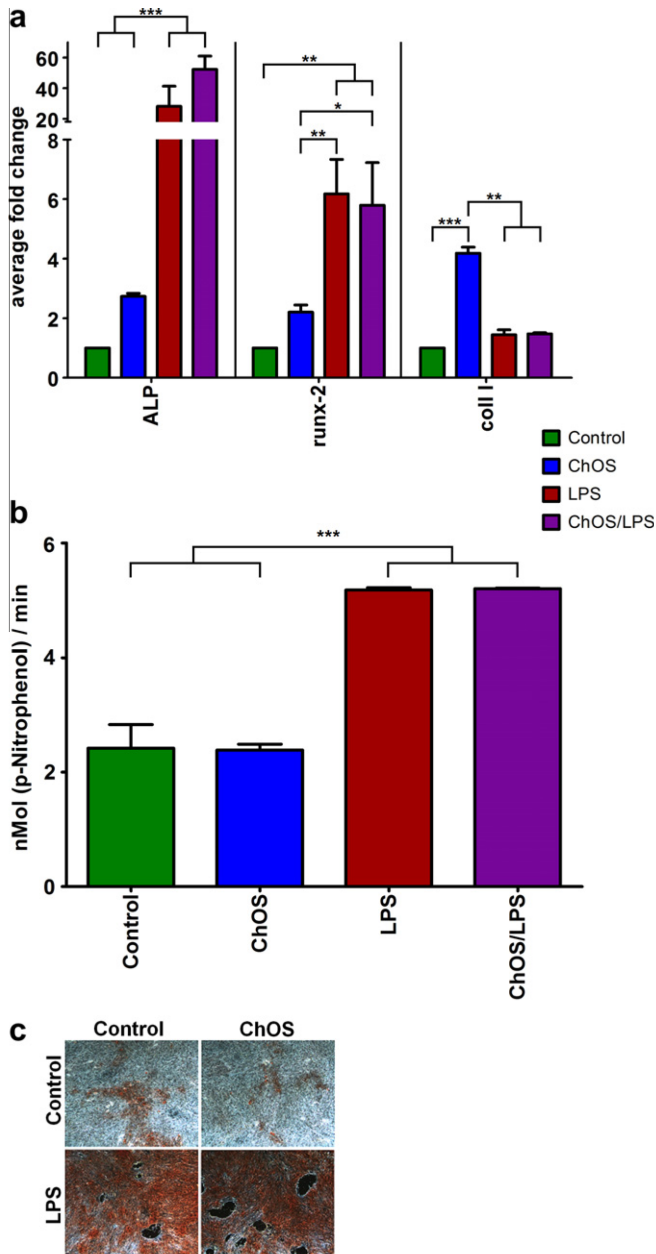

Fig. 3. Quality of osteogenic differentiation in hMSC. (a) Expression of alkaline phosphatase (*ALP*), runt-related transcription factor 2 (*RUNX-2*) and collagen type I (*COL1A2*) after 21 days of osteogenic differentiation. *ALP* and *RUNX-2* were significantly increased in the presence of endotoxins, whereas chitooligosaccharides alone but not in combination with LPS were able to significantly induce expression of *COL1A2*. Error bars are standard errors ($n = 6$) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$); (b) alkaline phosphatase activity at 18 days during osteogenesis. Alkaline phosphatase activity was significantly induced in the presence of endotoxins. Error bars are standard errors ($n = 9$) ($^{***}p < 0.001$); c, Alizarin Red staining of calcification at 21 days of osteogenesis. Red staining is specific for calcium deposition (a representative picture of three donors).

The increase in the expression of osteogenic marker genes is highly correlated with the increase in gene expression of *YKL-40* and *TLR3*, as demonstrated by the heatmap in Fig. 4b (also see Fig. 4a). Since *YKL-40* has been suggested to play a role in the remodeling of the extracellular matrix [12], the correlation of this gene with two osteogenic marker genes (*ALP* and *RUNX-2*) is not unexpected. The increase in *YKL-40* gene expression is furthermore consistent with increased secretion of YKL-40 protein into the cell culture medium (Fig. 4c).

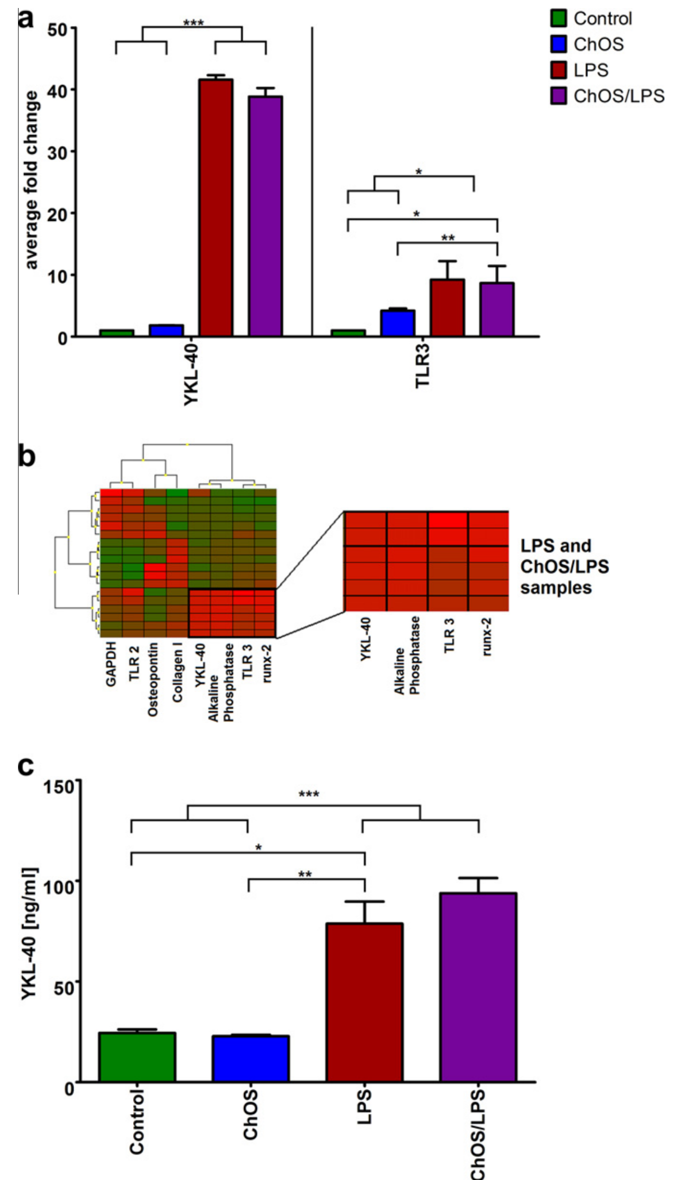

Fig. 4. Long-term effects of LPS and ChOS. (a) Expression of *YKL-40* and *TLR3*. Both genes were significantly increased in the presence of endotoxins. Error bars are standard errors ($n = 6$) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$); (b) heatmap and hierarchical clustering of gene expression data autoscaled to genes. High expression of *YKL-40* is correlated with the expression of the osteogenic genes alkaline phosphatase (*ALP*) and *RUNX-2*, as well as Toll-like-receptor 3 (*TLR3*). A red color indicates high expression and green ndicates low expression ($n = 6$); (c) YKL-40 protein secretion during osteogenic differentiation of hMSC. Secretion of YKL-40 protein into medium supernatants was significantly increased in the presence of endotoxins. Error bars are standard errors ($n = 6$) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

3.4. Effect of LPS on cytokine secretion during osteogenesis

As observed during the expansion of hMSC, the presence of 10 ng ml^{-1} endotoxins during osteogenic differentiation cannot be conclusively determined from the quality of osteogenesis, i.e. gene expression, ALP activity and mineralization, but requires the analysis of the cytokine secretion profile. With 10 ng ml^{-1} LPS, the secretion of IL-4 ($p < 0.001$), IL-12p(40) ($p < 0.001$) and the pro-inflammatory cytokine TNF-α ($p < 0.001$) is up-regulated as compared to the control samples and ChOS alone (Fig. 5a). Again, RANTES was only induced in endotoxin-spiked samples. In

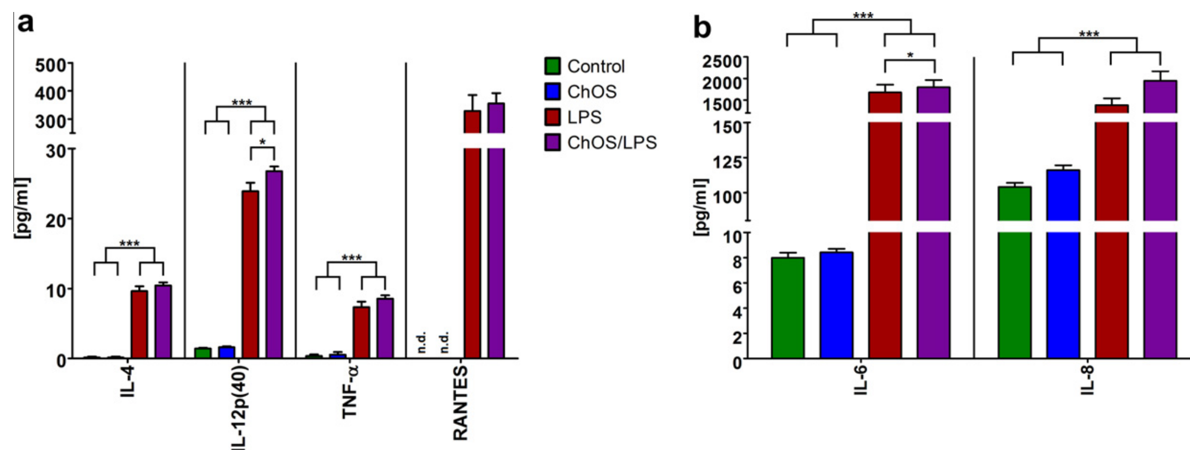

Fig. 5. Effect of endotoxins on cytokine secretion during osteogenesis. (a and b) Secretion of IL-4, IL-12p(40), TNF-a, RANTES, IL-6 and IL-8 at 20 days of osteogenic differentiation. Previously mentioned cytokines were significantly increased in the presence of endotoxins. RANTES secretion was only induced in samples containing LPS. Error bars are standard errors ($n = 6$) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

addition, a substantial increase ($p < 0.001$) in the secretion of IL-6 and IL-8, corresponding to the results obtained during short-term culturing, was observed (Fig. 5b).

4. Discussion

Here, we report the effects of 10 ng ml^{-1} endotoxin contamination on the in vitro evaluation of the bioactivity of chitosan derivatives and how the presence of endotoxins can modify the results of in vitro bioactivity testing, in general leading to false-positive results. During standard evaluation of bioactivity, i.e. proliferation, morphology and gene expression of selected genes, the presence of endotoxins can escape notice unless cytokine secretion patterns are analyzed.

After 3 days in culture, the presence of LPS induced a significant increase in the secretion of inflammatory cytokines in hMSC. The activation of the innate immune system through interaction of ligands with TLRs induces a signaling cascade that results in the secretion of cytokines and chemokines, such as IL-6, IL-8, TNF-α and IFN-γ [21]. The increase in expression of IL-6 and IL-8 is an important part of the host defense in regulating inflammatory responses, and can directly affect bone metabolism [22]. The secretion of inflammatory cytokines can in turn activate the adaptive immune system, including the mobilization of antigen-presenting cells, the maturation of dendritic cells and the differentiation of naive T cells into activated T-helper 1 cells [23,24]. The activation of TLRs creates an inflammatory environment that stimulates cells of the adaptive immune system to migrate to the site of inflammation and remove the invading pathogens [23]. Mesenchymal stem cells (MSC) are known to secrete factors that can alter immune responses [25]. Several studies have shown that MSC suppress T- and B-cell activation, and inhibit the action of antigen-presenting cells as well as dendritic cell maturation [26]. TLRs 1–7 are expressed in MSC in vitro, and triggering of these receptors can alter migration and immunomodulatory functions of these cells [27].

Among the cytokines induced after challenge with LPS, IL-8 and TNF-α are known to be up-regulated in the T-helper 1 cell response, whereas IL-4 and IL-6 are associated with T-helper 2 cell responses [28]. The proinflammatory cytokines TNF-α, IL-6 and IL-8 are expected to decrease wound healing by increasing cellular activation and chemotaxis [28]. IL-12p(40) is a potent regulator of cell-mediated immune responses and activates natural killer cells and T cells [29]. RANTES is known as a chemoattractant to monocytes, memory T cells and basophils, and has been shown to be increased in patients suffering from sepsis. This cytokine was identified as a key player in the uncontrolled expression of proinflammatory cytokines during inflammation, which is further supported by the notion that RANTES is rarely expressed in normal adult tissues but can be found at sites of inflammation [29–31].

TLRs were established as important parts of innate immunity, providing a link between innate and adaptive immune system activation [21]. The conserved structure of these receptors aids in the pattern recognition of conserved motifs in the make-up of invading microorganisms, and TLRs have been linked to epithelial homeostasis by inducing proliferation and tissue repair after injury [25]. We determined the effect of LPS on the expression of its direct receptor counterpart TLR4 and the closely related receptor TLR3, which is believed to be important in facilitating stress responses in hMSC [27]. TLR3 not only reacts to its known ligand, poly(I:C), but can also respond to ligands that bind to closely related TLRs [11]. In the present study, small amounts of LPS did not affect the expression levels of genes in hMSC previously shown to be sensitive to the presence of endotoxins.

With the aid of a heatmap based on hierarchical clustering, the correlation between gene expression and the presence of LPS was visualized [32]. The increase in the expression of the osteogenic marker genes *ALP* and *RUNX-2* is highly correlated to the gene expression levels of *YKL-40* and *TLR3*. *RUNX-2* is one of the key players involved in osteogenic differentiation [33]. It can be detected throughout osteogenic and chondrogenic differentiation, and is essential for the expression of downstream transcription factors [34]. Even though *RUNX-2* is not specific for osteogenic differentiation, it maintains a pool of undifferentiated osteoprogenitor cells and induces the expression of osteogenesis-related genes like *OPN* (osteopontin), *OCN* (osteocalcin) and *COL1A2* [35,36]. An early event during osteogenic differentiation of hMSC is the reduction of proliferative abilities, accompanied by increased expression of the early marker gene *ALP* (alkaline phosphatase) [37].

A large subset of signaling molecules and receptors expressed during bone turnover are shared with the immune system [38]. This is caused, in part, by the fact that osteoclasts, the bone resorbing cells, and immune cells are both derived from the hematopoietic lineage [39]. Osteoclasts can react to cytokines produced by macrophages and other immune cells during inflammation, and can activate bone remodeling pathways in vivo [40].

The expression of the osteogenic marker genes *ALP* and *RUNX-2* was not affected by ChOS alone, whereas the expression levels of *COL1A2* were significantly increased. The presence of LPS completely abrogated this effect on *COL1A2* gene expression. The up-regulation of *COL1A2* expression in the presence of ChOS alone might be explained by the heparin binding site found in the collagen type I protein. Heparin is a glycosaminoglycan with a variable

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structure, but one of the typical building blocks is the amino sugar D-N-acetylglucosamine [41]. This amino sugar comprises approximately 40% of the ChOS mixture used in this study and might be able to interact with the heparin binding site on the collagen type I protein. This effect is not observed when ChOS is used in combination with LPS. One possible explanation for this might lie in the direct interaction between the two compounds. Chitosan was shown to form stable complexes with LPS, based on the formation of hydrogen bonds and electrostatic interactions [7]. Water-soluble derivatives with a high degree of deacetylation, such as the ChOS used in this study, are expected to interact with the lipid A moiety of endotoxins and bind with high affinity [7]. A high degree of interaction of the chitosan derivative with endotoxins could sterically hinder possible binding sites of ChOS in the cell and might therefore be responsible for the abrogation of increased *COL1A2* expression. However, the effect of the endotoxin itself on cellular functions should not be affected by this interaction. Even under optimal interaction conditions, approximately 30% of the endotoxin will be unbound and able to interact with cellular receptors [42].

Chitosan is considered to be a promising candidate for applications in regenerative medicine and tissue engineering, partly due to recent reports on the positive influence of chitosan on osteogenesis in vitro and in vivo [3,4]. Lahiji et al. [43] showed that human osteoblasts grown on chitosan membranes with a 90% degree of deacetylation sustained a spherical morphology as compared to spindle-shaped cells on tissue culture plastic, and preserved collagen type I expression during short-term culture. Similarly, Amaral et al. [5] demonstrated that chitosan membranes could promote the differentiation of osteoprogenitor cells and aid in bone formation. Neither study reported endotoxin testing of the chitosan starting material and both focused on the use of chitosan as a biological growth substrate for cells in the form of membranes and bioactive coatings. ChOS, as used in the present study, are low-molecular-weight derivatives of chitosan that has undergone regioselective enzymatic degradation. Since the biological potential strongly depends on chemical properties, differences in bioactivity between chitosan and ChOS can be assumed [44]. Additionally, we have recently shown that pure, endotoxin-free hexamer fractions of chitosan and chitin oligomers increase the expression of *TLR3*, *RUNX-2* and *COL1A2* during osteogenic differentiation of hMSC, but do not affect the mineralization process [45]. Mass production of pure oligomer fractions with a well-defined degree of polymerization is laborious and expensive, which is why many studies focus on the use of ChOS mixtures to evaluate bioactivity [46,47]. This introduces a higher level of complexity despite advanced analysis methods, since both active and inhibitory oligomer fractions might be present simultaneously, affecting the biological activity [48].

The presence of LPS resulted in increased osteogenic marker gene expression, elevated ALP activity and increased deposition of calcium hydroxyapatite crystals. Improvement of osteogenic differentiation in MSC in the presence of LPS has been described in several studies and was shown to be coupled with increased activation of the ERK pathway and dependent on the duration of endotoxin exposure [49,50]. The main event connecting the presence of endotoxins to bone biology is the induction of proinflammatory cytokines such as IL-6 after ligation with TLR4. IL-6 was tightly linked to osteoclast-induced bone resorption [22,51]. This also explains why an increase in osteogenic differentiation of hMSC can be observed in vitro, whereas increased rates of bone-turnover are induced in vivo or in co-culture with preosteoclasts.

5. Conclusion

We have shown that the presence of endotoxins in chitosan derivatives can profoundly alter the performance of a potential biomaterial in vitro. Endotoxin-contaminated ChOS will improve osteogenic differentiation and deposition of calcium hydroxyapatite crystals without affecting cell viability of hMSC. This positive effect on osteogenesis is abrogated after appropriate endotoxin removal.

Endotoxin contamination is difficult to avoid completely during production and handling of natural substances. With the proper testing and handling of starting materials derived from natural materials and the evaluation of cytokine secretion patterns, such materials can be useful tools in biomaterials research. Spiking samples with known amounts of endotoxins can be beneficial, as well as testing bioactivity in the presence of endotoxin inhibitors such as polymyxin B [52]. The inclusion of simple quality control procedures to standard evaluation protocols of biomaterials will reduce the potential discrepancies between in vitro and in vivo studies, where a formerly bioactive and successful biomaterial has reduced bioactivity because of the strict rules for material safety in clinical practice.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1–5, are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi: http://dx.doi.org/10.1016/j.actbio.2012.08.043.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2012.08.043.

References

[1] Goodman SB, Gómez Barrena E, Takagi M, Konttinen YT. Biocompatibility of total joint replacements: a review. J Biomed Mater Res A 2009;90:603–18.
[2] Bumgardner JD, Wiser R, Elder SH, Jouett R, Yang Y, Ong JL. Contact angle, protein adsorption and osteoblast precursor cell attachment to chitosan coatings bonded to titanium. J Biomater Sci Polym Ed 2003;14:1401–9.
[3] Kurita K. Chitin and chitosan: functional biopolymers from marine crustaceans. Mar Biotechnol (NY) 2006;8:203–26.
[4] Di Martino A, Sittinger M, Risbud MV. Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. Biomaterials 2005;26:5983–90.
[5] Amaral IF, Lamghari M, Sousa SR, Sampaio P, Barbosa MA. Rat bone marrow stromal cell osteogenic differentiation and fibronectin adsorption on chitosan membranes: the effect of the degree of acetylation. J Biomed Mater Res A 2005;75:387–97.
[6] Ermak IM, Davydova VN, Gorbach VI, Berdyshev EL, Kuznetsova TA, Ivanushko IA, et al. Modification of biological activity of lipopolysaccharide in the complex with chitosan. Bull Exp Biol Med 2004;137:379–81.
[7] Naberezhnykh GA, Gorbach VI, Likhatskaya GN, Davidova VN, Solov'eva TF. Interaction of chitosans and their N-acylated derivatives with lipopolysaccharide of Gram-negative bacteria. Biochemistry (Mosc) 2008;73:432–41.
[8] Lamb Machado R, de Arruda EJ, Costapinto Santana C, Alves Bueno SM. Evaluation of a chitosan membrane for removal of endotoxin from human IgG solutions. Process Biochem 2006;41:2252–7.
[9] Beutler B. Endotoxin, Toll-like receptor 4, and the afferent limb of innate immunity. Curr Opin Microbiol 2000;3:23–8.
[10] Gorbet MB, Sefton MV. Endotoxin: the uninvited guest. Biomaterials 2005;26:6811–7.

[11] Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;4:499–511.

[12] Bleau G, Massicotte F, Merlen Y, Boisvert C. Mammalian chitinase-like proteins. EXS 1999;87:211–21.

[13] Lee CG. Chitin, chitinases and chitinase-like proteins in allergic inflammation and tissue remodeling. Yonsei Med J 2009;50:22–30.

[14] Ling H, Recklies AD. The chitinase 3-like protein human cartilage glycoprotein 39 inhibits cellular responses to the inflammatory cytokines interleukin-1 and tumour necrosis factor-alpha. Biochem J 2004;380:651–9.

[15] Haglund L, Bernier SM, Onnerfjord P, Recklies AD. Proteomic analysis of the LPS-induced stress response in rat chondrocytes reveals induction of innate immune response components in articular cartilage. Matrix Biol 2008;27:107–18.

[16] Recklies AD, Ling H, White C, Bernier SM. Inflammatory cytokines induce production of CHI3L1 by articular chondrocytes. J Biol Chem 2005;280:41213–21.

[17] Yoon HJ, Moon ME, Park HS, Im SY, Kim YH. Chitosan oligosaccharide (cos) inhibits LPS-induced inflammatory effects in RAW 264.7 macrophage cells. Biochem Biophys Res Commun 2007;358:954–9.

[18] Yermak IM, Davidova VN, Gorbach VI, Luk'yanov PA, Solov'eva TF, Ulmer AJ, et al. Forming and immunological properties of some lipopolysaccharide–chitosan complexes. Biochimie 2006;88:23–30.

[19] Lieder R, Reynisdóttir ST, Thormódsson F, Ng C-H, Einarsson JM, Gíslason J, et al. Glucosamine increases the expression of YKL-40 and osteogenic marker genes in hmsc during osteogenic differentiation. Nat Prod Bioprospect 2012;2:87–91.

[20] Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, et al. Repair of large bone defects with the use of autologous bone marrow stromal cells. New Engl J Med 2001;344:385–6.

[21] Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. Microbes Infect 2004;6:1382–7.

[22] Sammons J, Ahmed N, El-Sheemy M, Hassan HT. The role of BMP-6, IL-6, and BMP-4 in mesenchymal stem cell-dependent bone development: effects on osteoblastic differentiation induced by parathyroid hormone and vitamin D(3). Stem Cells Dev 2004;13:273–80.

[23] Roeder A, Kirschning CJ, Rupec RA, Schaller M, Korting HC. Toll-like receptors and innate antifungal responses. Trends Microbiol 2004;12:44–9.

[24] Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol 2001;1:135–45.

[25] Liotta F, Angeli R, Cosmi L, Filì L, Manuelli C, Frosali F, et al. Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing notch signaling. Stem cells 2008;26:279–89.

[26] Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815–22.

[27] Tomchuck SL, Zwezdaryk KJ, Coffelt SB, Waterman RS, Danka ES, Scandurro AB. Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. Stem cells 2008;26:99–107.

[28] Brodbeck WG, Nakayama Y, Matsuda T, Colton E, Ziats NP, Anderson JM. Biomaterial surface chemistry dictates adherent monocyte/macrophage cytokine expression in vitro. Cytokine 2002;18:311–9.

[29] Pirog KA, Stabinsky Y, Goldman R. PEPROTECH cytokine index. Rocky Hill, NJ: PeproTech Inc.; 2010.

[30] Ness TL, Carpenter KJ, Ewing JL, Gerard CJ, Hogaboam CM, Kunkel SL. CCR1 and CC chemokine ligand 5 interactions exacerbate innate immune responses during sepsis. J Immunol 2004;173:6938–48.

[31] Volin MV, Shah MR, Tokuhira M, Haines GK, Woods JM, Koch AE. RANTES expression and contribution to monocyte chemotaxis in arthritis. Clin Immunol Immunopathol 1998;89:44–53.

[32] Bergkvist A, Rusnakova V, Sindelka R, Garda JM, Sjögreen B, Lindh D, et al. Gene expression profiling – clusters of possibilities. Methods 2010;50:323–35.

[33] Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 2002;108:17–29.

[34] Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. Nature 2003;423:349–55.

[35] Komori T. Regulation of osteoblast differentiation by transcription factors. J Cell Biochem 2006;99:1233–9.

[36] Mackie EJ. Osteoblasts: novel roles in orchestration of skeletal architecture. Int J Biochem Cell Biol 2003;35:1301–5.

[37] Thomas GP, Baker SU, Eisman JA, Gardiner EM. Changing RANKL/OPG mRNA expression in differentiating murine primary osteoblasts. J Endocrinol 2001;170:451–60.

[38] Takayanagi H. Mechanistic insight into osteoclast differentiation in osteoimmunology. J Mol Med (Berl) 2005;83:170–9.

[39] Arron JR, Choi Y. Bone versus immune system. Nature 2000;408:535–6.

[40] Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 1999;20:345–57.

[41] Sweeney SM, Guy CA, Fields GB, San Antonio JD. Defining the domains of type I collagen involved in heparin-binding and endothelial tube formation. Proc Natl Acad Sci USA 1998;95:7275–80.

[42] Davydova VN, Yermak IM, Gorbach VI, Krasikova IN, Solov'eva TF. Interaction of bacterial endotoxins with chitosan. Effect of endotoxin structure, chitosan molecular mass, and ionic strength of the solution on the formation of the complex. Biochemistry (Mosc) 2000;65:1082–90.

[43] Lahiji A, Sohrabi A, Hungerford DS, Frondoza CG. Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. J Biomed Mater Res 2000;51:586–95.

[44] Xiong C, Wu H, Wei P, Pan M, Tuo Y, Kusakabe I, et al. Potent angiogenic inhibition effects of deacetylated chitohexaose separated from chitooligosaccharides and its mechanism of action in vitro. Carbohydr Res 2009;344:1975–83.

[45] Lieder R, Thormodsson F, Ng CH, Einarsson JM, Gislason J, Petersen PH, et al. Chitosan and chitin hexamers affect expansion and differentiation of mesenchymal stem cells differently. Int J Biol Macromol 2012;51:675–80.

[46] Qin C, Gao J, Wang L, Zeng L, Liu Y. Safety evaluation of short-term exposure to chitooligomers from enzymic preparation. Food Chem Toxicol 2006;44:855–61.

[47] Wu GJ, Tsai GJ. Chitooligosaccharides in combination with interferon-gamma increase nitric oxide production via nuclear factor-kappaB activation in murine RAW264.7 macrophages. Food Chem Toxicol 2007;45:250–8.

[48] Aam BB, Heggset EB, Norberg AL, Sørlie M, Vårum KM, Eijsink VG. Production of chitooligosaccharides and their potential applications in medicine. Mar Drugs 2010;8:1482–517.

[49] Hwa Cho H, Bae YC, Jung JS. Role of Toll-like receptors on human adipose-derived stromal cells. Stem Cells 2006;24:2744–52.

[50] Lombardo E, DelaRosa O, Mancheño-Corvo P, Menta R, Ramírez C, Büscher D. Toll-like receptor-mediated signaling in human adipose-derived stem cells: implications for immunogenicity and immunosuppressive potential. Tissue Eng Part A 2009;15:1579–89.

[51] Kikuchi T, Matsuguchi T, Tsuboi N, Mitani A, Tanaka S, Matsuoka M, et al. Gene expression of osteoclast differentiation factor is induced by lipopolysaccharide in mouse osteoblasts via Toll-like receptors. J Immunol 2001;166:3574–9.

[52] Magalhães PO, Lopes AM, Mazzola PG, Rangel-Yagui C, Penna TC, Pessoa A. Methods of endotoxin removal from biological preparations: a review. J Pharm Pharm Sci 2007;10:388–404.

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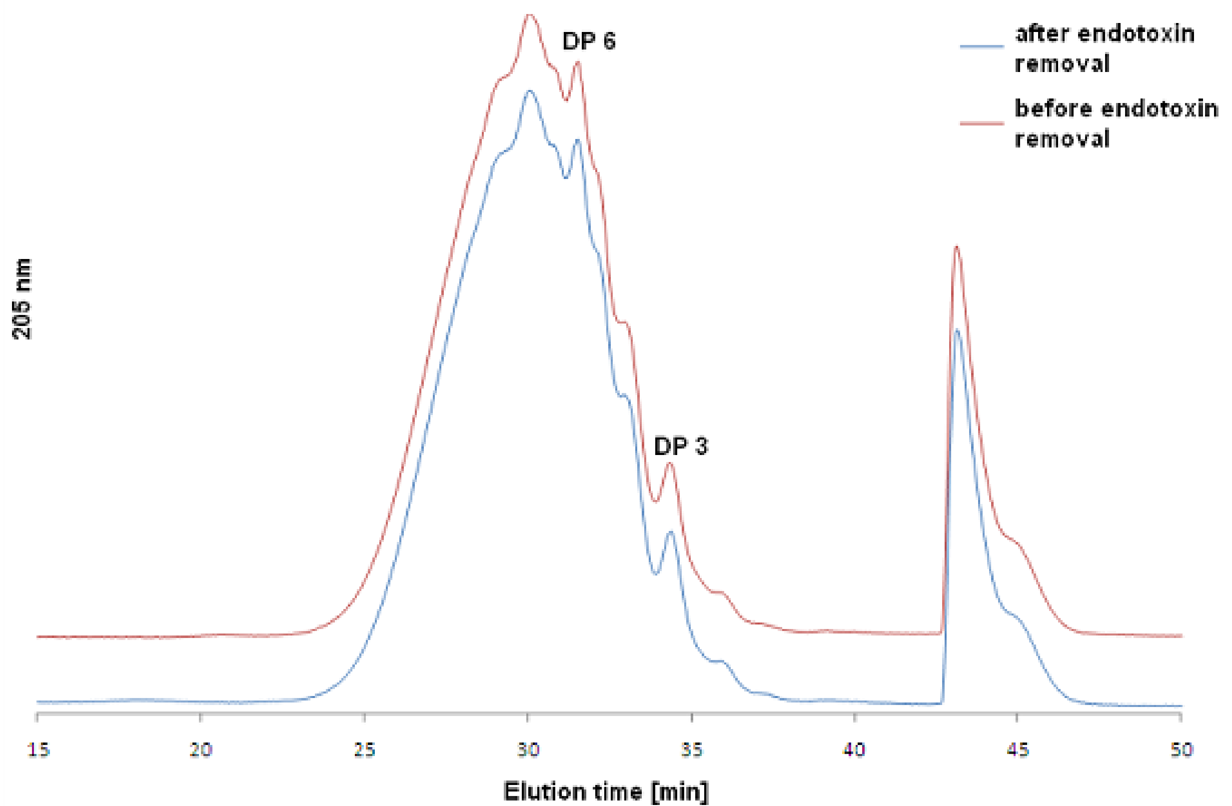
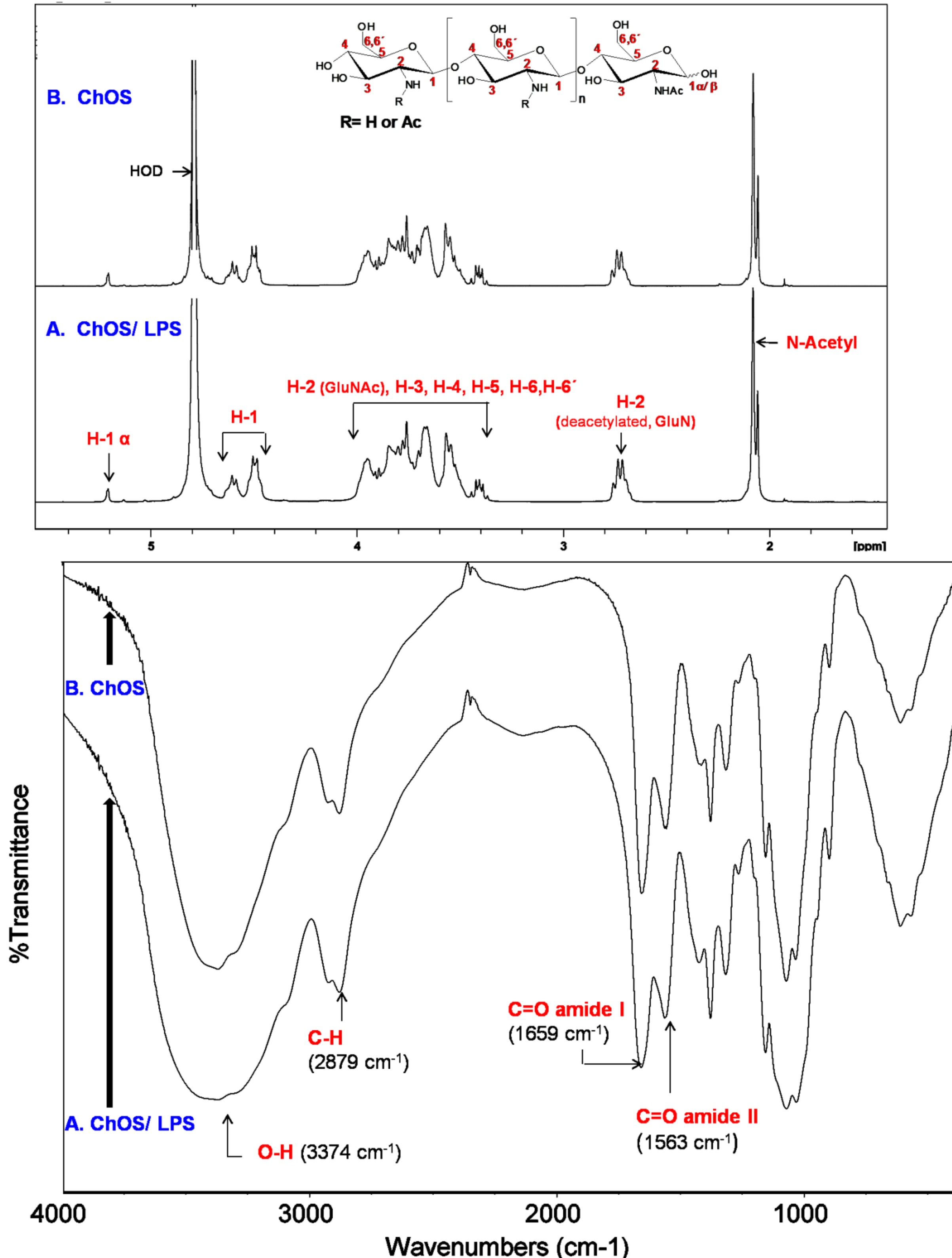
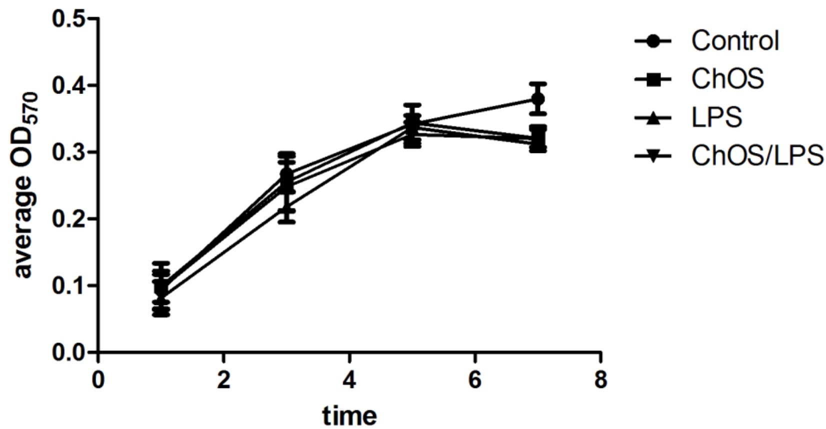
Supplementary Figures



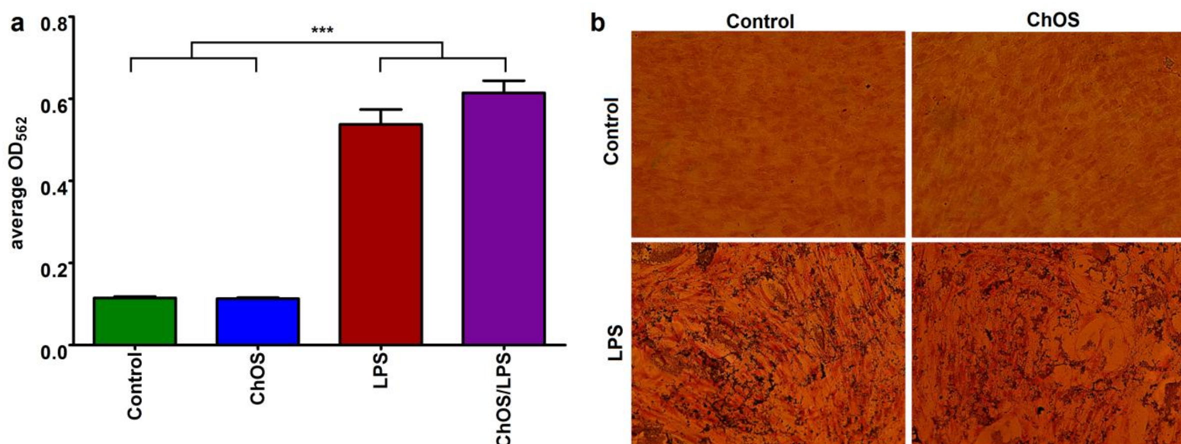
Supplementary Fig. 1. HPLC characterization of endotoxin removal procedures on the degree of polymerization of chitooligosaccharides. Distribution and quantity of different degree of polymerization oligomers was determined with HPLC using TSK-Oligo column. DP = degree of polymerization. No effect on the distribution and quantity of different DP oligomers could be accounted to the endotoxin removal treatment.



Supplementary Fig. 2. ^{1}H-NMR analysis and FT-IR analysis of oligosaccharides. ^{1}H-NMR analysis overlay of endotoxin cleaned chitooligosaccharides (ChOS) and chitooligosaccharides with LPS (ChOS/LPS). Measurements were performed at 400.14 MHz at 298°K without water suppression. D_2O or D_2O/DCl were used as solvents; IR measurements were performed with 2-5 mg of samples that were thoroughly mixed with KBr and then pressed into pellets.



Supplementary Fig. 3. Proliferation of hMSC during expansion. Proliferation was determined using the MTT proliferation assay at days 1, 3, 5 and 7 in three independent donors. No effect of chitooligosaccharides or LPS either alone or in combination was observed. Error bars are standard error (n=15).



Supplementary Fig. 4. Validation of successful osteogenic differentiation in hMSC. a, Alizarin Red Quantitation with cetyl-pyridinium chloride. Significant accumulation of Alizarin Red Stain was observed in the presence of endotoxins. Error bars are standard error (n=9) (***$p<0.001$); **b,** Von Kossa staining of mineral deposition during osteogenic differentiation. Brown color depicts mineralization. Pictures are representative from three donors.

	(ChOS endotoxin removed) ChOS	(ChOS before endotoxin removal) ChOS + LPS
^{1}H-NMR	(400 MHz, D_2O): δ 2.08 (s, CH_3C=O), 2.67-2.76 (m, H-2 GluN), 3.37–3.99 (m, H-2 GluNAc, H-3, H-4, H-5, H-6, H-6´), 4.46-4.64 (m, H-1), 5.21 (weak s, H-1 α) ppm. ^{1}H NMR (400 MHz, D_2O/ DCl): δ 2.09 (s, CH_3C=O), 3.14-3.23 (m, H-2 GluN), 3.46–3.96 (m, H-2 GluNAc, H-3, H-4, H-5, H-6, H-6´), 4.61-4.96 (m, H-1), 5.21 (weak s, H-1 α) ppm.	(400 MHz, D_2O): δ 2.08 (s, CH_3C=O), 2.67-2.76 (m, H-2 GluN), 3.37–3.99 (m, H-2 GluNAc, H-3, H-4, H-5, H-6, H-6´), 4.46-4.64 (m, H-1), 5.21 (weak s, H-1 α) ppm. ^{1}H NMR (400 MHz, D_2O/ DCl): δ 2.09 (s, CH_3C=O), 3.14-3.23 (m, H-2 GluN), 3.46–3.96 (m, H-2 GluNAc, H-3, H-4, H-5, H-6, H-6´), 4.61-4.96 (m, H-1), 5.21 (weak s, H-1 α) ppm.
FT-IR	v 3369 (br, OH), 2879 (m, C–H), 1657 (vs, C=O amide I),1557 (vs, C=O amide II) cm^{-1}.	v 3374 (br, OH), 2879 (m, C–H), 1659 (vs, C=O amide I),1563 (vs, C=O amide II) cm^{-1}.

Supplementary Table 1

Paper IV

"Aerodynamically, the bumblebee shouldn't be able to fly,
But the bumblebee doesn't know it, so it goes on flying anyway."

Mary Kay Ash, American business woman (1918-2001)

Society For Biomaterials

In vitro bioactivity of different degree of deacetylation chitosan, a potential coating material for titanium implants

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Abstract: Clinical treatment of orthopaedic tissue injuries often involves the use of titanium and titanium alloys with considerable research focusing on the surface modification of these materials. Chitosan, the partly deacetylated form of chitin, is one of the materials under investigation as surface coating for orthopaedic implants in order to improve osteointegration and cellular attachment. In this study, we determined the effects of the degree of deacetylation (DD) of chitosan membranes on attachment, proliferation and osteogenic differentiation of MC3T3-E1 mouse preosteoblasts. Chitosan membranes were coated with fibronectin to promote biocompatibility and cellular attachment. Membranes were characterized in terms of wettability and surface topography using water contact angle measurements and atomic force microscopy. The results in this study indicate that the surface roughness and fibronectin adsorption increase with increased DD. A higher DD also facilitates attachment and proliferation of cells, but no induction of spontaneous osteogenic differentiation was observed. Lower DD chitosan membranes were successfully prepared to sustain attachment and were modified by crosslinking with glutaraldehyde to promote long-term studies. The chitosan membranes used in this study are suitable as a potential coating for titanium implants. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2012.

Key Words: Chitosan membrane, degree of deacetylation, MC3T3-E1 mouse preosteoblasts, fibronectin, osteogenic differentiation

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INTRODUCTION

Clinical treatment of orthopaedic tissue injuries often involves the use of titanium or titanium alloys with considerable research focusing on the surface modification of these materials.[1,2] The integration of the implant and its performance at the bone-biomaterial interface crucially determine the progress of the fixation.[3] Successful integration depends on surface characteristics including surface chemistry, topography, charge, and wettability, as well as the mechanical properties of the implant.[3,4] During implantation, damage to the bone environment can lead to the failure of the implant by inducing the formation of a fibrous tissue capsule before osteoblastic cells can attach to the implant surface.[5] Cell-based approaches furthermore increased the desire to coat implants with suitable biodegradable materials to promote cell attachment, proliferation and possibly a guided differentiation into the desired phenotype.[6]

At the bone-biomaterial interface, the surface characteristics determine the initial amount and conformation of proteins adsorbed to the implant surface.[7] This layer of adsorbed proteins constitutes the primary interaction site for the cells, as interaction on that level only occurs at the outermost atomic layer, and critically regulates integrin signalling and the ability of cells to regenerate the damaged tissue.[8,9] Materials under investigation as bioactive coatings for titanium and titanium alloys are the polysaccharide chitosan, starch, and mucopolysaccharides.[10–12] Chitosan can be derived from chitin, a polymer of β-(1→4)-linked-2-acetamido-2-deoxy-D-glucopyranose, which is a structural component in the cell walls of bacteria and fungi, as well as crustaceans and insects.[13,14] Because chitin is the second

Additional Supporting Information may be found in the online version of this article.
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most abundant organic compound in nature, the production of chitosan is economically and ecologically profitable.[13] N-deacetylation of chitin using aqueous alkali yields chitosan with three functional groups that allow for chemical modification to obtain modified chitosan derivatives.[13,15] The key properties of chitosan include that it is nontoxic, biodegradable, and biocompatible.[16] Fungistatic and bacteriostatic effects as well as an influence on tumour growth have been reported.[13] For applications in various systems, chitosan can be pressed into various forms and combined with different biomaterials, thereby offering to be a promising polymer in regenerative medicine, for scaffolds in tissue engineering, gene therapy, and drug delivery systems.[13,17] In addition, chitosan has been shown to have favorable effects on osteogenesis *in vitro* and *in vivo*, by promoting cell attachment and supporting the formation of a natural extracellular matrix, which is critical for bone regeneration.[10] The cationic charge of chitosan can be held responsible for negatively charged cytokines and growth factors to bind to its surface which in turn can induce polymorphonuclear cells to migrate to the implantation site to aid in the tissue regeneration process.[18] Via the amino group, chitosan can be chemically bonded to titanium implants thereby improving cellular attachment over uncoated implants with oligosaccharides as degradation products that can take part in the regeneration of the mineralized bone matrix.[18] Because there is no distinct definition on the degree of deacetylation (DD) that constitutes chitosan but rather a general consideration that less than 50% N-acetyl-glucosamine in the chain is considered to be chitosan, the influence of this property was reported repeatedly to alter cellular behavior with different effects on different cell types.[19,20] A higher DD is generally considered to increase cell attachment and proliferation but a lower DD has the promising ability to induce healing without scar tissue formation.[21,22]

The aim of this study was to investigate the effects of different DD of chitosan in the form of chitosan membranes, as a potential coating for titanium implants. For this purpose, the influence on cellular attachment, proliferation and gene expression of MC3T3-E1 mouse preosteoblastic cells was determined. Membranes were characterized in terms of wettability and surface topography, using water contact angle measurements and atomic force microscopy. Lower DD chitosan membranes were successfully prepared to sustain attachment and were modified by crosslinking with glutaraldehyde to promote long-term cultures.

METHODS AND MATERIALS

Analysis of chitosan powder

DD of chitosan powder was determined using ^{1}H-NMR in Bruker AVANCE 400 (Bruker Biospin GmbH, Karlsruhe, Germany) at 400.14 MHz at 298K. D_2O/DCl (deuterium hydrochloride) or D_2O/TFA (trifluoro acetic acid) was used as solvent and measurements were taken without water suppression. Sample concentration was 10–15 mg/mL.

Preparation of chitosan membranes

Shrimp shell chitosan with a DD of 47, 68, 87, and 94% (DD47, DD68, DD87, and DD94, respectively) (Genis ehf., Reykjavik, Iceland) and crab shell chitosan with a DD of 87% (Sigma DD87) (Sigma Aldrich, St. Louis, MO) were used. A 1% (w/w) chitosan solution in deionized H_2O (dH_2O) with 50 mM 100% acetic acid was prepared and stirred until solution was clear. Chitosan solutions were centrifuged before casting to remove undissolved particles and to ensure homogeneous membrane casting. 0.1 mL chitosan solution/cm^2 was cast into tissue culture treated plates (Falcon) and dried over night in an incubator at 37°C. Chitosan membranes were neutralized with 0.1 M NaOH and sterilized with 70% ethanol. Membranes were additionally sterilized under UV-light and then incubated with a 5 μg/mL fibronectin solution (Gibco, Carlsbad, CA) in sterile PBS for 3 h. Before cell seeding, chitosan membranes were equilibrated in DMEM/F12 media (Gibco) supplemented with penicillin/streptomycin (Invitrogen) and 10% heat-inactivated FBS (Gibco).

For osteogenic differentiation experiments, DD47 and DD68 chitosan membranes were additionally internally crosslinked with 0.02% glutaraldehyde (Sigma) before coating with fibronectin to prevent degradation of the chitosan membranes during long-term experiments.

Water contact angle measurements

Water contact angles were determined using a KSV CAM 200 optical contact angle meter (KSV Instruments) and a droplet volume of 5 μL distilled water. The contact angle measurement was started 10 s after drop down and calculated using the Laplace & Young equation. Measurements were performed at room temperature and ambient humidity.

AFM surface topography

Surface topography of chitosan membranes was evaluated using an XE-100 atomic force microscope (Park Systems) with a scan size of 5 μm and a scan rate of 0.15 Hz in noncontact mode. Two samples were analysed per DD and mean surface roughness determined from eight measurements.

Fibronectin adsorption studies

Chitosan membranes and tissue culture plastic controls were incubated with a 5 μg/mL fibronectin solution in PBS over night at 4°C. Samples were then washed four times with PBS and nonspecific adsorption was blocked with 1% BSA (Sigma) in PBS for 2 h. After washing three times with PBS, samples were incubated with rabbit anti-fibronectin antibody (1:15000) (Sigma) for 2 h. Samples were then washed three times with PBS and incubated with goat anti-rabbit IgG Alkaline Phosphatase-conjugated antibody (1:50000) for 2 h. Samples were then washed three times with PBS and incubated with the substrate p-nitrophenyl phosphate (Sigma) for 30 min. Optical density was measured at 405 nm in a MultiSkan spectrometer (Thermo Scientific, Vantaa, Finland).

Cell cultures

MC3T3-E1 (CRL 2593, subclone 4, ATCC) cells were maintained in α-MEM media (Gibco) supplemented with

penicillin/streptomycin and 10% heat-inactivated FBS. During experiments on chitosan membranes, expansion media was replaced by DMEM/F12 media supplemented with penicillin/streptomycin and 10% heat-inactivated FBS to enhance attachment and proliferation (observation from pilot studies).

To induce osteogenic differentiation, MC3T3-E1 cells were maintained in DMEM/F12 media supplemented with penicillin/streptomycin, 10% heat-inactivated FBS, 2 mM β-glycerophosphate (Sigma) and 0.1 mM ascorbic acid (Sigma). For qPCR and alkaline phosphatase activity assays, 6-well plates and 12-well plates were used respectively, seeded with 4000 cells/cm^2.

Attachment and morphology

Cells were periodically checked in an inverted microscope (Leica DM IRB) for phenotype consistency and morphology. Attachment and morphology was determined in 6-well plates seeded with 5000 cells/cm^2. Images were taken after 24 and 48 h as well as periodically during osteogenic differentiation with IC Capture 2.0 Software. 0.5% crystal violet solution (Sigma Aldrich) in methanol was used to stain cells and images were taken in an inverted microscope with IC Capture 2.0 software.

Proliferation studies

Proliferation was assessed using the MTT proliferation assay (ATCC bioproducts, Boras, Sweden) with the optimal cell density at 3000 cells/well in a 96-well plate. Ten microliters of MTT reagent (ATCC bioproducts) was added and the plate incubated at 37°C in the dark for 4 h. After that, 100 μL of MTT detergent (ATCC bioproducts) was added and the plate incubated at 37°C on a shaker for 3 h in the dark. Colorimetric analysis was performed by measuring optical density at 570 and 650 nm in a MultiSkan spectrometer (Thermo Scientific). Measurements were corrected by the blank (tissue culture plastic without cells) and the values from the OD_{650}. Chitosan membranes were clear and did not affect the colorimetric measurement.

Gene expression

RNA isolation was performed using Qiagen BioRobot workstation (Qiagen, Hilden, Germany) and the EZ-1 RNA Cell Mini Kit (Qiagen), following manufacturer's instructions. Samples from osteogenic differentiation experiments were homogenized in a FastPrep instrument using Lysing Matrix D tubes (MP Biomedicals) before RNA isolation. High-Capacity cDNA Reverse Transcription Kit was purchased from Applied Biosystems (Foster City, CA) and was used in all experiments. A master mix was prepared, containing 2.0 μL 10x RT buffer, 0.8 μL 25xdNTP (100 mM), 2.0 μL 10x Random Primers RT, 1.0 μL Multiscribe Reverse Transcriptase, 1.0 μL RNase Inhibitor and 3.2 μL Nuclease free H_2O per sample. Ten microliters of the master mix and 10 μL of RNA samples were mixed and then transferred to a Thermal Cycler 2720 (Applied Biosystems). Cycling conditions were as following: 25°C for 10 min, 37°C for 120 min, 85°C for 5 s and then cooled down to 4°C.

To quantify gene expression of selected genes, qPCR was performed in a StepOne Real Time PCR System (Applied Biosystems). 10 μL Taqman master mix (Applied Biosystems) with 1 μL of Taqman assay (Applied Biosystems) and 9 μL of 1:10 diluted sample cDNA was prepared for each sample. GAPDH was used as a reference gene. Taqman assays included alkaline phosphatase, runx-2, and osteopontin. Data were analysed in GenEX 5.3.2.13 software (MultiD Analyses, Gothenburg, Sweden) and calculated relative to the control sample at 7 days during osteogenic differentiation.

Alkaline phosphatase activity

Alkaline phosphatase activity assay was performed to determine the quality of osteogenic differentiation with and without osteogenic stimulus after 4 and 7 days. To determine alkaline phosphatase activity, cell lysates were incubated for 30 min in a solution of 1 mg/mL pNPP (p-Nitrophenyl phosphate) and 0.2 M Tris buffer prepared in dH_2O and optical density measured at 400 nm in a MultiSkan spectrometer. Protein content of cell lysates was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) in a microplate design according to manufacturer's instructions. Alkaline phosphatase activity is presented as nMol (p-Nitrophenol)/min*mg/mL protein.

Statistical analysis

Data are presented as mean and standard deviation. Statistical analysis was performed using Prism 5.01 software (GraphPad Software) and for qPCR analysis using GenEx 5.3.2.13 software. To evaluate the effect of different DD chitosan membranes, one-way ANOVA with Tukey's Multiple Comparison Post-Test was used, for the effect of time on the expression of osteogenic genes, *t*-test was used; $p < 0.05$ was considered statistically significant.

RESULTS

Analysis of chitosan powder

Integral values of ^{1}H-NMR peaks were used to evaluate the DD of chitosan material. This was based on the ratio between the integral of the sugar backbone protons H-2, H-3, H-4, H-5, H-6, and H-6' in the range δ3.1–4.0 ppm and the integral of the N-acetyl (CH_3) peak at 2.08. DD was calculated according to following equation:

$$\mathrm{DA}(\%) = ([\mathrm{CH_3}(\text{N-acetyl})]/[\text{H-2} - \text{H-6}']) \times (6/3) \times 100$$

DD was confirmed to be 47, 68, 87, 87, and 94% for DD47, DD68, DD87, Sigma DD87, and DD94, respectively (Supporting Information Figure 1).

Water contact angle measurement

Water contact angle measurements were performed on chitosan membranes of different DD coated with fibronectin (Figure 1). Contact angles for DD47 (68.3 ± 3.6°) and DD68 (70.1 ± 3.5°) were comparable to Sigma DD87 (72.7 ± 2.4°) values, whereas DD87 (78.9 ± 3.9°) and DD94 (77.9 ± 2.2°) contact angle values were higher ($p < 0.05$).

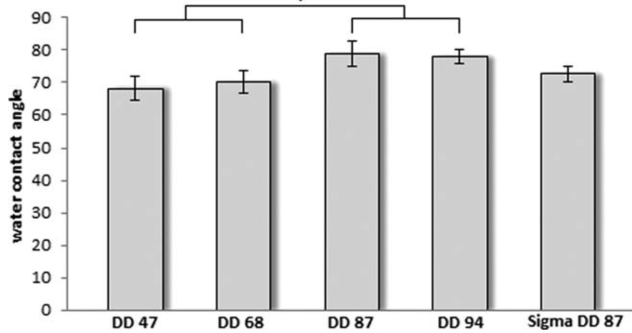

FIGURE 1. Water contact angle measurements on chitosan membranes with different degree of deacetylation coated with fibronectin. $n = 4$ (*$p < 0.05$).

Overall, a trend of increase of the contact angle can be observed from lowest to highest DD.

AFM surface topography

Surface roughness analysis showed, that DD47 (1.49 ± 0.11 nm) to DD87 (1.56 ± 0.16 nm) chitosan membranes displayed a mean surface roughness (mean R_a) similar to the tissue culture plastic control (1.57 ± 0.21 nm) (Figure 2). DD68 (1.63 ± 0.19 nm) and Sigma DD87 (1.73 ± 0.10 nm) membranes were rougher but not statistically different. DD94 displayed a much smoother surface than any other membranes tested with a mean R_a of 0.91 ± 0.07 nm. Even though surface roughness did not vary significantly between membranes and the tissue culture plastic, surface topography showed differences in surface patterns. Tissue culture plastic control displayed long fibres stretching over the whole surface with even distribution whereas chitosan membranes of different DD showed a more valley and hill like structure without fibre formation.

Fibronectin adsorption studies

Highest fibronectin adsorption was observed on DD87 chitosan membranes (0.707 ± 0.055; $p < 0.001$) (Figure 3). Relative adsorption to DD94 (0.583 ± 0.036), and Sigma DD87 chitosan membranes (0.542 ± 0.055) was comparable to the tissue culture plastic control (0.522 ± 0.059; $p =$ not significant). DD68 (0.359 ± 0.027; $p < 0.001$) and DD47 (0.129 ± 0.012; $p < 0.001$) showed decreased fibronectin adsorption as compared to the other chitosan membranes, with DD47 chitosan membranes adsorbing the least amount of fibronectin.

Cell attachment, morphology, and proliferation

Staining of cell bodies with crystal violet after 24 and 48 h showed attachment of MC3T3-E1 cells on all chitosan membranes and the tissue culture plastic control [Figure 4(A)]. Cell layers grew until almost confluent after 48 h and could be sustained for at least 7 days in culture (data not shown). Morphology on DD47 was more round after initial attachment and cells started to grow in star-like structures. After 7 days, cell layers on DD47 chitosan membranes were grown to almost confluency and cells showed the typical spindle-shaped morphology (data not shown). Cells on tissue culture plastic and chitosan membranes, except for DD47, were spindle-shaped after 24 h and a change of cell shape could be observed over a period of 14 days. Cells appeared to be more cuboidal in shape and 100% confluent [Figure 4(A)]. Attachment on lower DD chitosan membranes slightly decreased after 10–14 days and could not be sustained more than 24 days in culture (data not shown).

Highest proliferation was shown in the tissue culture plastic control, followed by tissue culture plastic coated with fibronectin and chitosan membranes DD94, DD87, and Sigma DD87 [Figure 4(B)]. Fibronectin coated tissue culture

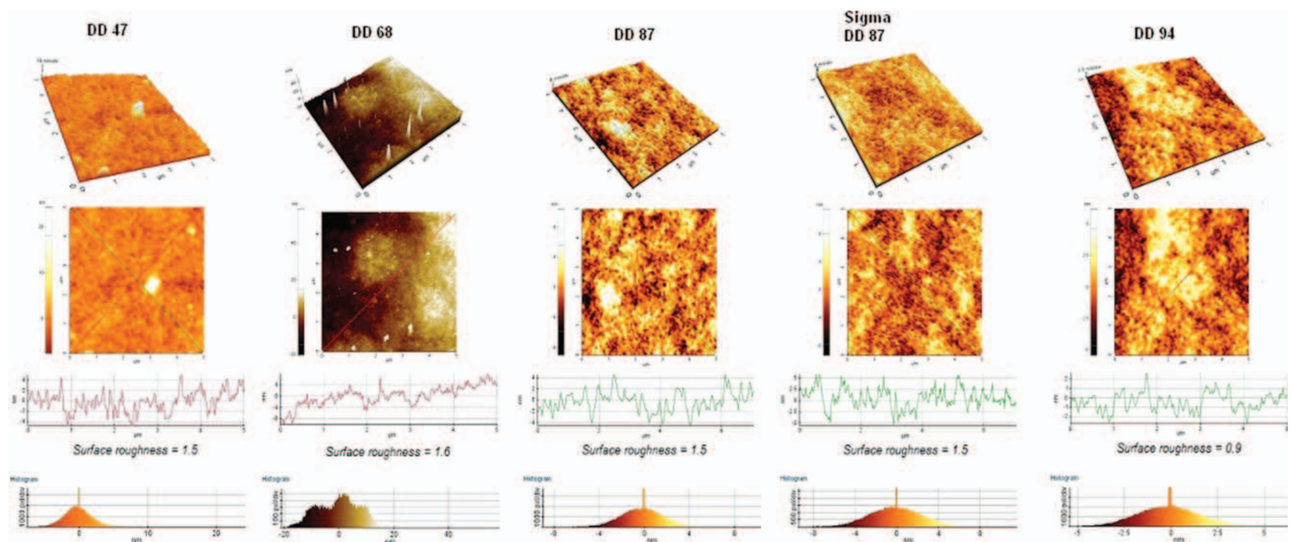

FIGURE 2. Average surface topography of chitosan membranes with different DD. Surface roughness was determined in an XE-100 atomic force microscope with a scan size of 5 μm and a scan rate of 0.15 Hz in noncontact mode. Representative images of each DD chitosan membrane are presented. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

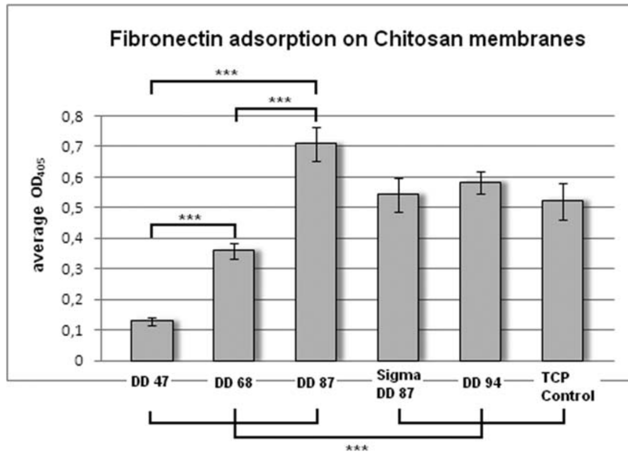

FIGURE 3. Fibronectin adsorption assay on chitosan membranes with different degree of deacetylation. Fibronectin adsorption was detected by ELISA. $n = 8$ (***$p < 0.001$)

plastic lowered the proliferation rate of the cells similar to the values observed with cells grown on chitosan membranes. Proliferation on DD68 membranes was slightly lower, followed by minimal proliferation on DD47 membranes.

Gene expression

qPCR analysis of the osteogenic marker genes alkaline phosphatase, osteopontin and runx-2 showed that fibronectin did not affect the expression of these genes. When no osteogenic stimulus was provided during the culture period, no induction of spontaneous osteogenic differentiation was observed on chitosan membranes (data not shown).

The presence of ascorbic acid and β-glycerophosphate for 7 and 14 days, respectively, did not show any statistical difference in gene expression patterns between the tissue culture plastic control and chitosan membranes. Fibronectin coating could be excluded to affect osteogenic gene expression. Chitosan membranes of different DD did not negatively

FIGURE 4. (A) Attachment of mouse preosteoblastic MC3T3-E1 cells on chitosan membranes with different DD coated with fibronectin. Cells were stained with crystal violet solution and pictures taken after 24 and 48 h. Osteogenic differentiation of MC3T3-E1 mouse preosteoblasts for 14 days; pictures were taken in an inverted microscope. $n = 5$ (B) Proliferation of mouse preosteoblastic MC3T3-E1 cells grown on chitosan membranes with different DD coated with fibronectin for 1, 3, 5, and 7 days. $n = 30$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

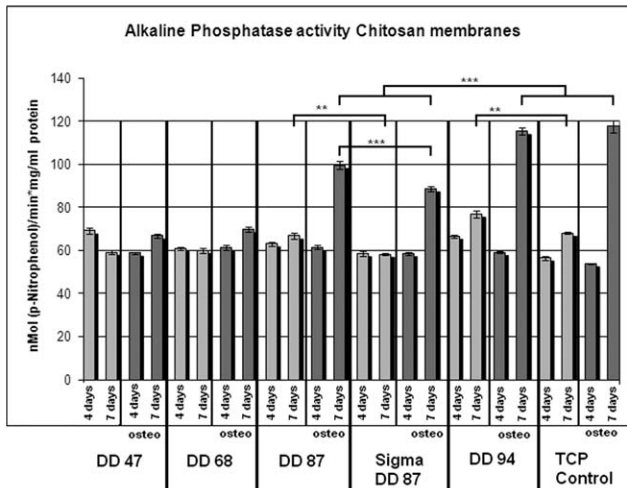

FIGURE 5. Alkaline phosphatase activity of mouse preosteoblastic MC3T3-E1 cells grown on chitosan membranes with different DD for 4 and 7 days, with or without osteogenic stimulus. $n = 2$ (** $p < 0.01$; *** $p < 0.001$).

affect osteogenic differentiation (Supporting Information Figure 2).

Alkaline phosphatase activity

Alkaline phosphatase activity assay was performed to determine the quality of osteogenic differentiation with and without osteogenic stimulus after 4 and 7 days (Figure 5). At day 4, no difference in the activity of alkaline phosphatase was found between the different DD chitosan membranes and the tissue culture plastic control, both with and without osteogenic stimulus. After 7 days of culture, the activity of alkaline phosphatase was significantly higher ($p < 0.01$) in cultures supplemented with ascorbic acid and β-glycerophosphate than without osteogenic stimulus. DD94 induced significantly higher ($p < 0.01$) alkaline phosphatase activity without osteogenic stimulus than did the tissue culture plastic control. No difference was found in the remaining chitosan membranes as compared to the tissue culture plastic control, when no osteogenic stimulus was present. Highest induction of alkaline phosphatase activity was detected in tissue culture plastic control and DD94 chitosan membranes ($p < 0.001$), followed by DD87 and Sigma DD87 chitosan membranes. There, the induction of DD87 chitosan membranes was significantly higher after seven days in osteogenic media than in Sigma DD87 chitosan membranes ($p < 0.001$). The same trend was observed for the induction of alkaline phosphatase in these membranes without osteogenic stimulus ($p < 0.01$).

DISCUSSION

In this study, the effect of a wide range of DD chitosan membranes, as potential coating for titanium implants, was evaluated in terms of surface characteristics and bioactivity. Surface roughness was shown to be comparable to tissue culture plastic, except for DD94 chitosan membranes, which were much smoother. Water contact angle measurements revealed that DD87 and DD94 showed similar contact angle values whereas DD47, DD68, and Sigma DD87 were more hydrophilic. Despite the difference in water contact angles, the ability of high DD chitosan membranes (DD87, DD94, and Sigma DD87) to adsorb fibronectin was similar. The lower the contact angle and the more acetylated the membrane, the less likely fibronectin is supposed to be retained at the surface to promote attachment and proliferation of cells. This observation could be explained by the number of amine groups available for interaction with fibronectin and should be addressed in further research by the determination of the number of amine groups as a function of the chitosan deacetylation degree. DD47 and DD68 chitosan membranes showed low adsorption of fibronectin, whereas Sigma DD87 and DD94 could adsorb comparable amounts of fibronectin as the tissue culture plastic control. Highest adsorption of fibronectin was observed for DD87 chitosan membranes, which was significantly higher than the tissue culture plastic control.

During bioactivity studies, it was observed that MC3T3-E1 mouse preosteoblastic cells were able to attach to all DD chitosan membranes and formed a confluent cell layer after 1 week in culture, even though DD47 and DD68 chitosan membranes showed reduced adsorption of fibronectin and lower water contact angle values. Proliferation, as compared to tissue culture plastic controls, was decreased but similar to fibronectin coated controls. Fibronectin coating increases cell attachment, which in turn decreases the ability of the cells to proliferate. Even though proliferation on lower DD membranes was low, a confluent cell layer was obtained after 7 days and attachment could be sustained for at least 24 days in culture.

To determine, whether preosteoblastic cells will undergo spontaneous osteogenic differentiation due to surface characteristics of chitosan membranes, osteogenic genes were analysed for their expression. No statistical difference was found between chitosan membranes and tissue culture plastic controls, except for an indication that some sort of action was taking place, as cells grown on chitosan membranes underwent the morphological changes associated with osteogenic differentiation. Analysis of the activity of alkaline phosphatase, an early enzyme active during osteogenic differentiation, showed that the enzyme was not active in membranes with a lower DD (DD47 and DD68), independent of the presence of an osteogenic stimulus. When cells were cultured in osteogenic media, alkaline phosphatase activity was present in chitosan membranes with a higher DD. Highest activity was observed in the tissue culture plastic control and DD94 chitosan membranes, but the latter also induced significantly higher induction of alkaline phosphatase activity without the presence of an osteogenic stimulus. As this was only a momentary snapshot, upregulation of alkaline phosphatase activity during earlier or later time points on lower DD membranes could not be excluded.

In the presence of ascorbic acid and β-glycerophosphate to induce osteogenic differentiation of MC3T3-E1 preosteoblasts, no negative effect of chitosan membranes of different DD was indicated. No effect could be attributed to

the presence of fibronectin used to increase attachment on chitosan membranes nor to crosslinking with glutaraldehyde in membranes prepared from DD47 and DD68 chitosan. Crosslinking with glutaraldehyde is commonly used in biomaterials research to prolong the stability and improve the properties of biomaterials, that is, degradation, chemical resistance, porosity and mechanic properties.[23,24] In chitosan materials, the use of a low percentage of glutaraldehyde crosslinking has the advantage to enhance the biomineralization abilities of the biomaterial.[23] This is in accordance with our experience in the use of glutaraldehyde in crosslinking processes. Lower degrees of crosslinking improve cell survival and proliferation on the membranes, which might be due to decreased leakage of glutaraldehyde into the cell culture media and less modification of surface characteristics (unpublished observations). The notion of chitosan materials with a DD of around 50% dissolving in aqueous solutions is not new. Better solubility of these materials was attributed to the degradation of secondary structures and the increase in hydrophilicity.[25] Therefore, we crosslinked chitosan membranes with low DD during long-term experiments with low levels of glutaraldehyde, since experiments in stimulated body wet environment showed that crosslinked chitosan formulations are stable for at least 60 days after an initial release of low molecular mass fractions.[26]

Furthermore, the physicochemical characteristics of chitosan cannot only be attributed to the DD, but are also dependent on the natural source of chitin.[27] In this study, two preparations of chitosan with the same DD (87%) were used, one derived from shrimp shells (Genis ehf.) and the other one from crab shells (Sigma Aldrich). Crab shell chitosan served as positive control in this study to evaluate the bioactivity and surface characteristics of different DD chitosan membranes derived from shrimp shells, as it has been successfully used by several researchers. A direct comparison of the physicochemical characteristics of chitosan preparations from different natural sources of chitin revealed a strong dependence on the taxonomy of the chitin source, affecting both the DD and the molecular weight. The preparation of crab shell chitosan with a DD of 87% requires a longer deacetylation process as compared to shrimp shell chitosan, which results in the hydrolysis of the polysaccharide backbone (decrease in molecular weight), thereby ultimately influencing performance during the evaluation of bioactivity.[27]

Fibronectin coating was used in this study to promote cellular attachment to chitosan membranes. The fibronectin layer on the surface, which is the initial interaction site for the cells, can alter integrin signalling, thereby affecting gene expression and the osteoblastic phenotype.[28] High adsorption of fibronectin to chitosan membranes can be explained by the high hydrophilicity and positive surface charges of chitosan. Thereby, the membranes can adsorb comparable or even higher amounts of fibronectin than the tissue culture plastic control. Water contact angle and the thereby indicated hydrophilicity and hydrophobicity can take an impact on the conformation of the fibronectin adsorbed to the surface. Hydrophilic surfaces favor a more flexible and extended conformation, whereas hydrophobic surfaces support a more rigid structure.[29] The conformation of the fibronectin adsorbed to the chitosan membranes was not determined during this study, but it seems highly unlikely that the small difference in water contact angle measurements observed would be enough to cause a large conformational change in fibronectin structure. Especially, since best attachment and high fibronectin adsorption was observed in the most hydrophobic surfaces. Nevertheless, since fibronectin is negatively charged under physiological conditions, the adsorption could be influenced by the presence of cationic groups, which in turn decreases the likelihood of denaturation.[30,31] The conformation of adsorbed fibronectin and the orientation of the cell binding domain should still be addresses in further research to exclude the possibility of minor conformational changes affecting the cell interaction properties. The results obtained during this study regarding the adsorption of fibronectin can be compared to the results published previously in the literature. It has been shown by Amaral et al.[32] that a high DD correlates with a significantly higher adsorption of fibronectin as compared to membranes with a lower DD, including a higher specificity of chitosan to fibronectin as in comparison to tissue culture plastic. These results also correlate with the notion that self-assembling monoloayers with amine groups can adsorb higher levels of fibronectin than monolayers with methyl groups. Because cell culture experiments in our study were performed in media containing 10% FBS, the interference of the proteins present in the serum, that is, albumin, vitronectin and fibronectin, needs to be evaluated in further studies.

The contribution of surface topography and roughness to osteoblastic cell attachment and differentiation are controversial.[33] Osteoblastic cell attachment was shown to be elevated on smooth surfaces even though rougher surfaces have been associated with increased differentiation.[34] As there were only small differences observed in surface roughness and only minimal variations in the visual examination of surface topography, except for DD94 chitosan membranes, it seems more likely that the differences in bioactivity in our study were due to differences in the surface chemistry, the water contact angle values and the ability to adsorb fibronectin to the surface. It is generally accepted that a higher DD favors cellular attachment associated with the increase of amine groups but that more acetyl groups could have the promising ability to induce healing without scar tissue formation.[21,22]

The amine groups in the chitosan polymer chains allow the membrane to be covalently bonded to titanium via silanization and in employing its bioactivity and positive effects on wound healing and tissue regeneration make it an interesting tool to coat titanium implants for orthopaedic applications. The effect of the difference in DD on bonding strength and degradation rate has not been investigated yet.[11]

CONCLUSION

We conclude that the surface roughness and fibronectin adsorption increase with increased DD. A higher DD also facilitates attachment and proliferation of preosteoblastic

MC3T3-E1 cells without inducing spontaneous osteogenic differentiation. No negative effects on osteogenic differentiation in the presence of ascorbic acid and β-glycerophosphate were observed.

Lower DD chitosan membranes were successfully prepared to sustain attachment and were modified by crosslinking with glutaraldehyde to promote long-term studies. The possibility of preparing chitosan membranes by selection from chitosan types with a wide range of DD and thereby displaying different bioactivity will be a useful advancement in the coating of titanium for orthopaedic applications.

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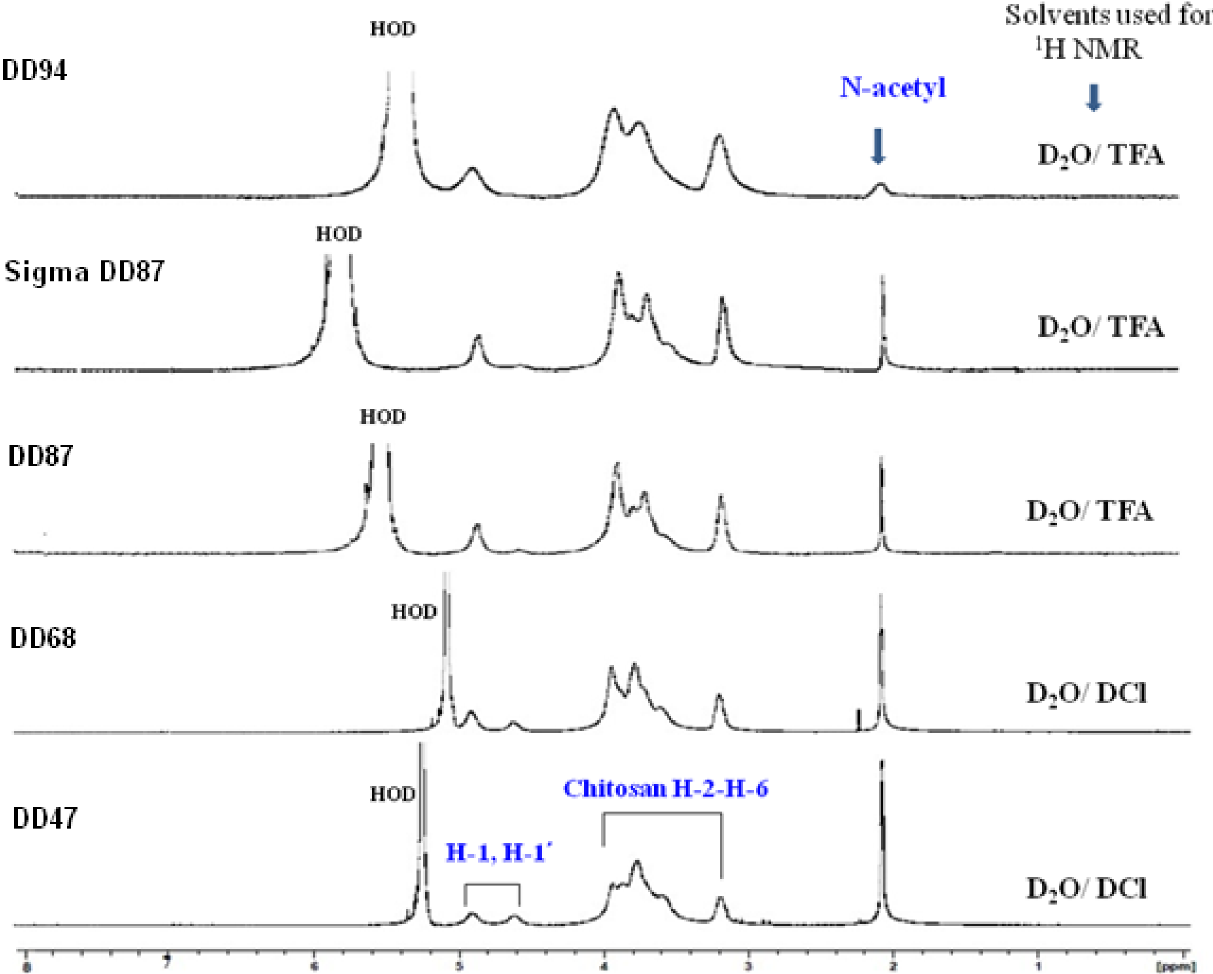
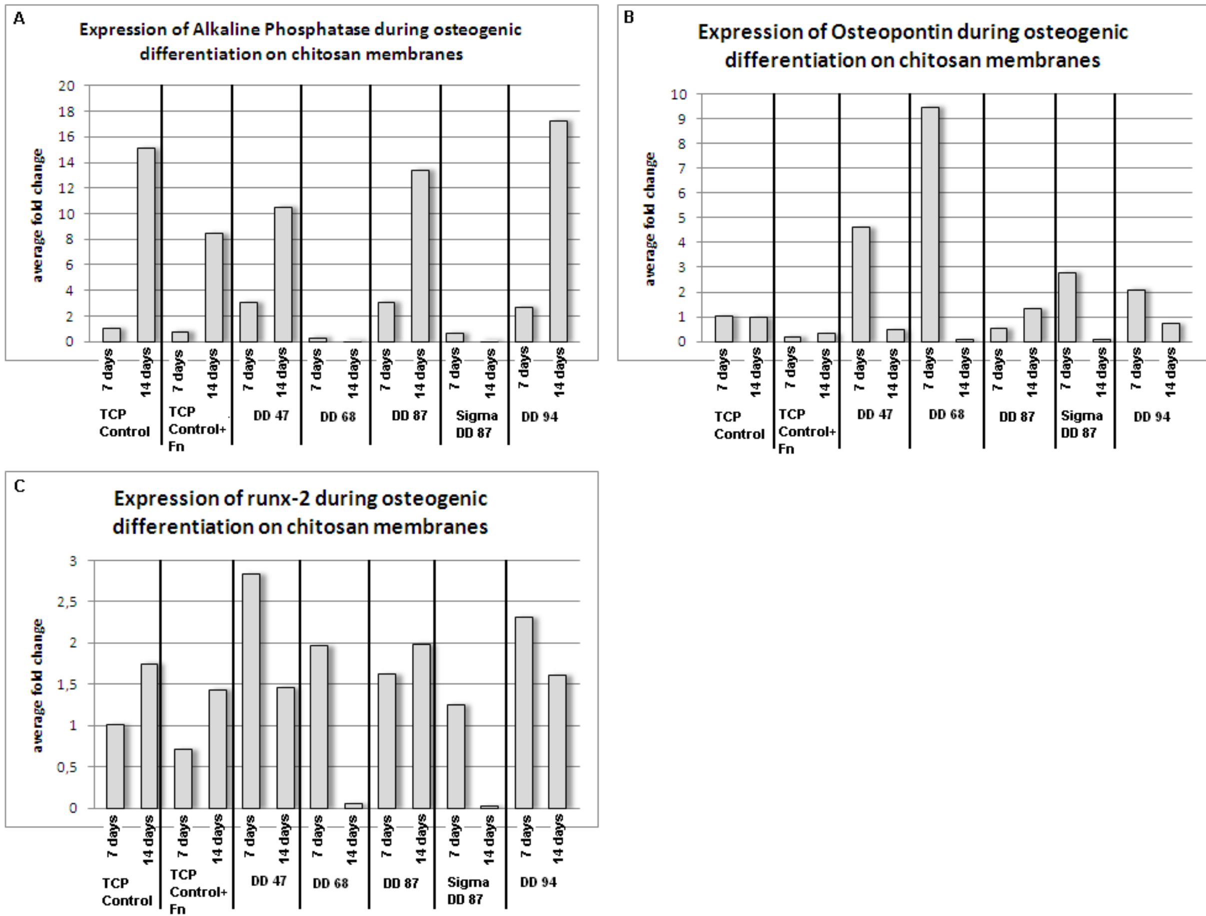
REFERENCES

1. Guarino V,Causa F, Ambrosio L. Bioactive scaffolds for bone and ligament tissue. Expert Rev Med Devices 2007;4:405–418.
2. Goodman SB, Gómez Barrena E, Takagi M, Konttinen YT. Biocompatibility of total joint replacements: A review. J Biomed Mater Res A 2009;90:603–618.
3. Esposito M, Coulthard P, Thomsen P, Worthington HV. The role of implant surface modifications, shape and material on the success of osseointegrated dental implants. A Cochrane systematic review. Eur J Prosthodont Restor Dent 2005;13:15–31.
4. Siebers MC, ter Brugge PJ, Walboomers XF, Jansen JA. Integrins as linker proteins between osteoblasts and bone replacing materials. A critical review. Biomaterials 2005;26:137–146.
5. Puleo DA, Nanci A. Understanding and controlling the bone-implant interface. Biomaterials 1999;20:2311–2321.
6. Amaral IF, Cordeiro AL, Sampaio P, Barbosa MA. Attachment, spreading and short-term proliferation of human osteoblastic cells cultured on chitosan films with different degrees of acetylation. J Biomater Sci Polym Ed 2007;18:469–85.
7. García AJ, Vega MD, Boettiger D. Modulation of cell proliferation and differentiation through substrate-dependent changes in fibronectin conformation. Mol Biol Cell 1999;10:785–98.
8. Ito Y, Kajihara M, Imanishi Y. Materials for enhancing cell adhesion by immobilization of cell-adhesive peptide. J Biomed Mater Res 1991;25:1325–1337.
9. Boyan BD, Hummert TW, Dean DD, Schwartz Z. Role of material surfaces in regulating bone and cartilage cell response. Biomaterials 1996;17:137–146.
10. Bumgardner JD, Wiser R, Elder SH, Jouett R, Yang Y, Ong JL. Contact angle, protein adsorption and osteoblast precursor cell attachment to chitosan coatings bonded to titanium. J Biomater Sci Polym Ed 2003;14:1401–1409.
11. Yuan Y, Chesnutt BM, Wright L, Haggard WO, Bumgardner JD. Mechanical property, degradation rate, and bone cell growth of chitosan coated titanium influenced by degree of deacetylation of chitosan. J Biomed Mater Res B Appl Biomater 2008;86:245–252.
12. Tomihata K, Ikada Y. In vitro and in vivo degradation of films of chitin and its deacetylated derivatives. Biomaterials 1997;18: 567–575.
13. Kurita K. Chitin and chitosan: Functional biopolymers from marine crustaceans. Mar Biotechnol (NY) 2006;8:203–226.
14. Debono M, Gordee RS. Antibiotics that inhibit fungal cell wall development. Annu Rev Microbiol 1994;48:471–497.
15. Athanasiou KA, Shah AR, Hernandez RJ, LeBaron RG. Basic science of articular cartilage repair. Clin Sports Med 2001;20: 223–247.
16. Khor E, Lim LY. Implantable applications of chitin and chitosan. Biomaterials 2003;24:2339–2349.
17. Shahidi F, Abuzaytoun R. Chitin, chitosan, and co-products: Chemistry, production, applications, and health effects. Adv Food Nutr Res 2005;49:93–135.
18. Di Martino A, Sittinger M, Risbud MV. Chitosan: A versatile biopolymer for orthopaedic tissue-engineering. Biomaterials 2005; 26(30):5983–5990.
19. Dornish M, Kaplan D, Skaugrud O. Standards and guidelines for biopolymers in tissue-engineered medical products: ASTM alginate and chitosan standard guides. American Society for Testing and Materials. Ann N Y Acad Sci 2001;944:388–397.
20. Anselme K. Osteoblast adhesion on biomaterials. Biomaterials 2000;21:667–681.
21. Chatelet C, Damour O, Domard A. Influence of the degree of acetylation on some biological properties of chitosan films. Biomaterials 2001;22:261–268.
22. Mi FL, Shyu SS, Wu YB, Lee ST, Shyong JY, Huang RN. Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing. Biomaterials 2001;22:165–173.
23. Feng F, Liu Y, Zhao B, Hu Ka. In Vitro Biomineralization of Glutaraldehyde Crosslinked Chitosan Films. J Wuhan Univ Technol Mater Sci Ed 2005;20:20–23.
24. Jameela SR, Jayakrishnan A. Glutaraldehyde cross-linked chitosan microspheres as a long acting biodegradable drug delivery vehicle: Studies on the in vitro release of mitoxantrone and in vivo degradation of microspheres in rat muscle. Biomaterials 1995;16:769–775.
25. Sannan T, Kurita K, Iwakura Y. Studies on chitin, 2. Effect of deacetylation on solubility. Die Makromolekulare Chemie 1976; 177:3589–3600.
26. Silva RM, Silva GA, Coutinho OP, Mano JF, Reis RL. Preparation and characterisation in simulated body conditions of glutaraldehyde crosslinked chitosan membranes. J Mater Sci Mater Med 2004;15:1105–1112.
27. Rhazi M, Desbrières J, Tolaimate A, Alagui A, Vottero P. Investigation of different natural sources of chitin: influence of the source and deactylation process on the physicochemical characteristics of chitosan. Polym Int 2000;49:337–344.
28. Moursi AM, Globus RK, Damsky CH. Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation in vitro. J Cell Sci 1997;110:2187–2196.
29. Conti M, Donati G, Cianciolo G, Stefoni S, Samorì B. Force spectroscopy study of the adhesion of plasma proteins to the surface of a dialysis membrane: Role of the nanoscale surface hydrophobicity and topography. J Biomed Mater Res 2002;61: 370–379.
30. Pellenc D, Berry H, Gallet O. Adsorption-induced fibronectin aggregation and fibrillogenesis. J Colloid Interface Sci 2006;298: 132–144.
31. Benesch J, Tengvall P. Blood protein adsorption onto chitosan. Biomaterials 2002;23:2561–2568.
32. Amaral IF, Lamghari M, Sousa SR, Sampaio P, Barbosa MA. Rat bone marrow stromal cell osteogenic differentiation and fibronectin adsorption on chitosan membranes: The effect of the degree of acetylation. J Biomed Mater Res A 2005;75:387–397.
33. Marquis ME, Lord E, Bergeron E, Drevelle O, Park H, Cabana F, Senta H, Faucheux N. Bone cells-biomaterials interactions. Front Biosci 2009;14:1023–1067.
34. Bächle M, Kohal RJ. A systematic review of the influence of different titanium surfaces on proliferation, differentiation and protein synthesis of osteoblast-like MG63 cells. Clin Oral Implants Res 2004;15:683–692.

Supplementary Figures



Supplementary Figure 1. ^{1}H NMR analysis of chitosan powder at 400.14 MHz at 298K. D_2O/DCl (deuterium hydrochloride) or D_2O/TFA (trifluoro acetic acid) was used as solvent and measurements were taken without water suppression. Sample concentration was 10-15 mg/ml.



Supplementary Figure 2. Effect of different DD Chitosan membranes on the expression of osteogenic genes at 7 and 14 days after osteogenic induction of MC3T3-E1 mouse pre-osteoblasts. (A) Average expression of Alkaline Phosphatase, (B) average expression of Osteopontin and (C) average expression of runx-2. Bars describe average fold change at days 7 and 14. n=2.

Paper V

5

"The most exciting phrase to hear in science,
the one that heralds new discoveries,
is not "Eureka" but "That's funny"."

Isaac Asimov, Professor of Biochemistry (1919-1992)

Solution Casting of Chitosan Membranes for *in vitro* Evaluation of Bioactivity

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Abstract

Considerable research is focusing on the surface modification of titanium implants for the treatment of orthopaedic tissue injuries to increase the success of orthopaedic fixations. Chitosan is one of the natural materials under investigation based on several favourable properties. Numerous techniques have been described for the preparation of chitosan membranes, including solution casting methods for the investigation of bioactivity before applying coatings onto potential titanium implants. Solution casting enables the easy in-house evaluation of chitosan membranes and allows for the selection of promising chitosan materials. We present a method for the standardized and easily applied preparation of chitosan membranes by solution casting. This protocol is suitable for chitosan materials spanning a wide degree of deacetylation, being derived from different chitin sources and chitosan derivatives with novel properties. We detail the preparation and quality control methods in order to prepare membranes with favourable bioactivity, sustaining cell attachment and proliferation for extended culture periods.

1. Introduction

Titanium implants for the treatment of orthopaedic tissue injuries are recommended for a number of load-bearing applications, but still lack improvement at the bone-biomaterial interface [1, 2]. Research focusing on the surface modification of these materials could considerably increase the success of orthopaedic fixations. Chitosan, the partly deacetylated configuration of chitin, is one of the natural materials under investigation to improve implant integration and cellular attachment [3-5]. Several promising properties are attributed to chitosan, including biocompatibility, non-toxicity and *in vivo* degradation [6]. Based on the chemical nature of chitosan, negatively charged cytokines and growth factors can be retained at its surface and exert favourable effects on osteogenesis *in vitro* and *in vivo* [7, 8]. The straight-forward use of chitosan and the easy molding abilities have long been recognized, and make this polymer an attractive tool for tissue engineering and regenerative medicine applications [9, 10].

Several properties of chitosan have been reported to strongly influence cell attachment and bioactivity *in vitro*, including the degree of deacetylation (DD), origin of the chitin source and the surface characteristics of the final membrane/coating [11, 12]. The effect on cellular behaviour was shown to be cell-type specific, but generally a lower degree of deacetylation is thought to induce healing without scar tissue formation, whereas higher degrees of deacetylation are more beneficial for cell attachment and proliferation [13, 14].

Numerous techniques have been developed for the preparation of chitosan membranes, including solution casting, layer-by-layer-self-assembly and silanization methods for bonding to titanium implants [3, 15, 16]. Solution casting of chitosan membranes on tissue culture plastic is a widely used method for the *in vitro* evaluation of bioactivity before applying coatings onto potential titanium implants. This technique provides an easy in-house investigation of cell attachment following standard laboratory protocols and allows for the selection of promising chitosan materials in accordance to general requirements for coated implants in tissue engineering applications. However, there are a vast number of protocols available for solution casting of chitosan membranes, often restricted to the use of a specific degree of deacetylation [17-19]. Additionally, cell attachment and proliferation are frequently significantly lower than on traditionally used tissue culture plastic and cannot be sustained for extended periods of time [1].

We have recently developed a standardized and easily applied protocol for the solution casting of chitosan membranes spanning a wide degree of deacetylation, displaying favourable bioactivity by sustaining cell attachment and proliferation for extended culture time. This protocol is suitable for the use of chitosan materials derived from varying chitin sources and the investigation of chitosan derivatives with modified properties.

2. Materials

2.1. Solution Casting of Chitosan Membranes

1. Chitosan powder or flakes (e.g. Chitosan from crab shells; Cat. No.50494, Sigma)
2. 100% Glacial Acetic Acid (Merck)
3. Transfer pipettes (Cat. No. 86.1172.010, Sarstedt)
4. Glass test tubes (Sigma)
5. 15 ml plastic conical tubes (Falcon)
6. Centrifuge with buckets for 15 ml tubes (5 000 rpm acceleration)
7. Flat bottom plates with low evaporation lid; tissue culture treated by vacuum gas plasma (Falcon) (*See* **Note 1**)
8. Incubator heated at 37°C, no CO_2 injection, no humidity control
9. Sodium hydroxide pellets (Cat. No. 6482.5000, Merck). Dissolve pellets in water to obtain 0.5 M solution.
10. 96% ethanol. Dilute in dH_2O to obtain 70% ethanol.
11. Fibronectin from Human plasma (Cat. No. 356008, BD Biosciences). Aliquot (100 µl/ eppendorf) and store frozen at -20°C.

2.2. MC3T3-E1 Culture on Chitosan Membranes

12. Mouse pre-osteoblastic cell line MC3T3-E1 (subclone 4; Cat. No. ATCC-CRL-2593, ATCC)
13. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12 1:1) with GlutaMAX (Cat. No. 31331, Gibco)
14. Minimum Essential medium alpha (αMEM) without ascorbic acid (Cat. No A10490, Gibco)
15. Heat-inactivated fetal bovine serum (FBS; Cat. No. 10500, Gibco). Aliquot and store frozen at -20°C.
16. Antibiotics: Penicillin-streptomycin- glutamine mix (100x solution; Cat. No. 10378, Gibco). Aliquot and store frozen at -20°C.
17. β-glycerophosphate disodium salt hydrate (Cat. No. G9422, Sigma)
18. L-Ascorbic acid (Cat. No. A4403, Sigma)
19. 75 cm^2 cell culture flasks (Nunc)
20. 2-well chamber slides with cover (Cat. No. 177429, Lab-Tek)
21. Cell incubator set at 95% humidity, 37°C and 5% CO_2 in air

2.3. Materials needed for Quality Control

22. Glutaraldehyde Solution Grade I 50% (Cat. No. G7651, Sigma Aldrich). Store frozen at -20°C and thaw in the dark.
23. Alizarin Red S powder (Cat. No. A5533, Sigma Aldrich)
24. Ammonium hydroxide (Merck). Prepare 0.5% solution.
25. Crystal Violet powder (Cat. No. C3886, Sigma Aldrich)
26. Phosphate buffered saline (1x PBS; pH 7.2; Cat. No 10010-015, Gibco)

27. Bovine Serum Albumin (BSA; Cat. No. A-4503, Sigma)
28. Rabbit anti-fibronectin antibody (Cat. No. F3648, Sigma Aldrich)
29. Goat anti-rabbit IgG Alkaline Phosphatase conjugated antibody (Cat. No. A9919, Sigma Aldrich)
30. Sigma FAST p-Nitrophenyl phosphate tablets (1mg/ml p-Nitrophenyl phosphate and 0.2 M Tris buffer; Cat. No. N2770, Sigma)

3. Methods

3.1. Solution Casting of Chitosan Membranes - Day 1

The preparation of chitosan membranes using solution casting methods follows a three day procedure. For best results, the procedure should be performed without stopping points in order to ensure best bioactivity results. However, after initial sterilization on day 3, chitosan membranes can be stored for several weeks until use in experiments or analysis of surface characteristics. The preparation of chitosan membranes can be performed on the bench. An overview of the work flow required for membrane preparation is shown in Figure 1.

We have found that the preparation of chitosan solution for membrane casting should follow slightly different procedures depending on the volume needed (*also see* **Note 2**). For optimal thickness of chitosan membranes, 0.1 ml of chitosan solution are cast per cm^2 of tissue culture plastic. At least 10% extra should be prepared to account for losses during the preparation. Both chitosan powder and chitosan flakes with different degree of deacetylation (DD) can be used in this protocol. However, chitosan flakes are generally more difficult to handle than chitosan powders. Depending on the molecular weight of chitosan, the solutions will differ in viscosity, with less viscous solutions being easier to process. The protocol can be easily scaled to the volume required, and is here described for “high volume” (*See* **Subsection 3.1.1.**) and “low volume” (*See* **Subsection 3.1.2**.) set-up. Solution casting methods are described for the coating of 6-well plates. Respective volumes needed for smaller sized tissue culture plastic are described in Table 1.

3.1.1. High Volume (4-20 ml)

1. Weigh in 10 mg of chitosan material per ml of solution to be prepared in a small plastic cup (~50-100 ml flat bottom cup)
2. Add 0.985 g/ml of dH_2O using a transfer pipette
3. Add 5 µl/ml of 100% Acetic Acid in a fume hood
4. Stir solution on a magnetic stirrer. This may take between 5 min to 1 h depending on the chitosan material. Cover the plastic cup with aluminium foil and assure that stirring is not too strong, to avoid splashing of the solution onto the sides of the cup. (*See* **Note 3**).
5. Transfer chitosan solution into 15 ml Falcon tubes.
6. Centrifuge at 5 000 rpm for 1 h to remove air bubbles and un-dissolved particles.
7. (*See* **Section 3.5.** for glutaraldehyde cross-linking of membranes with low DD)

8. For coating a 6-well plate, carefully place 1 ml of Chitosan solution into the middle of the well. Air bubbles should be avoided.
9. Spread chitosan solution to the corners of the well with a continuous smooth hand movement. Be sure to completely cover the well with solution.
10. Occasional bubbles in the solution after casting can be removed by using a needle or a small pipette tip. All bubbles need to be removed before drying the membranes.
11. Dry membranes over night in an incubator at 37°C without CO_2 injection or humidity control. Plates need to be uncovered to allow evaporation.

3.1.2. Low Volume (1-3 ml)

1. For 2 ml of Chitosan Solution, weigh in 10 mg/ml of Chitosan powder or flakes in a clear test tube.
2. Add 0.7 g/ml of dH_2O using a transfer pipette.
3. Add 100 µl/ml of 100% Acetic Acid in a fume hood (*See* **Note 2**).
4. Re-suspend chitosan solution using a transfer pipette to wash off any chitosan material from the test tube walls.
5. Cover test tube with aluminium foil.
6. Heat test tube in a water bath at 50°C for at least one hour or until dissolved.
7. Transfer chitosan solution into 15 ml Falcon tubes.
8. *See* **Subsection 3.1.1** and follow Steps 6-10.

3.2. Neutralization of Chitosan Membranes – Day 2

Proper casting of the chitosan membranes can be inspected by visual examination of the plastic ware against a light source. Small areas of in-homogenous casting then become visible. Since Acetic Acid is used to decrease the pH of the chitosan solution and enable solubility, chitosan membranes need to be neutralized after the drying process to render them water insoluble.

1. Add 2 ml of 0.5 M NaOH to each well.
2. Incubate for 30 min on a shaker at room temperature. Shaking should be slow and solution should be just moving. To account for the increased concentration of acetic acid during “low volume” preparations, neutralization time needs to be increased to 4 h.
3. Invert plates to remove NaOH and tap on a piece of paper to remove excess liquid.
4. Wash three times with 2 ml of dH_2O for 10 min on a shaker at room temperature.
5. Invert plates to remove dH_2O and tap on a piece of paper to remove excess liquid.
6. Dry membranes over night in an incubator at 37°C, without CO_2 injection or humidity control. Pates should be uncovered to allow for evaporation.

3.3. Sterilization and Fibronectin Coating of Chitosan Membranes – Day 3

In order to not compromise cell culture studies, chitosan membranes follow a dual sterilization procedure, using both 70% ethanol and UV-light. After sterilization, all work with chitosan coated plastic ware should be performed in a sterile fume hood. Coating with the adhesion protein fibronectin is used to promote initial cell attachment to the chitosan surface and is therefore essential for the success of the *in vitro* cultures.

3.3.1. Sterilization of Chitosan Membranes

1. Prepare 70% ethanol for sterilization.
2. Add 2 ml of 70% ethanol into each well and incubate 30 min at room temperature without shaking.
3. Invert plates to remove ethanol and tap on a piece of paper to remove excess liquid.
4. Dry membranes for 1 h in an incubator at 37°C without CO_2 injection or humidity control. Plates should be uncovered to allow for evaporation.
5. At this point, chitosan coated plastic ware can be covered with parafilm for storage at 4°C in the dark until further use.

6. Place uncovered chitosan coated plastic ware under the UV-light lamp in a fume hood for 30 min.
7. (*See* **Subsection 3.6.1.** for fibronectin adsorption studies).

3.3.2. Fibronectin Coating of Chitosan Membranes

1. Prepare a 5 µg/ml Fibronectin solution in 1x PBS into each well, by diluting frozen 1 mg/ml fibronectin stock solution.
2. Add 2 ml of Fibronectin solution into each well and incubate on a shaker for 3 h at 37°C. Solution should be just moving on the shaker to allow fibronectin to attach to the surface.
   Invert the plate to remove fibronectin solution and tap plastic ware on a paper to remove excess liquid.
3. (*See* **Section 3.6.** for surface characterization of chitosan membranes).

4. Equilibrate chitosan membranes for 20 min in DMEM/F12 media supplemented with 10% FBS and penicillin/streptomycin. Culture media will turn pink due to the high pH of the fibronectin solution. Equilibration is needed both for removal of excess fibronectin and buffering of pH.
5. Discard equilibration media before cell seeding.

3.4. MC3T3-E1 Cell Seeding and Culture – Day 3 - Day 24

The mouse pre-osteoblastic cell line MC3T3-E1 can be grown on chitosan membranes in an undifferentiated state using basic growth media or induced to undergo osteogenic differentiation. Cell attachment can be maintained for up to 24 days under differentiation conditions. Generally, it is sufficient to set-up MC3T3-E1 cultures two days before initiation of the chitosan casting protocol, at a density of 3 500 cells/cm^2. In our experience, subclone 4 of this particular cell line is best suited for

induction of osteogenic differentiation. Representative images of cells grown on different sources of chitin and chitosan derivatives used for chitosan membrane casting are shown in Figure 2 and Figure 3.

1. Expand MC3T3-E1 cells in αMEM media supplemented with 10% FBS and streptomycin/penicillin until 80% confluent.
2. 5 000 cells/cm^2 are required for seeding on chitosan coated plastic ware.
3. Trypsinize and count cells.
4. Re-suspend cells in DMEM/F12 media supplemented with 10% FBS and streptomycin/penicillin (basic expansion media).
5. Place 5 000 cells/cm^2 into the well and cover with appropriate volume of basic expansion media (*see* Table 1).
6. Incubate cell cultures at 37°C, 95% humidity and 5% CO_2.
7. Fully replace cell culture media every second day. (*See* **Note 4**).

8. To induce osteogenic differentiation of MC3T3-E1 cells, incubate cells for one night in basic expansion media to allow for initial attachment.
9. Osteogenic induction media is prepared from basic expansion media by addition of 2 mM β-Glycerophosphate and 50 µl/ml ascorbic acid. Mix thoroughly on a vortexer.
10. Remove basic expansion media and replace with osteogenic induction media. Again, culture media should be fully replaced every second day. First signs of osteogenic differentiation will be visible after 7-10 days (calcification, collagen type I deposition, cell contraction and change in phenotype).

3.5. Glutaraldehyde Cross-linking

The lower the DD, the more soluble chitosan membranes generally become based on the degradation of secondary structures [20]. From our experience with membranes prepared from a low DD (40-70%), cross-linking with glutaraldehyde is required to avoid dissolution during long term cultures. Membranes prepared from low DD materials will only be stable for one week in culture media before substantial dissolution can be observed. In order to avoid glutaraldehyde-related toxicity ("leaking of glutaraldehyde into the culture media") and still provide sufficient stability, a fine balance concerning the amount of glutaraldehyde needs to be achieved. We have found that chitosan membranes with low DD that are internally cross-linked with 0.02% glutaraldehyde are stable for long-term cultures and still retain favourable bioactivity and cell attachment.

1. *See* **Subsection 3.1.1.** and **Subsection 3.1.2.** for "high" and "low volume" preparation. Follow the procedure until Step 6.
2. During the centrifugation time (Step 6), thaw glutaraldehyde solution in the dark
3. Prepare an eppendorf tube containing 100 µl distilled water. (*See* **Note 5**).

4. Prepare a 0.02% glutaraldehyde solution (0.02% of the final volume of chitosan solution). Add into the eppendorf tube containing 100 µl distilled water. Mix well!
5. Transfer chitosan solution after centrifugation into a fresh 15 ml Falcon Tube.
6. Add the glutaraldehyde – distilled water solution to the chitosan solution.
7. Mix thoroughly on a vortexer.
8. Cast Membranes according to Step 8-10 in Section 3.1.1. (*See* **Note 6**).
9. *See* **Section 3.2.** and **Section 3.3.** for neutralization and sterilization of cross-linked chitosan membranes

3.6. Surface Characterization of Chitosan Membranes

Bioactivity and cell attachment are not only dependent on the DD and the origin of the chitin source, but are strongly influenced by surface characteristics, water contact angles and the ability of chitosan membranes to retain fibronectin [11, 12, 21, 22]. Fibronecting adsorption can be determined by in-house ELISA.

Water contact angle measurements are best performed on microscopy slides to avoid unnecessary manipulation of the specimen. We have found that 2-well chamber slides from LabTek are well suited for solution casting of chitosan membranes, providing easy handling and the appropriate tissue culture plastic surface.

In order to determine the average surface roughness and topography, chitosan membranes can be prepared in 12-well plates. This decreases the amount of sample needed and still enables successful analysis after cutting the plastic sides off the well. Since the area studied during Atomic Force Microscopy is very small, the cutting does not affect the surface characteristics in the middle of the well.

3.6.1. Fibronectin Adsorption studies

Fibronectin adsorption can be examined by simple in-house ELISA and measured in a spectrophotometer at 400 nm. This protocol has been adapted from Uygun et al. [19] and modified appropriately.

1. Prepare chitosan membranes in a 96-well plate following the procedure described in **Section 3.1., Section 3.2** and **Section 3.3** (Step 6).
2. Prepare a 5 µg/ml Fibronectin solution in 1x PBS, by diluting frozen 1 mg/ml fibronectin stock solution
3. Add 100 µl of fibronectin solution into each well and incubate over night at 4°C. Include non-coated tissue culture plastic as positive control for fibronectin adsorption.
4. Invert the plate to remove fibronectin solution and tap on paper to remove excess liquid.
5. Wash four times for 30 min with 200 µl 1x PBS at room temperature.
6. Tap plates on paper to remove excess liquid in-between washing steps.
7. Block unspecific adsorption by incubation with 1% BSA in 1x PBS for 2 h at room temperature.
8. Wash three times for 10 min with 200 µl 1x PBS at room temperature.

9. Tap plates on paper to remove excess liquid in-between washing steps.
10. Prepare a 1:15 000 dilution of primary antibody (rabbit anti-fibronectin antibody).
11. Add 100 µl of primary antibody and incubate for 2 h at room temperature.
12. Wash three times for 10 min with 200 µl 1x PBS at room temperature.
13. Tap plates on paper to remove excess liquid in-between washing steps.
14. Prepare a 1:50 000 dilution of secondary antibody (goat anti-rabbit IgG Alkaline Phosphatase-conjugated antibody).
15. Add 100 µl of secondary antibody and incubate for 2 h at room temperature
16. Wash three times for 10 min with 200 µl 1x PBS at room temperature
17. Tap plates on paper to remove excess liquid in-between washing steps.
18. Prepare p-nitrophenyl phosphate solution in dH_2O and mix thoroughly until fully dissolved. Store at 37°C in the dark until used.
19. Add 100 µl of p-Nitrophenyl phosphate solution and incubate for 30 min at 37°C in the dark.
20. If necessary, reaction can be stopped by addition of 3 M sodium hydroxide.
21. Measure optical density at 400 nm in a spectrophotometer.

3.7. Quality Control

3.7.1. Alizarin Red Staining

The *in vitro* success of chitosan membranes used for cell attachment strongly depends on the homogeneity of the final membrane. This can be easily assessed using a modified Alizarin Red Staining protocol. Based on the chemical characteristics of chitosan, acidic dyes, including Alizarin Red S, are robustly retained. Thereby, defects in the membrane casting can be easily spotted. This method is also useful for the investigation of dissolution after extended culture periods on chitosan membranes. Representative images of homogeneously and in-homogeneously distributed chitosan membranes after staining with Alizarin Red are shown in Figure 4.

1. Prepare a 2% Alizarin Red Solution and mix thoroughly on a vortexer until completely dissolved.
2. Adjust pH to 4.2 by adding 0.5% ammonium hydroxide (*See* **Note 7**).
3. Wash chitosan coated culture ware three times with 2 ml dH_2O for 5 min on a shaker.
4. Add 2 ml of Alizarin Red solution and incubate for 5 min on a shaker at room temperature.
5. Carefully remove Alizarin Red Solution using a pipette.
6. Wash four times for 5 min with 2 ml dH_2O on a shaker at room temperature.
7. Dry upside down over night.
8. Images can be taken in an inverted microscope equipped with a camera.

3.7.2. Crystal Violet Staining

Cells grown on chitosan membranes can be easily visualized by standard Crystal Violet Staining. Based on the chemical characteristics of the Crystal Violet Dye, chitosan membranes will remain

unstained. However, lipophilic chitosan derivates can react with the triphenyl methane structure, which will result in dark violet staining of chitosan membranes. In that case, cell bodies are best observed using an inverted microscope equipped with a camera.

1. Remove cell culture media.
2. Wash carefully with 2 ml 1x PBS.
3. Remove PBS and add 2 ml of 0.5% crystal violet solution.
4. Incubate for 30 min at room temperature without shaking.
5. Carefully remove crystal violet solution without disturbing the cell layer.
6. Wash four times with 2 ml 1x PBS.
7. Wash once with 2 ml tap water.
8. Dry upside down over night.
9. Images can be taken in an inverted microscope equipped with a camera.

4. Notes

1. Appropriate surface characteristics of tissue culture plastic used for coating with chitosan membranes is essential for the outcome of the experiment. We have tested three commercially available brands/surface treatments for tissue culture plastic, including “Primaria” (surface modified polystyrene; Falcon), “Nunclone” (Nunc) and non-tissue culture treated polystyrene plates (Falcon). Cell attachment on membranes prepared on these plates could not be sustained for more than a few days, whereas the identical procedure on the vacuum gas plasma treated plates allows for several weeks of cell attachment. Since the chitosan membranes prepared with this protocol are very thin, the surface of the tissue culture plastic can certainly affect elementary surface characteristics [19]. Furthermore, the differences in surface treatment could result in slight changes in the charge of the plate, thereby affecting the bonding of chitosan membrane to the plate surface.
2. Since chitosan solution during the “low volume” preparation process is not directly stirred, but rather heated, a higher concentration of acetic acid is required for full dissolution. We have found that concentrations up to 10% of acetic acid do not affect bioactivity of MC3T3-E1 cells *in vitro*.
3. The suitability of chitosan derivatives for solution casting of membranes can be assessed by their ability to completely dissolve during this step. Dispersion of particles, even after increasing the concentration of acetic acid to 10% -using either low or high volume preparation steps-, will result in sedimentation during the centrifugation step and finally in in-homogeneously cast membranes. Generally, in-homogeneously cast membranes do not sustain cell attachment. However, as long as the chitosan solution is clear, any differences in colour do not affect bioactivity.
4. We have found that complete replacement of cell culture media every second day is critical to sustain cell attachment on chitosan membranes, especially with lower degree of deacetylation preparations. The use of DMEM/F12 media instead of the generally recommended αMEM media for this cell type, results in better bioactivity of chitosan membranes and increased cell attachment. Nevertheless,

expansion of MC3T3-E1 cells before seeding on chitosan membranes should be performed in αMEM media for best growth behaviour.

5. Since the volume of glutaraldehyde used for 0.02% cross-linking reactions is generally very low (~4 µl), preparing a 100 µl mixture in water will provide better distribution in the relatively high volume of chitosan solution. The small amount of 100 µl distilled water will not statistically affect the final concentration of the chitosan solution.
6. Chitosan membranes cross-linked with 0.02% glutaraldehyde turn slightly yellow/orange based on the reaction between the primary amino group of chitosan and the aldehyde group of glutaraldehyde, resulting in the formation of an imine bond.
7. A pH of 4.2 is recommended for the use of Alizarin Red to stain calcium deposits [23]. However, we have observed that Alizarin Red strongly stains chitosan membranes also in the pH range of 4.1 – 4.7.

8. Conclusions

The possibilities associated with the use of chitosan in tissue engineering applications are far from being exhausted and numerous challenges remain prior to successful translation into the clinics. However, a vast body of conflicting literature is available describing the attachment, proliferation and osteogenic differentiation of osteo-progenitor cells grown on chitosan membranes with different degrees of deacetylation [24]. This is mainly due to the lack of consistency in providing sufficient data on molecular weight, source of chitosan and sample preparation in order to compare and draw conclusions [24]. The often scarcely detailed methodological sections in biomaterials-related publications strongly impede reproducibility.

Here we describe a protocol for the solution casting of chitosan membranes that is suitable for the use of chitosan from different chitin sources, chitosan with a wide range of degree of deacetylation and chitosan derivatives with novel properties. We provide a step-by-step procedure that results in cellular attachment comparable to tissue culture plastic controls and allows for the maintenance of cultures for extended periods of time. Based on our experience, we have developed simple in-house methods for quality control of homogeneous membrane casting and early prediction of successful experimental outcome.

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References

[1] I.F. Amaral, A.L. Cordeiro, P. Sampaio, M.A. Barbosa, J Biomater Sci Polym Ed, 18 (2007) 469-485.
[2] V.K. Balla, A. Bhat, S. Bose, A. Bandyopadhyay, J Mech Behav Biomed Mater, 6 (2012) 9-20.
[3] Y. Yuan, B.M. Chesnutt, L. Wright, W.O. Haggard, J.D. Bumgardner, J Biomed Mater Res B Appl Biomater, 86 (2008) 245-252.
[4] S.N. Danilchenko, O.V. Kalinkevich, M.V. Pogorelov, A.N. Kalinkevich, A.M. Sklyar, T.G. Kalinichenko, V.Y. Ilyashenko, V.V. Starikov, V.I. Bumeyster, V.Z. Sikora, L.F. Sukhodub, J Biomed Mater Res A, 96 (2011) 639-647.
[5] A.G. Moutzouri, G.M. Athanassiou, Ann Biomed Eng, 39 (2011) 730-741.
[6] E. Khor, L.Y. Lim, Biomaterials, 24 (2003) 2339-2349.
[7] A. Di Martino, M. Sittinger, M.V. Risbud, Biomaterials, 26 (2005) 5983-5990.
[8] J.D. Bumgardner, R. Wiser, S.H. Elder, R. Jouett, Y. Yang, J.L. Ong, J Biomater Sci Polym Ed, 14 (2003) 1401-1409.
[9] K. Kurita, Mar Biotechnol (NY), 8 (2006) 203-226.
[10] F. Shahidi, R. Abuzaytoun, Adv Food Nutr Res, 49 (2005) 93-135.
[11] M. Dornish, D. Kaplan, O. Skaugrud, Ann N Y Acad Sci, 944 (2001) 388-397.
[12] K. Anselme, Biomaterials, 21 (2000) 667-681.
[13] C. Chatelet, O. Damour, A. Domard, Biomaterials, 22 (2001) 261-268.
[14] F.L. Mi, S.S. Shyu, Y.B. Wu, S.T. Lee, J.Y. Shyong, R.N. Huang, Biomaterials, 22 (2001) 165-173.
[15] K. Cai, A. Rechtenbach, J. Hao, J. Bossert, K.D. Jandt, Biomaterials, 26 (2005) 5960-5971.
[16] J.D. Bumgardner, B.M. Chesnutt, Y. Yuan, Y. Yang, M. Appleford, S. Oh, R. McLaughlin, S.H. Elder, J.L. Ong, Implant Dent, 16 (2007) 66-79.
[17] I.F. Amaral, M. Lamghari, S.R. Sousa, P. Sampaio, M.A. Barbosa, J Biomed Mater Res A, 75 (2005) 387-397.
[18] R.M. Silva, G.A. Silva, O.P. Coutinho, J.F. Mano, R.L. Reis, J Mater Sci Mater Med, 15 (2004) 1105-1112.
[19] B.E. Uygun, T. Bou-Akl, M. Albanna, H.W. Matthew, Acta Biomater, 6 (2010) 2126-2131.
[20] T. Sannan, K. Kurita, Y. Iwakura, in, Die Makromolekulare Chemie, 1976, pp. 3589-3600.
[21] M. Rhazi, J. Desbrières, A. Tolaimate, A. Alagui, P. Vottero, Polymer International, 49 (2000) 337-344.
[22] A.M. Moursi, R.K. Globus, C.H. Damsky, J Cell Sci, 110 (Pt 18) (1997) 2187-2196.
[23] M.J. Taylor, C.J. Hunt, Br J Ophthalmol, 65 (1981) 815-819.
[24] V. Hamilton, Y. Yuan, D.A. Rigney, A.D. Puckett, J.L. Ong, Y. Yang, S.H. Elder, J.D. Bumgardner, J Mater Sci Mater Med, 17 (2006) 1373-1381.

Figure Legends

Figure 1. Work flow chart for the solution casting of chitosan membranes. The preparation scheme is visualized stepwise for the successful casting of chitosan membranes on tissue culture plastic. Additional analysis and quality control are indicated where appropriate.

Figure 2. Images of MC3T3-E1 pre-osteoblastic cells grown on chitosan membranes prepared from different chitin sources. A) Is an image of cells after 48 h grown on crab shell derived chitosan with 87% degree of deacetylation. B) Is an image of cells after 48 h grown on shrimp shell derived chitosan with 87% degree of deacetylation. Images were taken in an inverted microscope.

Figure 3. Images of MC3T3-E1 pre-osteoblastic cells grown on chitosan membranes prepared from chitosan derivatives. A) Is an image of cells grown on a chitosan free base derivative for 7 days. B) Is an image of cells grown on Chitosan p-Toluensulfonic acid-salt (PTSA salt) for 7 days. C) Is an image of cells grown on Chitosan-Bromide salt for 7 days. Cells were stained with crystal violet and images were taken in an inverted microscope.

Figure 4. Images of chitosan membranes stained with Alizarin Red Stain for comparison of homogeneous and in-homogenous membrane casting. A) Is an image of a homogeneously distributed chitosan membrane prepared from crab shell chitosan with 87% degree of deacetylation. B) Is an image of an in-homogeneously distributed chitosan membrane prepared from modified crab shell chitosan with 87% degree of deacetylation. A spiral-like distribution of the membrane can be observed. C) Is an image of an in-homogeneously distributed chitosan membrane prepared from N-lauroyl chitosan derivatives with a degree of substitution of 0.05%.

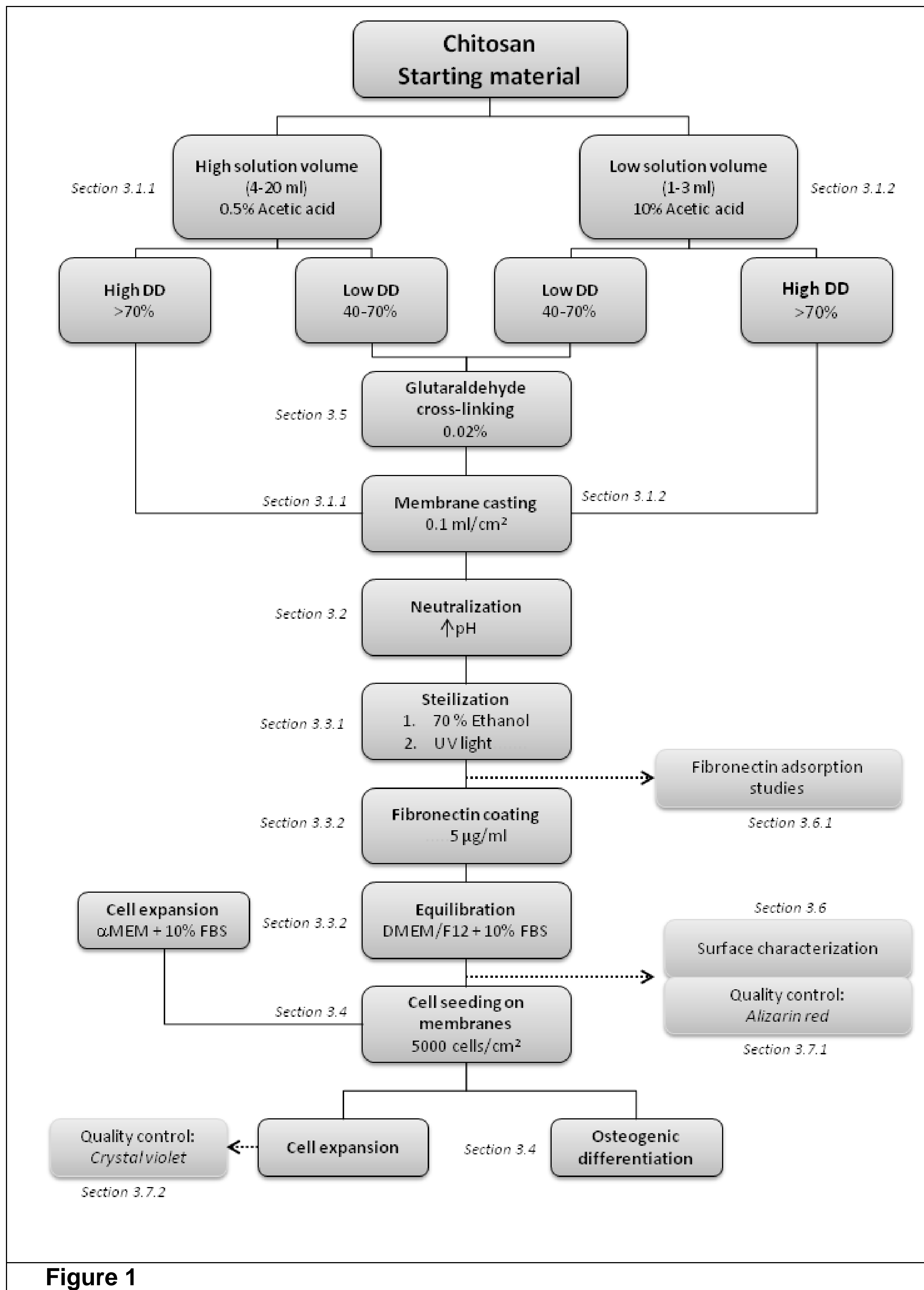

Figure 1

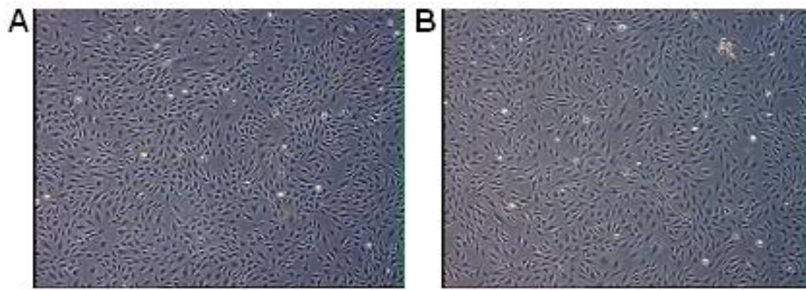

Figure 2

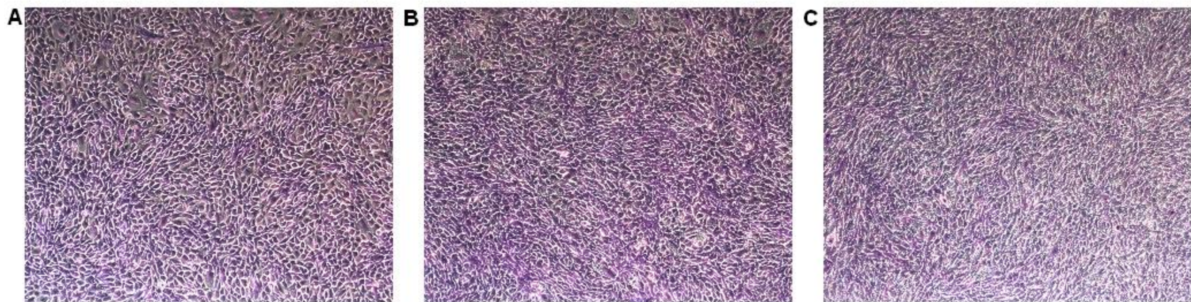

Figure 3

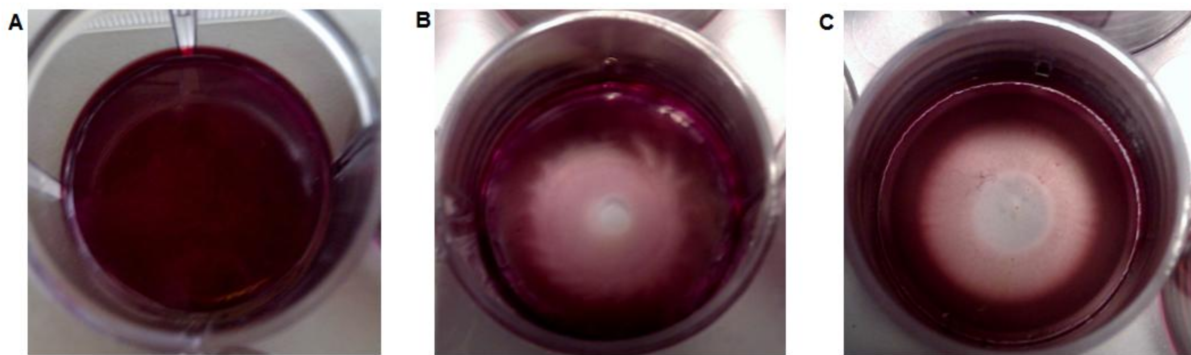

Figure 4

	Chitosan Solution	NaOH (neutralization)	70% ethanol (sterilization)	Fibronectin solution	Cell culture media	Alizarin Red Stain	Crystal Violet Stain
6-well plate	1 ml	2 ml	2 ml	2 ml	3 ml	2 ml	2 ml
12-well plate	500 µl	1 ml	1 ml	1 ml	1.5 ml	1 ml	1 ml
24-well plate	250 µl	500 µl	500 µl	500 µl	1 ml	1 ml	1 ml
96-well plate	100 µl	200 µl	200 µl	200 µl	100 µl	100 µl	100 µl
2-well slide chamber	500 µl	1 ml	1 ml	1 ml	2 ml	1 ml	1 ml

Table 1. Volume for preparation of chitosan membranes dependent on the type of culture plate.

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- Molecular biology, including RNA isolation, reverse transcription, Real-Time qPCR, gradient PCR end-point qPCR, investigation of gene expression patterns and statistical analysis
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Bone-Tec 2011 International Bone Tissue-Engineering Congress, 12-15th October 2011, Hannover, Germany, poster presentation

„Scaffolds Technology in Restoring Bone to Normality – from Engineering, Design, Regulatory Approval to Patient“ – Workshop by Prof. Dr. Swee-Hin Teoh, 12th October 2011, Hannover, Germany

„Advances in Regenerative Dentistry: BMP‘s for Alveolar Augmentation/Osseointegration and Periodontal Regeneration“ – Workshop by Prof. Dr. Dr. Dr. Ulf ME Wikesjö, 12th October 2011, Hannover, Germany

„Experimental design and statistical data analysis for qPCR“ - Workshop, TATAA Biocenter, 4-6th May 2011, Göteborg, Sweden

„Preclinical Models and Imaging in Muscoloskeletal Tissue Engineering“ – Winterschool Expertissues/Termis, 16-19th January 2011, Radstadt, Austria

Publications

Ramona Lieder, Mariam Darai, Margrét Björk Thor, C.-H. Ng, Jón M. Einarsson, Sveinn Gudmundsson, Benedikt Helgason, Vivek Sambhaji Gaware, Már Másson, Jóhannes Gíslason, Gissur Örlygsson and Ólafur E. Sigurjónsson. „In vitro bioactivity of different degree of deacetylation chitosan, a potential coating material for titanium implants“, J. Biomed. Mater. Res. Part A (2012), 100A, 3392-3399

Ramona Lieder, Sigrídur Thóra Reynisdóttir, Finnbogi Thormódsson, C.-H. Ng, Jon M. Einarsson, Jóhannes Gíslason, Jóhannes Björnsson, Sveinn Gudmundsson, Pétur H. Petersen and Ólafur E. Sigurjónsson. „Glucosamine increases the expression of YKL-40 and osteogenic marker genes in hMSC during osteogenic differentiation“, Nat. Prod. Bioprospect. (2012), 2, 87-91

Ramona Lieder, Sigrídur Thóra Reynisdóttir, Finnbogi Thormódsson, C.-H. Ng, Jon M. Einarsson, Jóhannes Gíslason, Jóhannes Björnsson, Sveinn Gudmundsson, Pétur H. Petersen and Ólafur E. Sigurjónsson. „Chitosan and Chitin Hexamers affect short-term expansion and osteogenic differentiation of mesenchymal stem cells differently“, Int. J. Biol. Macromol. (2012), 51, 675-680

Ramona Lieder, Vivek S. Gaware, Finnbogi Þormóðsson, Jon M. Einarsson, C.-H. Ng, JohannesGislason, Johannes Bjornsson, Pétur H. Petersen and Olafur E. Sigurjónsson. „

„Endotoxins affect the bioactivity of chitooligosaccharides in cultures of human, bone marrow-derived mesenchymal stem cells“, Acta Biomater. (2013), 9, 4771-4778

Ramona Lieder and Olafur E. Sigurjónsson. "Chitosan as a coating material for titanium implants" In: Chitin and Chitosan Derivatives: Advances in Drug Discovery and Developments. Se-Kwon Kim, 1 ed. CRC Press LLC, Boca Raton; 2014

Ramona Lieder, Pétur H. Petersen and Olafur E. Sigurjónsson. „Endotoxins – the invisible companion in biomaterials research. Tissue Eng. Part B (2013), doi:10.1089/ten.teb.2012.0636

Ramona Lieder and Olafur E. Sigurjónsson. „Stem cells and tissue engineering – the ultimate combo for clinical therapy?“ (editorial), Drug Design (accepted for publication)

Abstracts

Ramona Lieder, Vivek S. Gaware, Finnbogi Þormóðsson, Jon M. Einarsson, C.-H. Ng, JohannesGislason, Johannes Bjornsson, Pétur H. Petersen and Olafur E. Sigurjónsson. „ „Endotoxins – the real reason for bioactivity in your in vitro cell cultures?“, Termis World Congress 2012

Ramona Lieder, Mariam Darai, Margrét Björk Thor, C.-H. Ng, Jón M. Einarsson, Sveinn Gudmundsson, Benedikt Helgason, Vivek Sambhaji Gaware, Már Másson, Jóhannes Gíslason, Gissur Örlygsson and Ólafur E. Sigurjónsson. Bone-Tec 2011 International Bone Tissue-Engineering Congress

Ramona Lieder, Mariam Darai, Margrét Björk Thor, C.-H. Ng, Jón M. Einarsson, Sveinn Gudmundsson, Benedikt Helgason, Vivek Sambhaji Gaware, Már Másson, Jóhannes Gíslason, Gissur Örlygsson and Ólafur E. Sigurjónsson. Termis EU Conference 2010

Manuscripts in preparation

Ramona Lieder, Mariam Darai, Gissur Örlygsson and Olafur E. Sigurjónsson. "Solution casting of chitosan membranes for *in vitro* evaluation of bioactivity" (submitted manuscript)

Sandra M. Jónsdóttir-Buch, **Ramona Lieder**, Salome Jónsdóttir, Brendon Noble, Olafur E. Sigurjónsson. "Outdated platelet lysates can replace the use of fetal bovine serum in the culture of human, bone marrow-derived mesenchymal stem cells", (Manuscript in preparation)