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(71) **Applicant** *(for all designated States except US)*: **CHI2GEL LTD.** [IL/IL]; 38 Habarzel Street, 69710 Tel Aviv (IL).

(72) **Inventors; and**
(75) **Inventors/Applicants** *(for US only)*: **BEN-SHALOM, Noah** [IL/IL]; 12 Revadim Street, Ramat Hachayal, 69278 Tel Aviv (IL). **NEVO, Zvi** [IL/IL]; 11 Yair Stern Street, 46412 Herzliya (IL). **PATCHORNIK, Avraham** [IL/IL]; 9 Zrubavel Street, 70400 Ness-Ziona (IL). **ROBINSON, Dror** [IL/IL]; Kfar Shmuel 100, 99788 (IL).

(74) **Agents**: **WEBB & ASSOCIATES** et al.; P.O. Box 2189, 76121 Rehovot (IL).

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(54) **Title**: INJECTABLE HYDROGEL FORMING CHITOSAN MIXTURES

(57) **Abstract**: The present invention relates to combinations of chitosans that form hydrogels in a pH-dependant and thermo-sensitive manner, aqueous solutions used to form such hydrogels, and methods of use thereof.

INJECTABLE HYDROGEL FORMING CHITOSAN MIXTURES

FIELD OF THE INVENTION

The present invention relates to combinations of chitosans that form hydrogels in a pH-dependent and thermo-sensitive manner, aqueous solutions used to form such hydrogels, and methods of use thereof.

BACKGROUND OF THE INVENTION

Hydrogels are highly hydrated, macromolecular networks, dispersed in water or other biological fluids. Hydrogels that are thermo-sensitive (or thermosetting) exhibit increased viscosity upon increases in temperature. Such hydrogels have been shown to have favorable application properties and longer survival periods at the site of application as compared to non-thermosensitive hydrogels, and are therefore advantageous as vehicles for slow-release drug delivery systems.

Various polymers, including chitosan, may be used for hydrogel preparation. Chitosan occurs as a family of polymers, obtained by partial to substantial N-deacetylation of chitin, the latter being a linear polysaccharide of N-acetyl-D-glucosamine (GlcNAc) units in β-1,4 linkage. Chitin is present in abundance in invertebrates, particularly in the exoskeletons of crustaceans (e.g. shrimps, crabs, lobsters). Commercial deacetylation processes used for chitosan production generally involve treatment of shellfish food processing waste products with concentrated alkaline solutions, usually sodium hydroxide. According to the extent of deacetylation achieved during its production, chitosan may be a heteropolymer of D-glucosamine (GlcN) and GlcNAc units, or it may be a homopolymer formed exclusively of GlcN units. Various techniques are available for varying the extent of deacetylation, and for re-acetylating the polymer in order to obtain chitosan having a desired degree of acetylation. The deacetylation process results in free amino groups along the polymer chain, which render the polymer soluble in selected aqueous acid systems. The degree of solubility of chitosan with a given degree of deacetylation depends on multiple parameters, including polymer molecular weight, temperature, and concentration and nature of the acid solvent.

Accordingly, one parameter used to characterize chitosan is the proportion of GlcNAc units to GlcN units, expressed as the degree of acetylation (DA), or as the

reciprocal degree of deacetylation (DD or DDA). The structure of chitosan may be further characterized by the mode of distribution of the GlcNAc and GlcN units within the polymer chain, wherein for example, a non-homogeneously deacetylated chitosan has deacetylated units occurring in blocks of variable size and/or location along the chain, whereas a homogeneously deacetylated chitosan has a randomly distribution of deacetylated units along the polymer chain. Further, the molecular weight is an important parameter, which together with the degree, and distribution mode of acetylation, determine properties of chitosan, such as solubility, biodegradability and viscosity.

Chitosan is bioadhesive, biocompatible, non-toxic, and non-immunogenic, allowing its use in the medical, pharmaceutical, cosmetic and tissue construction fields. For example, the use of chitosan for topical ocular application, intraocular injection and and transplantation in the vicinity of the retina has been disclosed (Felt et al., 1999; Patashnik et al., 1997; Song et al., 2001). Further, uniform submicron chitosan fibers prepared by electro-wet-spinning technology, and their use in development of artificial muscles, biosensors, and artificial organ components has been disclosed (Lee et al. 2006). Since chitosan is specifically recognized and cleaved by certain enzymes, e.g. lysozyme, it is therefore bioerodable and biodegradable (Muzzarelli, 1997; Koga, 1998).

Various hydrogels comprising cross-linked chitosan and/or chitosan in combination with additional polymers are known, including for example, those comprising: chitosan chlorides or chitosan glutamates cross-linked with genipin (Mwale et al., 2005), reportedly useful as scaffolds for the encapsulation of invertebral disc cells (Roughley et al., 2006); chitosan-graft-poly(N-isopropylacrylamide), reportedly useful for entrapping chondrocytes and meniscus cells (Chen et al., 2006); a chitosan/poly(acrylic acid) pH-sensitive hydrogel (Shi et al., 2004); chitosan, β-glycerol phosphate and hydroxyethyl cellulose (Li et al., 2002); a chitosan/polyvinyl pyrrolidone pH-sensitive hydrogel (Risbud et al., 2000); N-acetylchitosan and tropcollagen (Hirano et al., 2000): chondroitin sulfate and chitosan (Sechriest et al., 2000); palmitoyl glycol chitosan (Noble et al., 1999); poly(caprolactone)-co-poly(ethylene glycol)-co-poly(caprolactone) diacrylate and chitosan (Zhu et al., 2005); oxidized and N-carboxyethyl chitosan (Weng et al, 2007).

U.S. Patent No. 6,344,488 discloses a polysaccharide based gel solution comprising 0.1 to 5.0% by weight of chitosan; and 1.0 to 20% by weight of a monophosphate dibasic salt of polyol or sugar; wherein said gel solution is a solution at

pH between 6.5 and 7.4 at a temperature below 20 °C, and forms a gel within a temperature range from 20 to 70 °C. According to the disclosure, the thermosensitive chitosan hydrogel may be prepared by neutralizing a chitosan having a deacetylation degree of about 80% with β-glycerophosphate.

Thermosensitive chitosan hydrogels containing β-glycerophosphate include those referred to under the trade names BST-CarGel™, intended for filling cartilage defects and cartilage repair; BST-DermOn™, intended for wound healing; and BST-InPod™, intended for treatment of heel pain.

The aforementioned prior art products are associated with a number of disadvantages, including limited degradation rates, and the limitations imposed by use of glycerophosphate or similar plasticizing salts. β-glycerophosphate is a negatively charged molecular entity that can react with positively charged drugs, leading to their precipitation, or to the disturbance of their release from the hydrogel. Therefore, the presence of β-glycerophosphate decreases the range of drugs with which chitosan/β-glycerophosphate hydrogels can be used.

Further, the modulation of the properties of the hydrogel, such as gelation time and viscosity, depends on the concentration of glycerophosphate, and is therefore limited by the solubility of β-glycerophosphate. In particular, a high concentration of β-glycerophosphate is required for a low gelation time, and for avoiding the rapid elimination of the hydrogel after its administration. However, a high concentration of β-glycerophosphate also decreases the viscosity of the hydrogel. Accordingly, these hydrogels lack the desirable combination of a low gelation time together with high viscosity. Further, the high concentration of β-glycerophosphate may induce precipitation of the hydrogel at the administration site. In addition, chitosan/β-glycerophosphate gels are hampered by turbidity, thus rendering them inappropriate for particular applications such as ocular or topical administration.

Preparation of transparent chitosan/β-glycerophosphate hydrogels using deacetylated and acetic anhydride reacetylated chitosan has been disclosed (Berger et al., 2004). According to the disclosure, turbidity of chitosan/β-glycerophosphate hydrogels is modulated by the degree of deacetylation of chitosan and by the homogeneity of the medium during reacetylation, which influences the distribution mode of the glucose amine monomers. The preparation of transparent chitosan/β-glycerophosphate hydrogels therefore reportedly requires a homogeneously reacetylated chitosan with a degree of deacetylation between 30 and 60%.

WO 2005/097871 discloses a pseudo-thermosetting neutralized chitosan composition, which comprises 0.1 to 2.0 wt/v %, preferably 0.5 to 1 wt/v %, based on the total composition, of a homogeneously reacetylated chitosan derived from a chitosan having a deacetylation degree of 80 to 90%, having a molecular weight of not smaller than 200 kDa, preferably not smaller than 600 kDa, and a deacetylation degree of 30 to 60%, preferably 45 to 55%, neutralized with an hydroxylated base, such as NaOH, wherein said composition forms a phosphate-free transparent hydrogel at a temperature higher than 5 °C. According to the disclosure, the homogeneous distribution of the acetylated and deacetylated monomers of chitosan is an essential criterion for obtaining the subject hydrogels. It is furthermore disclosed that the consistency of the subject hydrogels may be improved by the addition of a diol, in particular 1,3-propandiol.

U.S. Patent No. 4,738,850 discloses a controlled release formulation comprising a drug selected from angiotensin converting enzyme inhibitors and ascorbic acid, in combination with chitosan, wherein a gel-like complex is formed of the drug and the chitosan in environments ranging from neutral to acidic. According to the disclosure, the chitosan is 80 to 90% deacetylated and is present in the formulation at a concentration from about 5 to about 70% by weight.

U.S. Patent No. 6,140,089 discloses an encapsulation device comprising viable cells dispersed in a three-dimensional particulate, essentially non-cross-linked, chitosan core matrix encapsulated in a thermoplastic semipermeable membrane, wherein the chitosan core matrix is formed by precipitation of a chitosan solution containing said cells. According to the disclosure, chitosan precipitation can be achieved by any method which removes or masks the charge of a sufficient number of the free amino groups, such as by placing chitosan-containing capsules in a buffered solution containing monovalent ions, or by adjusting the pH of a chitosan solution.

U.S. Patent No. 6,486,140 discloses use of a combination of an agent comprising chitosan and heparin immobilized to the chitosan for prevention of undesirable tissue adhesion. According to the disclosure, the chitosan has a degree of N-acetylation of no more than about 90%, and the agent may be applied in the form of *inter alia* a solution or a gel.

U.S. Patent Application Publication No. 2005/0042265 discloses a composition comprising a chitosan hydrogel, having a degree of acetylation no greater than about 40%, and preferably between about 2 and about 6%, for use in repair and cicatrization of cutaneous lesions of chronic or acute wounds.

U.S. Patent No. 6,521,243 discloses an ionic chitosan iodine complex in the form of solutions and hydrogels, comprising chitosan or a derivative thereof; an aqueous vehicle; elemental iodine; and an iodide source. According to the disclosure, the chitosan has an average molecular weight between 10 to 1000 kDa, preferably 100 to 800 kDa, and most preferably 250 to 750 kDa, and a degree of deacetylation of 40% to 95%, preferably 60% to 90%.

U.S. Patent No. 6,858,222 discloses a drug releasing fiber, comprising segments of chitosan having different degrees of deacetylation, and a method of production thereof. This disclosure does not specify any particular chitosan degree of deacetylation.

U.S. Patent No. 6,329,337 discloses an adhesive for biological tissue comprising a recombinant human serum albumin as a glue agent, and a bifunctional or multifunctional aldehyde as a cross-linking agent. According to the disclosure, the glue agent may contain an additional component *inter alia* a partially-acetylated chitosan, such as a 50% acetylated chitosan, and further, an aqueous solution of the glue agent may be lyophilized, and, prior to use, reconstituted with water or saline for injection.

WO 03/011912 teaches a process of preparing chitosan comprising subjecting particulate chitin to low temperature swelling with an aqueous solution, e.g. 10 N alkali, for a period of at least 36 hours, followed by reacting the swollen particulate chitosan with an alkaline solution at an elevated temperature so as to cause deacetylation. According to the disclosure, the swelling is carried out at a temperature of up to 30 °C, and the deacetylation is carried out at a temperature that is at least 5 °C and preferably at least 25 °C higher than the swelling stage, and acetylation is effected to give a chitosan product having a degree of acetylation of 0.2 to 0.7, especially 0.45 to 0.6. The disclosed method further comprises a washing step following deacetylation, and the product may be optionally further modified e.g. by gel or solution formation. According to the disclosure, the chitosan product is fully water soluble i.e. more than 97% by weight may be dissolved in a dilute acid solution.

WO 2004/069230 discloses a pharmaceutical composition comprising a release sustaining or mucoadhesive agent, and a physiologically active agent, wherein the release sustaining or mucoadhesive agent comprises at least two chitosans having different degrees of acetylation (F_A), wherein at least one chitosan has an F_A value in the range from 0.25 to 0.80 (e.g., 0.30 to 0.60 or 0.33 to 0.55). Also disclosed is a mixture of two or more chitosans having F_A values differing by at least 0.1, and preferably by at

least 0.2, and that the chitosans comprise 5 to 98% by weight of the composition. According to the disclosure, the compositions are in a form suitable for administration into the gastrointestinal tract, e.g. orally or rectally, and may include gels.

WO 2004/068971 teaches foodstuffs comprising a nutritional food substance and a chitosan having an acetylation degree (F_A) of from 0.25 to 0.80, or a mixture of chitosans.

WO 2004/069230 and WO 2004/068971 do not teach injectabgle solutions or ge forming solutions. These publications fail to teach chitosan hydrogels of defined viscosity, and further fail to describe preparation of hydrogels from mixtures of chitosans. In fact, the compositions and products exemplified in WO 2004/069230 and WO 2004/068971 comprise either only highly deacetylated chitosans which are not capable of forming hydrogels under physiological conditions since they will precipitate at pH values in the neutral range, or only highly acetylated chitosans which remain soluble at neutral pH and at 37 °C.

WO 2008/072230, published after the priority date of the present invention, discloses a chitosan composition comprising at least one type of chitosan having a degree of acetylation in the range of from about 30% to about 60%, and at least one type of chitosan having a degree of acetylation of at least 70%, wherein the composition undergoes pH- and temperature-dependent gelation to form a hydrogel. According to the disclosure, the molecular weight of the each of the highly deacetylated and the highly acetylated chitosans is in the range of 10 kDa to about 4000 kDa, wherein the highly deacetylated chitosan preferably has a molecular weight of greater than about 200 kDa and the highly acetylated chitosan preferably has a molecular weight of greater than about 60 kDa.

There remains an unmet need for hydrogel forming chitosan compositions that combine the properties of viscosity, mechanical integrity, favorable rates of gel formation and degradation at physiological conditions, and predictable drug release profiles, which can be utilized for development of slow release drug formulations and for scaffolds and implants for regenerative medicine.

SUMMARY OF THE INVENTION

The present invention provides a pH- and temperature-dependent hydrogel forming chitosan composition, gels comprising mixtures of chitosans and uses thereof.

The present invention is based, in part, on the finding that a composition comprising two different types of chitosans, which differ with respect to their degree of acetylation/deacetylation and having specific molecular weight ranges, forms a hydrogel at physiological conditions of pH and temperature, yet remains in a liquid state at pH values below the neutral range and/or at low temperatures, e.g. under refrigeration.

In particular, the composition comprises: a first type of chitosan having a degree of acetylation in the range of about 20% or less (also referred to interchangeably herein as "highly deacetylated chitosan" or "type 1 chitosan"); and a second type of chitosan having a degree of acetylation in the range from about 40% to about 60% (also referred to interchangeably herein as "highly acetylated chitosan" or "type 2 chitosan"). In other words, the degree of deacetylation of each of the first and the second type of chitosan are respectively in the range from about 80% to about 100%, and in the range from about 40% to about 60%.

The chitosan hydrogels of the present invention are advantageous over prior art chitosan gel-forming compositions comprising a single type of chitosan. In particular, the use of a combination of chitosans as described herein confers improved physical, chemical and pharmacokinetic properties of the resultant hydrogel, as compared to gels or hydrogels comprising a single type of chitosan.

Without wishing to be bound by any theory or mechanism of action, the present invention utilizes the ability of highly acetylated chitosan to interact with highly deacetylated chitosan and thus avoid precipitation of the highly deacetylated chitosan, which would normally occur under the same pH conditions, in the absence of the highly acetylated chitosan. More specifically, highly deacetylated chitosans precipitate in aqueous solutions at pH values higher than about 6.5 due to protonization of the free amine groups, and are therefore unsuitable on their own for injection into human subjects for hydrogel formation. On the other hand, highly deacetylated chitosans are advantageous for hydrogel formation as they confer mechanical strength and rigidity, and exhibit relatively long term stability since they are poor substrates for lysozyme and are therefore slowly degraded. Highly acetylated chitosans can be used to complement the properties of highly deacetylated chitosans, since the former remain soluble at physiological pH, contribute elasticity and softness to hydrogels comprising them, and are more rapidly degraded by lysozyme. Interaction of the highly acetylated chitosans with highly deacetylated chitosans, presumably via a combination of hydrogen bonds,

hydrophobic bonds and van der Waals forces, enables the formation of a stable hydrogel at pH values in the neutral range, which maintains its solubility even when the pH of its microenvironment is greater than the pKa value of the highly deacetylated chitosans. The disclosed hydrogel forming composition can be utilized in various medical applications, in particular, slow release drug formulations, viscoelastic treatment of degenerative conditions such as osteoarthritis, and implants and scaffolds for tissue regeneration.

According to one aspect, the present invention provides a chitosan composition comprising a highly acetylated chitosan having a degree of acetylation in the range of from about 40% to about 60%, and a highly deacetylated chitosan having a degree of acetylation of no greater than about 20%, wherein at 25 °C and at pH in the neutral range the composition has a viscosity of at least about 100 mPa·s at a shear rate of 50 rpm. According to particular embodiments, at 25 °C and at pH in the neutral range, the composition has a viscosity of no greater than about 70 mPa·s at a shear rate of 200 rpm.

Neutral pH as used herein refers to pH 7.0 ± 0.2.

According to a particular embodiment, at 25 °C and at pH in the neutral range, the composition has a viscosity of at least about 400 mPa·s at a shear rate of 20 rpm, and a viscosity of no greater than about 200 mPa·s at a shear rate of 100 rpm.

According to particular embodiments, at 25 °C and pH in the neutral range, the composition has a viscosity of at least 2 fold, preferably at least 3 fold that of the same composition at pH 6.0, wherein the viscosity is determined at a shear rate of 20 rpm.

According to particular embodiments, at 1 °C and at pH in the neutral range the composition is in the form of a liquid.

According to particular embodiments, at 25 °C and at pH of 6.5 ± 0.2 or less, the composition is in the form of a liquid.

According to another aspect, the invention further provides a chitosan composition in the form of a hydrogel, the composition comprising a highly acetylated chitosan having a degree of acetylation in the range of from about 40% to about 60%, and a highly deacetylated chitosan having a degree of acetylation of no greater than about 20%, wherein at 25 °C and at pH 7.0 ± 0.2 the composition has a viscosity of at least 100 mPa·s at a shear rate of 20 rpm.

According to particular embodiments, at 25 °C and at pH 7.0 ± 0.2, the composition has a viscosity of at least about 100 mPa·s at a shear rate of 50 rpm. According to particular embodiments, at 25 °C and at pH 7.0 ± 0.2, the composition has a viscosity of at least about 200 mPa·s at a shear rate of 50 rpm. According to particular embodiments, at 25 °C and at pH 7.0 ± 0.2, the composition has a viscosity of at least about 400 mPa·s at a shear rate of 20 rpm.

According to yet another aspect, the invention further provides a chitosan composition in the form of an injectable solution, the composition comprising a highly acetylated chitosan having a degree of acetylation in the range of from about 40% to about 60%, and a highly deacetylated chitosan having a degree of acetylation of no greater than about 20%, wherein at 25 °C and at pH below the neutral range the composition has a viscosity of no greater than about 150 mPa·s at a shear rate of 50 rpm.

According to particular embodiments, at 25 °C and at pH below the neutral range the composition has a viscosity of no greater than about 100 mPa·s at a shear rate of 50 rpm.

According to particular embodiments, the highly acetylated chitosan has a degree of acetylation in the range of from about 45% to about 55%, and the highly deacetylated chitosan has a degree of acetylation of about 15% or less.

According to particular embodiments, the highly acetylated chitosan is a homogenously reacetylated chitosan. According to particular embodiments, the highly deacetylated chitosan is a non-homogenously deacetylated chitosan.

According to particular embodiments, the highly deacetylated chitosan has a molecular weight of at least about 100 kDa, and the highly acetylated chitosan has a molecular weight of at least about 200 kDa.

According to particular embodiments, the highly deacetylated chitosan has a molecular weight in the range of from about 100 kDa to about 2000 kDa, and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 2000 kDa.

According to particular embodiments, the highly deacetylated chitosan has a molecular weight in the range of from about 100 kDa to about 700 kDa, such as for example, about 100 kDa to about 400 kDa, or about 400 kDa to about 700 kDa; and the

highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa.

According to particular embodiments, the highly deacetylated chitosan has a molecular weight in the range of from about 400 kDa to about 700 kDa, and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa. According to particular embodiments, the highly deacetylated chitosan has a molecular weight selected from the group consisting of about 100 kDa; about 400 kDa, and about 650 kDa; and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa.

According to particular embodiments, the highly deacetylated chitosan and the highly acetylated chitosan are each present at a concentration of about 0.2% to about 3% w/v of the total composition. According to another particular embodiment, the highly deacetylated chitosan and the highly acetylated chitosan are each present at a concentration of about 0.5 % to about 2% w/v of the total composition. According to another particular embodiment, the highly deacetylated chitosan and the highly acetylated chitosan are each present at a concentration of about 1% to about 1.2% w/v of the total composition.

According to a particular embodiment, the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is selected from the group consisting of 1:1; 1:2; 1:3 and 1:4.

According to a particular embodiment, each of the highly deacetylated chitosan and the highly acetylated chitosan are present at a concentration of about 1% to about 1.2 % w/v of the total composition, and the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is 1:1.

According to a particular embodiment, the highly deacetylated chitosan has a molecular weight in the range of from about 400 kDa to about 700 kDa, and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa, wherein each of the highly deacetylated chitosan and the highly acetylated chitosan are present at a concentration of from about 1% to about 1.2% w/v of the composition; and wherein the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is 1:1.

According to a particular embodiment, the highly deacetylated chitosan has a molecular weight of about 2000 kDa and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa, wherein the concentration

of the highly deacetylated chitosan is 0.5% w/v of the total composition, and wherein the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is selected from the group consisting of 1:2; 1:3 and 1:4.

According to a particular embodiment, the hydrogel has a degradation profile in which no more than about 50% of the hydrogel is degraded by 4 days following its formation.

According to a particular embodiment, the composition further comprises a lysozyme inhibitor, wherein the lysozyme inhibitor is selected from the group consisting of a protein and a saccharide. According to a particular embodiment, the saccharide is tri-N-acetyl-glucosamine. According to a particular embodiment, the lysozyme inhibitor is bound to the highly acetylated chitosan. According to a particular embodiment, the composition comprises tri-N-acetyl-glucosamine bound to the highly acetylated chitosan.

According to a particular embodiment, the chitosan composition further comprises at least one negatively charged substance selected from a polysaccharide, a phospholipid and combinations thereof. According to a particular embodiment, the negatively charged polysaccharide is selected from the group consisting of an animal-derived polysaccharide, a plant-derived polysaccharide, a glycosaminoglycan, and combinations thereof. According to a particular embodiment, the glycosaminoglycan is selected from the group consisting of chondroitin sulfate, dermatan sulfate, dextran sulfate, heparan sulfate, heparin, hyaluronic acid and keratan sulfate.

According to a particular embodiment, the phospholipid is phosphatidylcholine. According to a particular embodiment, the chitosan composition further comprising hyaluronic acid and phosphatidylcholine is for use in synovial fluid replacement and/or in viscoelastic treatment, for example in the treatment of osteoarthritis.

According to a particular embodiment, the composition comprises microspheres of chitosan encapsulating a drug and/or electrospun chitosan fibers embedded in the gel.

According to a particular embodiment, the composition further comprises at least one of a drug, a polypeptide and a cell (such as an animal cell or a plant cell).

According to a particular embodiment, the composition further comprises an emulsifier. In a particular embodiment, the chitosans and the emulsifier are in the form of nanoparticles. In a particular embodiment, the nanoparticles are encapsulated in the hydrogel.

According to a particular embodiment, the composition is formulated for administration by a route selected from direct instillation, injection and endoscopic administration.

According to a particular embodiment, an implantable device comprises the composition in the form of a hydrogel. Such an implantable device may be for a use selected from tissue repair, tissue reconstruction, tissue construction, and tissue replacement.

According to a particular embodiment, an anti-adhesion device comprises the composition of the invention, which may be used in applications such as cardiothoracic surgery and abdominal surgery.

According to a particular embodiment, a drug delivery device or system comprises the composition of the invention. The drug delivery device or system may be for slow release of an embedded medication. Non-limiting examples of drugs for use in this system include proteins and non-protein agents such as, for example, ACE-inhibitors, anti-inflammatory drugs, opthalmological drugs and urological drugs. In other embodiments, a cosmetic agent delivery device comprises the composition of the invention. An example of a suitable cosmetic agent is an anti-wrinkle agent The drug delivery device or system, or the cosmetic agent delivery device may also optionally comprise one or more of a mineral, a vitamin, a food additive or natural extract such as a plant derived extract. The hydrogel itself, optionally with an active ingredient, may be used as a food additive.

In particular embodiments, any of the drug, the cosmetic agent, the mineral, the vitamin, the food additive or the natural extract are in the form of nanoparticles, wherein the nanoparticles are encapsulated in the hydrogel of the invention.

According to a particular embodiment, a three-dimensional gel construct comprises the hydrogel of the invention, wherein the hydrogel is a support for cells. According to a particular embodiment, the cells are endogenous cells or exogenous cells, and the construct optionally further comprises exogenous growth factors. According to a particular embodiment, a cell-loaded artificial matrix comprises the hydrogel of the invention. According to a particular embodiment, the cells are selected from the group consisting of chondrocytes, fibrochondrocytes, ligament fibroblasts, skin fibroblasts, tenocytes, myofibroblasts, mesenchymal stem cells and keratinocytes.

According to a particular embodiment, there is provided a use of the composition of the invention for the preparation of a medicament for treating heel pain. According to

a particular embodiment, there is provided a use of the composition of the invention for the preparation of a medicament for wound healing. According to a particular embodiment, there is provided a use of the composition of the invention for the preparation of a medicament for disrupting biofilm. According to a particular embodiment, there is provided a use of the composition of the invention for the preparation of a medicament for preventing or treating surgical adhesions. According to a particular embodiment, there is provided a use of the composition of the invention for the preparation of a medicament for treating rotator cuff damage, including rotator cuff tears, wherein the medicament optionally further comprises autologous cells. According to a particular embodiment, there is provided a use of the composition of the invention for the preparation of a medicament for treating osteoarthritis, wherein the composition further optionally includes hyaluronic acid and phosphatidylcholine as a synovial fluid replacement, as described herein.

According to some embodiments, the chitosan hydrogel may be used as a lubricating agent for treatment of conditions such as vaginal atrophy, dry eyes, conjunctivitis sicca, dry nose following upper respiratory infections, as well as a general soothing agent for various abrasions.

According to a further aspect, the invention provides a chitosan composition comprising a highly deacetylated chitosan having a molecular weight in the range of from about 100 to about 4000 kDa, such as about 100 kDa to about 2000 kDa, and having a degree of acetylation of no greater than about 20%, and a saccharide oligomer having a molecular weight in the range of from about 200 to about 20000 Da, wherein the composition is in a form of an aqueous solution.

In particular embodiments, the saccharide oligomer is selected from the group consisting of a chitosan oligomer; a D-glucosamine oligomer and an N-acetyl-D-glucosamine oligomer. In a particular embodiment, the chitosan oligomer is selected from the group consisting of a highly deacetylated chitosan oligomer having a degree of acetylation of no greater than about 20%, and a highly acetylated chitosan oligomer having a degree of acetylation of from about 40% to about 60%. According to particular embodiments, the highly acetylated chitosan oligomer has a degree of acetylation in the range of from about 45% to about 55%. According to particular embodiments, the highly deacetylated chitosan oligomer has a degree of acetylation of about 15% or less. According to particular embodiments, the N-acetyl-D-glucosamine oligomer has up to about 7 units. According to particular embodiments, the D-glucosamine oligomer has

between 3 and about 100 units. In a particular embodiment, the D-glucosamine oligomer has between 3 and about 50 units.

In particular embodiments, the ratio of the saccharide oligomer and the highly deacetylated chitosan having a molecular weight in the range of from about 100 kDa to about 2000 kDa is greater than 1:1.

In particular embodiments, the ratio ranges from 2:1 to 20:1.

In particular embodiments, at 25 °C and at pH below the neutral range the composition has a viscosity of no greater than 150 mPa·s at a shear rate of 50 rpm. In particular embodiments, at 25 °C and at pH below the neutral range the composition has a viscosity of no greater than 100 mPa·s at a shear rate of 50 rpm. In particular embodiments, at 25 °C and at pH in the neutral range the composition has a viscosity of no greater than 70 mPa·s at a shear rate of 200 rpm.

According to another aspect, the present invention provides a method for the production of a stable hydrogel, wherein the method comprises dissolving in an acidic aqueous solution at least one highly acetylated chitosan having a degree of acetylation in the range of from about 40 to about 60%, and at least one highly deacetylated chitosan having a degree of acetylation of no greater than about 20%, so as to form a composite solution; adjusting the pH of the composite solution to a value of 6.5 to 7.2; and increasing the temperature of the composite solution to 37 °C while raising the pH to a value of 7.0 to 7.6; so as to produce a hydrogel which at 25 °C has a viscosity of at least 100 mPa·s at a shear rate of 50 rpm.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for

purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

FIG. 1 illustrates the formation of a hydrogel according to some embodiments of the present invention from a liquid composition comprising two different types of chitosan.

FIG. 2 illustrates degradation over time of different hydrogel compositions formed from a combination of type 1 and type 2 chitosans. FIG 2A shows degradation of compositions comprising a type 2 chitosan (MW 220 kDa; DA 50) in combination with a type 1 chitosan (MW 65 kDa; DA 15) in a ratio of 1:1 (diamond symbols); 1:2 (square symbols), and 1:3 (triangle symbols), expressed as a percentage of the weight of the gel remaining undegraded. FIG. 2B shows degradation over time of hydrogel compositions formed from a combination of a type 2 chitosan (MW 220 kDa; DA 50) and a type 1 chitosan of molecular weight of either 65 kDa and DA 15 (triangle symbols) or 100 kDa and DA 9 (square symbols), at a ratio of 1:1.

FIG. 3 illustrates release of hemoglobin from a hydrogel composition formed from a combination of a type 1 chitosan (MW 65 kDa; DA 15) and a type 2 chitosan (MW 220 kDa; DA 50), as measured by the amount of protein (μg/ml) in eluent. The systems represented by diamond, square, triangle, star and asterisk symbols correspond to repetitions of the experiment, and the circle symbols represent the mean of the experiments.

FIG. 4 illustrates release of bovine serum albumin (BSA) from a hydrogel composition formed from a combination of a type 1 chitosan (MW 65 kDa; DA 15) and a type 2 chitosan (MW 220 kDa; DA 50), as measured by optical density (OD) of the eluent. The systems represented by diamond, square, triangle, star and asterisk symbols correspond to repetitions of the experiment, and the circle symbols represent the mean of the experiments.

FIG. 5 presents a bar chart illustrating release of BSA from a hydrogel formed from a combination of type 1 chitosan (MW 65 kDa; DA 15) and a type 2 chitosan (MW 220 kDa; DA 50).

FIG. 6 illustrates the degradation over time of hydrogel compositions formed from a combination of a type 1 chitosan (MW 65 kDa; DA 50) and a type 2 chitosan (MW 220 kDa; DA 50), either including (square symbols) or lacking BSA (diamond symbols), expressed as a percentage of the weight of the gel remaining undegraded.

FIG. 7 illustrates the integration of the release profile of BSA (square symbols) with the degradation profile (triangle symbols) of a hydrogel composition formed from a combination of a type 1 chitosan (MW 65 kDa; DA 15) and a type 2 chitosan (MW 220 kDa; DA 50) and BSA.

FIG. 8 shows histopathology of wound bed biopsies taken from rats, either treated with a hydrogel composition formed from a combination of a type 1 chitosan (MW 660 kDa; DA 15) and a type 2 chitosan (MW 220 kDa; DA 50) (Fig. 8A), or untreated (Fig. 8B).

FIG. 9 shows a graph of the results of treating inflicted wounds in diabetic rats with a hydrogel composition formed from a combination of a type 1 chitosan (MW 660 kDa; DA 15) and a type 2 chitosan (MW 220 kDa; DA 50) and acetylglucosamine oligomers (Gel), expressed as the surface area of wound bed biopsies on Day 7 following biopsy. In animals of the control group, the wound was either covered by a plaster bandage (Control + Cover) or left uncovered (Control no Cover).

FIG. 10 shows the results of in vivo experiments performed on rats for rotator cuff damage. Histological slices from rats treated with chitosan hydrogel containing bone marrow cells following suturing (Fig. 10A) show migration of inflammatory cells and repair in the defect area, whereas slices from rats that were untreated following suturing (Fig. 10B) show empty defect areas with no signs of repair.

FIG. 11 presents a schematic illustration of measuring a friction coefficient.

FIG. 12 shows the static friction coefficient measured between two layers of normal cartilage to which were applied: saline; a hydrogel composition formed from a combination of a highly deacetylated chitosan (MW 660 kDa; DA 15) and a highly acetylated chitosan (MW 220 kDa; DA 50) (Combination); the same chitosan combination further containing chondroitin sulfate (+CS); the same chitosan combination further containing chondroitin sulfate and phosphatidyl choline (+CS/PC); or hyaluronic acid (HA).

FIG.13 shows the viscosity of various chitosan compositions as a function of pH when measured at 25 °C. Compositions contained a highly acetylated chitosan (MW 220 kDa DA 50) and a highly deacetylated chitosan (MW 420 kDa DA 9), either each on its own or in combination.

FIG.14 shows the viscosity of compositions comprising two types of chitosans as a function of pH when measured at 25° C. Compositions contained a combination of a highly deacetylated chitosan (MW 65 kDa; DA 15) and a highly acetylated chitosan

(MW 220 kDa; DA 50) at a ratio of 1:1, or a combination of a highly deacetylated chitosan (MW 100 kDa; DA 9) and a highly acetylated chitosan (MW 220 kDa; DA 50) at a ratio of 1:1.

FIG.15 shows the viscosity of compositions comprising two types of chitosans at different pH values when measured at 25 °C and at varying shear rates. Compositions contained a combination of a highly deacetylated chitosan (MW 100 kDa; DA 9) and a highly acetylated chitosan (MW 220 kDa; DA 50) at a ratio of 1:1 at various pH values (Figure 15A); or a combination of a highly deacetylated chitosan (MW 65 kDa; DA 15) and a highly acetylated chitosan (MW 220 kDa; DA 50) at a ratio of 1:1 at various pH values (Figure 15B).

FIG.16 shows the viscosity of compositions comprising two types of chitosans at different pH values when measured at 1 °C and at varying shear rates. Compositions contained a combination of a highly deacetylated chitosan (MW 100 kDa; DA 15) and a highly acetylated chitosan (MW 220 kDa; DA 50) (SK10:50) at a ratio of 1:1 at various pH values; or a combination of a highly deacetylated chitosan (MW 65 kDa; DA 15) and a highly acetylated chitosan (MW 220 kDa; DA 50) (FVL10:50) at a ratio of 1:1 at various pH values.

FIG.17 shows the viscosity of compositions comprising a combination of a highly deacetylated chitosan (MW 100 kDa; DA 9) and a highly acetylated chitosan (MW 220 kDa; DA 50) at a ratio of 1:1 when measured at varying shear rates, either at 1 °C (Figure 17A); or at 25 °C (Figure 17B), both at various pH values.

FIG.18 shows the viscosity of compositions comprising a combination of a highly deacetylated chitosan (MW 65 kDa; DA 15) and a highly acetylated chitosan (MW 220 kDa; DA 50) at a ratio of 1:1 when measured at varying shear rates and at 1 °C (Figure 18A); or at 25 °C (Figure 18B), both at various pH values.

FIG.19 shows the viscosity of a composition comprising two types of chitosans at different pH values when measured at 25° C at varying shear rates. The composition contained a combination of a highly deacetylated chitosan (MW 660 kDa; DA 15) and a highly acetylated chitosan (MW 220 kDa; DA 50) at a ratio of 1:1 at various pH values.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The pH-dependent and thermosensitive chitosan hydrogels of the present invention are advantageous over prior art chitosan gel-forming compositions comprising

a single type of chitosan. In particular, the use of a combination of chitosans as described herein confers improved physical, chemical and pharmacokinetic properties of the resultant hydrogel, as compared to gels or hydrogels comprising a single type of chitosan. As used herein, a composition "comprising a single type of chitosan" refers to compositions in which either a highly deacetylated chitosan or a highly acetylated chitosan is the sole chitosan present in the composition. More particularly, hydrogels which contain only a highly acetylated chitosan, as described for example in WO 2005/097871, are mechanically much weaker than the hydrogels of the present invention, possibly due to the different interactions responsible for gel formation. In addition, gels formed solely from highly acetylated chitosans generally require a high chitosan concentration to effect gel formation, and/or require an additional agent, such as 1,3-propanediol for improving the viscoelastic behavior of the gel, as disclosed for example in WO 2005/097871. Furthermore, in some cases, compositions which contain only a highly acetylated chitosan will form gels only at low temperatures, which negates their use for in vivo applications. Finally, gels formed solely from highly acetylated chitosans are subject to rapid enzymatic degradation by serum enzymes such as lysozyme, for which they are favorable substrates. Accordingly, such gels have relatively shorter half-life in vivo in comparison to the hydrogels of the invention, and accordingly, the former are not suitable for preparation of slow release drug formations, implants and devices which require prolonged resilience and stability.

In addition, highly deacetylated chitosans on their own precipitate at pH values which are greater than their pKa, and therefore require an additional agent such as β-glycerophosphate for effecting their stability and gelation at pH values in the neutral range, as disclosed for example in U.S. Patent No. 6,344,488. Such gels are hampered by limitations with respect to degradation rate, gelation time and viscosity, as described in the Background.

Definitions

As used herein, the term "about" when used in reference to a numerical value means that value ± 10%.

As used herein, the term "pseudo-thermosetting" in connection with the composition of the present invention means that temperature does not induce the gelation of the composition but acts rather as a catalyst which dramatically shortens the gelation time when risen.

As used herein, the term "hydrogel" refers to a three-dimensional macromolecular network having a degree of hydration of at least 90%, and which exhibits substantially no flow when in the steady-state. The chitosan hyrogels of the present invention generally attain a steady state at pH values greater than about 6.5 and at temperatures greater than about 4 °C. It is to be expressly understood that a chitosan hydrogel according to the invention does not encompass a chitosan gel formed from a single type of chitosan such as a highly deacetylated chitosan in the swollen state.

As used herein, the term "viscosity" refers to a measure of the resistance of a fluid which is being deformed by either shear stress or extensional stress. Newtonian fluids exhibit constant viscosity over a wide range of shear rates i.e. they are independent of the shear rate, whereas non-Newtonian fluids (such as polymeric gels) exhibit varying viscosity in response to different shear rates. Non-Newtonian fluids exhibit a variety of different correlations between shear stress and shear rate. Generally, the viscosity of non-Newtonian fluids decreases at high shear rates (a phenomenon known as shear thinning), and the viscosity increases at low shear rates.

The SI physical unit of dynamic viscosity is the pascal-second (Pa·s), which is identical to $kg \cdot m^{-1} \cdot s^{-1}$. The millipascal (mPa·s) is 0.01 Pa·s. Another unit used to express viscosity in the poise (P), wherein the relationship between pascal-second and poise is $10\ P = 1\ kg \cdot m^{-1} \cdot s^{-1} = 1\ Pa \cdot s$. Viscosity of a non-Newtoninan fluid may be measured with a rheometer, which imposes a specific stress field or deformation to the fluid, and monitors the resultant deformation or stress.

As used herein, the term "non-homogeneous deacetylation" in reference to chitosan means that the deacetylated units of D-glucosamine occur in blocks of variable size and/or distribution. As used herein, the term "homogeneous deacetylation" in reference to chitosan means that the deacetylated units of D-glucosamine are randomly distributed along the polymer.

As used herein, the term "homogeneous reacetylation" in reference to chitosan means that deacetylated chitosan is reacetylated in a manner in which the N-acetylD-glucoasamine units and the D-glucosamine units are randomly distributed along the polymer.

As used herein, the terms "neutral pH", "pH in the neutral range", "neutralized", "physiological pH" and the like mean a pH of 7.0 ± 0.2.

As used herein, the term "saccharide oligomer" means a saccharide having a degree of polymerization of 3 to about 100 monomeric units. The oligomer may be

formed from a single type of saccharide unit, for example only D-glucosamine units, or it may contain different types of saccharide units, for example D-glucosamine units and N-acetyl-D-glucosamine units, as in a chitosan oliogmer.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Chitosans which are deacetylated to a degree of deacetylation (also referred to herein as DD or DDA) of about 70-100% (i.e. degree of acetylation, DA, of up to about 30%), such as commercially available chitosan, are termed herein type 1 chitosans or chitosans type 1. These chitosans are insoluble at physiological pH, and are poorly recognized by lysozyme. Such chitosans, when utilized in *in vivo* applications, are typically characterized by relatively slow biodegradation, which, depending on the degree of deacetylation, can last from a few days to a few months. Gels formed by chitosans of this type have a low degree of acetylation, such that the free amine groups participate in dense hydrogen bonds with many hydrophobic interactions.

The degradation rate of chitosans has been shown to be a function of the degree of deacetylation. Degradation of chitosan has an influence on cell proliferation and remodeling.

Highly homogeneously deacetylated or reacetylated chitosans (having a degree of acetylation of from about 30% to about 60%) are termed herein type 2 chitosans or chitosans type 2. Such chitosans are readily digested/degraded by lysozyme, thereby enabling, for example, controlled drug release of a drug encapsulated therein.

If the degree of deacetylation of chitosan is lower than 30%, the chitosan becomes a polymer close to chitin that is insoluble in acidic conditions and therefore not suitable for use in embodiments of the present invention. At a degree of deacetylation greater than 70%, precipitation of chitosan occurs.

The degree of deacetylation of chitosan may be determined by a spectrophotometric method such as described, for example, in the literature by R.A. Muzarelli and R. Richetti [Carbohydr. Polym. 5, 461-472, 1985 or R.A. Muzarelli and R. Richetti in "Chitin in Nature and Technology", Plenum Press 385-388, 1986]. Briefly, in the latter method for example, chitosan is solubilized in 1% acetic acid and the DD is determined by measuring its content of N-acetyl-glucosamine by UV at 200, 201, 202, 203 and 204 nm using N-acetyl-D-glucosamine solutions as standards.

According to one embodiment, the present invention relates to a polysaccharide chitosan composition comprising a combination of at least one highly acetylated chitosan (type 2) having a degree of acetylation in the range of from about 40% to about 60%, and at least one highly deacetylated chitosan (type 1), having a degree of acetylation of no greater than about 20%. The highly acetylated type 2 chitosans can interact through electrostatic, hydrogen and hydrophobic interactions with the highly deacetylated chitosans type 1. The extent of interaction increases with increasing pH. A composition comprising solutions of both types of chitosan can form a stable gel at physiological pH, without the need for glycerophosphate. The obtained composition is therefore devoid of glycerophosphate.

Thus, the chitosan composition described is either in the form of an aqueous solution, or a hydrogel. The transition from the liquid state to a hydrogel occurs at pH conditions above about 6.5. The hydrogel is stable at physiological conditions i.e. pH in the neutral range and 37 °C.

It is noted that the composition described herein can form a gel at room temperature or at lower temperatures (e.g., 4 °C). Nonetheless, the gel formation at such conditions is slow and may last from a few days to a few months, thus enabling to store and transfer the composition as an aqueous solution.

Type 1 chitosans in the unprotected state precipitate at a pH of about 6.5, which is less than physiological pH. Interaction of the highly hydrophobic, homogenously acetylated chitosan type 2 with chitosan type 1 prevents this precipitation of the non-homogenously acetylated type 1 chitosan, by formation of hydrogen and hydrophobic bonds, allowing the formation of a stable semi-solid hydrogel at pH 7.0 in the neutral range.

The secondary bonds which are formed allow the encapsulation of the non-homogenous chitosan chains and maintain its solubility at pH greater than its pKa value.

Generally, such secondary chain interactions are the main molecular forces involved in gel formation (Chenite et al., 2000; Berger et al., 2005).

Type 1 chitosans mainly contribute to the stability, strength and rigidity of the gel, and provide slow degradation, while type 2 chitosans contribute to the softness, elasticity and fast solubilization of the gel. The degradation profiles of compositions comprising type 1 and type 2 chitosans are discussed further in Example 2 below, and are shown in Figure 2. In particular, the present inventors have shown that a hydrogel formed from a type 1 chitosan having a molecular weight of about 100 kDa and a type 2 chitosan having a molecular weight in the range of 200 to 250 kDa provides a substantially linear degradation rate, wherein at least 50% of the hydrogel remains undegraded by 4 days following gel formation (Figure 2B). In contrast, gels formed from the same type 2 chitosan and a type 1 chitosan of lower molecular weight, display less desirable two-phase degradation patterns (Figure 2A). The type 2 chitosan can be regarded as a "protector" or "coating", which provides a shell around the type 1 chitosan and thus prevents its precipitation.

Furthermore, the type 2 chitosan is recognized by lysozyme. This feature enables control of the degradation rate of a hydrogel formed from the composition described herein. For example, binding a lysozyme inhibitor, which may be a protein or a saccharide, to the type 2 chitosan can slow the degradation rate of the formed hydrogel. A suitable example of a lysozyme inhibitor is the saccharide tri-N-acetylglucosamine. Alternatively, it may be desirable that the composition have a relatively rapid degradation rate, for example in wound healing, as disclosed in Example 4 herein.

The physical and chemical properties of the hydrogel formed from the described composition are altered by raising or lowering the molecular weight of the chitosans and/or their degree of acetylation, and by the natural acetylation diversity of chitosans from different sources. The properties of the gel can further be controlled by selection of the type of reacetylation (i.e. homogenous or non-homogenous), or by mapping the patterns of distribution of the deacetylated/acetylated sites.

Preferably, the highly acetylated chitosan is homogenously reacetylated. Further preferably, the highly deacetylated chitosan is non-homogenously deacetylated.

Generally, commercially available chitosan is industrially prepared by deacetylation of dry chitin flakes (Muzzarelli, 1986). Deacetylation preferentially occurs in the amorphous zones of the chitin molecules at the surface of the flakes,

resulting in non-homogeneous monomers with variable block size of deacetylated unit distribution (Aiba, 1991). In comparison, reacetylated chitosan under homogeneous conditions, adopts a random distribution of deacetylated monomers, which induces a decrease of the crystallinity of chitosan and in turn increases its solubility (Aiba, 1991, 1994; Ogawa and Yui, 1993; Milot et. al., 1998).

Methods for non-homogenous deacetylation are disclosed for example in U.S. Patent No. 4,195,175; Vårum et al., pages 127- 136 in "Advances in chitin chemistry", Ed. C. J. Brine, 1992; Ottoy et al., Carbohydrate Polymers 29:17-24 (1996); Sannan et al., Macromol. Chem. 176:1191-1195 (1975); Sannan et al, Macromol. Chem. 177: 3589-3600 (1976); Kurita et al., Chemistry Letters 1597-1598 (1989); and CA 2,101,079.

Homogeneous reacetylation of chitosan has the effect of increasing the number of hydrophobic sites by replacing amine groups with acetyl groups, and also reduces the crystalline structure that makes chitosan tend to fold, cumulating in increased solubility of the chitosan. Reacetylation prevents refolding of the polymer, maintaining the straight chain, and thus preventing the pH-related decrease in solubility. Generally, any commercial chitosan of pharmaceutical grade and sufficient molecular weight may be used in the preparation of reacetyated chitosan. Methods for homogeneous reacetylation are disclosed for example in WO 2005/097871 and WO 03/011912.

Following reacetylation, the product may be characterized with respect to the degree of deactylation, for example as measured by a UV method (Muzarelli et al. in "Chitin in Nature and Technology", Plenum Press, New York, 385- 388, (1986)), and with respect to the molecular weight, for example as determined by size exclusion chromatography (Felt et al. Int. J. Pharm. 180:185-193 (1999)).

In a particular embodiment, the chitosan composition disclosed herein has a viscosity of at least 100 mPa·s at a shear rate of 50 rpm when the composition is at 25 °C and at pH in the neutral range. At a higher shear rate of 200 rpm, the viscosity is not greater than 70 mPa·s, when the composition is at the same temperature and pH conditions. According to another embodiment, at 25 °C and at pH 7.0 ± 0.2 the composition has a viscosity of at least 100 mPa·s at a shear rate of 20 rpm. According to another embodiment, at 25 °C and at pH in the neutral range, the composition has a viscosity of at least 400 mPa·s at a shear rate of 20 rpm. According to another embodiment, at 25 °C and at pH in the neutral range, the composition has a viscosity of

no greater than 200 mPa·s at a shear rate of 100 rpm. According to another embodiment, at 25 °C and at pH 7.0 ± 0.2 the hydrogel composition has a viscosity of at least 100 mPa·s at a shear rate of 20 rpm. According to another embodiment, at 25 °C and at pH 7.0 ± 0.2 the hydrogel composition has a viscosity of at least 100 mPa·s, or at least 200 mPa·s, at a shear rate of 50 rpm. Preferably, at 25 °C and at pH in the neutral range, the composition has a viscosity that is at least 2 fold greater than that of the same composition at pH 6.0, when the viscosity is determined at a shear rate of 20 rpm. Also preferably, at 25 °C and at pH in the neutral range, the composition has a viscosity that is at least 3 fold greater than that of the same composition at pH 6.0, when the viscosity is determined at a shear rate of 20 rpm. According to another embodiment, at 25 °C and at pH below the neutral range, the liquid composition has a viscosity of no greater than about 150 mPa·s, at a shear rate of 50 rpm. According to another embodiment, at 25 °C and at pH below the neutral range, the liquid composition has a viscosity of no greater than about 100 mPa·s, at a shear rate of 50 rpm.

The demonstration that the viscosity of the composition increases as the shear force decreases, as shown for example in Figure 19, is an indication of non-Newtonian behavior, characteristic of gels, including hydrogels. That is, when such compositions are in the undisturbed state, they "set" into a gel having characteristics seemingly of a solid, while in fact due to their highly hydrated state they have density more similar to a liquid as compared to a solid. Further, when the compositions are subjected to a stronger applied force, they remain substantially in the liquid state. Furthermore, and as disclosed in Example 9, the chitosan hydrogels of the invention are formed only when the compositions are in the neutral pH range, since at lower pH values they exhibit low viscosity which is substantially independent of the shear force applied. In other words, at pH less than that of the neutral range, the compositions remain in substantially a liquid state, indicating their suitability for low pH and/or refrigeration storage in the liquid state prior to administration, for example by injection for in vivo gel formation. Increasing the molecular weight of the chitosan increases its viscosity, such that the polymer is highly hydrated and highly hydrophobic. This is demonstrated for example in Example 9 and Figure 19 which disclose that the viscosity of the composition FM80-50 1:1 comprising a type 1 chitosan of molecular weight 660 kDa is substantially higher than that of the composition SK10:50 1:1, the latter of which comprises a type 1 chitosan of molecular weight of only 100 kDa. More specifically, in their gelled states,

these compositions exhibit viscosities of about 420 and about 110 mPa·s, respectively. Accordingly, the invention enables formation of hydrogels of varying strength and water retention. This enables one of ordinary skill in the art to produce a hydrogel of selected degradation rate, drug release, and mechanical strength.

Preferably, each of the highly acetylated and highly deacetylated chitosans has a molecular weight of greater than about 100 kDa. In a particular embodiment, the highly deacetylated chitosan may have a molecular weight of at least about 100 kDa, and the highly acetylated chitosan may have a molecular weight of at least about 200 kDa. In another particular embodiment, the highly deacetylated chitosan has a molecular weight in the range of from about 100 kDa to about 2000 kDa, and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 2000 kDa. According to particular embodiments, the highly deacetylated chitosan has a molecular weight in the range of from about 100 kDa to about 700 kDa, such as for example, about 100 kDa to about 400 kDa, or about 400 kDa to about 700 kDa; and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa. According to a particular embodiment, the highly deacetylated chitosan has a molecular weight in the range of from about 400 kDa to about 700 kDa, and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa. According to a particular embodiment, the highly deacetylated chitosan has a molecular weight selected from the group consisting of about 100 kDa; about 400 kDa, and about 650 kDa; and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa.

Molecular weight of chitosan may be determined by size exclusion chromatography as reported for example by O. Felt, P. Purrer, J. M. Mayer, B. Plazonnet, P. Burri and R. Gurny in Int. J. Pharm. 180, 185- 193 (1999). The upper limit of MW is determined by the required ease of administration, which depends on the chosen application.

Increasing the degree of acetylation results in increased hydrophobicity in the range of 0 to 30% DA, but at higher values, such as 40 to 60% DA, the polymer become more soluble as the DA is increased. Furthermore, increasing the number of N-acetyl glucosamine groups increases the rate of degradation in the body, due to increased recognition sites for lysozyme. Hence, the rate of release of drug from the hydrogel can be controlled by varying the degree of chitosan acetylation.

Variations in the molecular weight, degree of deacetylation and the distribution of the acetylated sites, concentration and ratio of the two or more chitosans, affect the conditions (pH, temperature etc.) under which gel formation occurs; solubility; biodegradability; degree of reactivity with proteins, active pharmaceutical ingredients or other chemicals; hydrophobicity/hydrophilicity; degree of hydration; as well as biological and biocompatibility properties of the gel, such as effect on cell growth, proliferation and survival, ability of chitosans to function as inflammatory or anti-inflammatory mediators, and the effect of chitosans on acceleration or deceleration of wound healing.

For example, type 1 chitosans of higher molecular weight have higher hydrophobicity and higher viscosity, resulting in a stronger gel due to higher inter-molecular interactions. Type 1 chitosans of higher DDA have a lower rate of degradation. Type 1 chitosans having higher crystallinity have a lower degradation rate due to the fact that the crystalline form is non-soluble. Hence one skilled in the art can predict properties of the resultant gel mixture, and would therefore be able to create gels having desired characteristics, using unique combinations of the different types of chitosan.

As shown in Examples 7 and 9, variation of the molecular weight of the chitosans used, the concentration thereof and the ratio between the type 1 chitosan and type 2 chitosan affect the conditions at which gel formation occurs. Hence, one skilled in the art can be able to select the appropriate parameters in order to produce a hydrogel under physiological conditions.

Preferably, each of the highly acetylated and the highly deacetylated chitosans is independently present at a concentration of about 0.2% to 3% w/v of the total composition.

In some embodiments, each of highly acetylated and the highly deacetylated chitosans is independently present at a concentration of about 0.5% to 2% w/v of the total composition.

In some embodiments, each of highly acetylated and the highly deacetylated chitosans is independently present at a concentration of about 1% to 1.2% w/v of the total composition.

In some embodiments, the ratio between the highly acetylated and the highly deacetylated chitosans is 1:1, such that the above concentrations are for each of the highly acetylated and the highly deacetylated chitosans.

In other embodiments, and depending, for example, on the molecular weight of the chitosans used, as well as their concentration, the ratio can be 2:1, 3:1 and even 4:1. Also contemplated are ratios such as 1.1:1, 1.2:1, 1.5:1 1.8:1, and any other ratio in the range of from 1:1 to 4:1.

According to a particular embodiment, each of the highly deacetylated chitosan and the highly acetylated chitosan are present at a concentration of about 1% to about 1.2% w/v of the total composition, and the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is 1:1.

According to a particular embodiment, the highly deacetylated chitosan has a molecular weight in the range of from about 400 kDa to about 700 kDa, and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa, wherein each of the highly deacetylated chitosan and the highly acetylated chitosan are present at a concentration of from about 1% to about 1.2% w/v of the composition; and wherein the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is 1:1.

According to a particular embodiment, the highly deacetylated chitosan has a molecular weight of about 2000 kDa and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa, wherein the concentration of the highly deacetylated chitosan is 0.5% w/v of the composition, and wherein the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is selected from the group consisting of 1:2; 1:3 and 1:4.

In general, it can be assumed that increasing the MW of any of the chitosans used for forming the hydrogel, and particularly the type 1 chitosan, allows decreasing its concentration and vise versa, decreasing the MW of the chitosan requires increased concentration thereof, in order to form a hydrogel.

The composition described herein offers greater possibility of controlling the properties of the formed hydrogel, including, for example, the hydrogel strength, rate of degradation, and release rate, as compared for example to previously disclosed chitosan/β-glycerophosphate hydrogels.

The hydrogel of the present invention may further comprise a third chitosan, selected from either type 1 or type 2, having a different molecular weight or degree of deacetylation, thus extending control over the resultant hydrogel.

The polysaccharide hydrogel according to the present invention may optionally further comprise a negatively charged substance, such as a negatively charged

polysaccharide or a negatively charged phospholipid. Negatively charged polysaccharides include various animal-derived polysaccharides, plant-derived polysaccharides, glycosaminoglycans and combinations thereof. Glycosaminoglycans include for example, chondroitin sulfate, dermatan sulfate, dextran sulfate, heparan sulfate, heparin, hyaluronic acid, keratan sulfate and combinations thereof.

An exemplary suitable phospholipid is phosphatidylcholine. According to a particular embodiment, the chitosan composition further comprises hyaluronic acid and phosphatidylcholine. Such a composition is highly beneficial for use as a synovial fluid replacement in osteoarthritis treatment, as it lowers the friction between cartilage surfaces, as disclosed in Example 6.

In some embodiments, the chitosan composition described herein further comprises both a glycosaminoglycan and a phospholipid.

Thus, different compositions and mixtures based on these two types of chitosans may be used to provide hydrogels with suitable properties for a wide range of applications. Exemplary applications include, but are not limited to drug delivery systems e.g. for slow release of agents or medications, scaffolding of various consistencies, including gels for supporting cell growth or bone structural support; cartilage repair; tissue reconstruction; in wound-dressings, promoting scar free healing and macrophage activation; for production of artificial skin; as an artificial kidney membrane; for bone filling; and heel pain relief and as synovial fluid replacement compositions.

The hydrogel may be formed in situ (in vivo) sub-cutaneously, intra-peritoneally, intra-muscularly or within biological connective tissues, bone defects, fractures, articular cavities, body conduits or cavities, eye cul-de-sac, or solid tumors.

The polysaccharide solution may be introduced within an animal or human body by injection or endoscopic administration.

Drugs, polypeptides, living microorganisms, animal or human cells may be incorporated within the polysaccharide solution prior to gelation.

In accordance with the present invention there is also provided the use of the polysaccharide gel formed from the compositions described herein for producing biocompatible degradable materials used in cosmetics, pharmacology, medicine and/or surgery.

Herein, a hydrogel encompasses a semi-solid gel formed from the chitosan aqueous solutions described herein, upon subjecting these solutions to the physiological

conditions described herein. The hydrogel is preferably formed in vivo, upon administration of the chitosan composition, but can alternatively be formed ex vivo prior to its utilization, for example as an implant.

The gel may be incorporated as a whole, or as a component, into implantable devices or implants for repair, reconstruction and/or replacement of tissues and/or organs, either in animals or humans.

The gel may be used as a whole, or as a component of, implantable, transdermal or dermatological drug delivery systems.

The gel may be used as a whole, or as a component of, ophthalmological implants or drug delivery systems.

The gel may be used for producing cells-loaded artificial matrices that are applied to the engineering and culture of bioengineered hybrid materials and tissue equivalents.

The loaded cells may be selected from the group consisting of chondrocytes (articular cartilage), fibrochondrocytes (meniscus), ligament fibroblasts (ligament), skin fibroblasts (skin), tenocytes (tendons), myofibroblasts (muscle), mesenchymal stem cells, keratinocytes (skin), and neurons, as well as adipocytes or bone marrow cells. In fact cells from any tissue which are capable of proliferation may optionally be embedded in such a construct.

A major detriment to wound healing is the presence of biofilm. Biofilm is composed of at least 80 percent extracellular macromolecules that are usually positively charged, similar to chitosan. Chitosan may optionally be used as a biofilm disruptor thus helping wound hygiene and limiting the inhibitory effect of biofilm on destruction of bacteria. Chitosan gel mixed with lactoferrin may optionally act as a slow release reservoir to destroy biofilm in any chronic wound or a wound that may become chronic. Chitosan gel mixed with xylitol may optionally also be a specific biofilm disruptor.

In accordance with the present invention there is also provided the use of loaded polysaccharide gel as injectable or implantable gel biomaterials which act as supports, carriers, reconstructive devices or substitutes for the formation in situ of bone-like, fibrocartilage-like or cartilage-like tissues at a physiological location of an animal or a human.

For example, chitosan gels according to the present invention may be useful as a sustained delivery drug-system for treatment of the eye. Results based on the ocular irritation test of chitosan compounds have indicated that chitosan preparations are

suitable for use as ophthalmic gels based on their excellent tolerance (Molinaro et. al., 2002).

In accordance with a further embodiment of the present invention, a slow release drug delivery hydrogel system is provided comprising highly acetylated type 1 chitosans and highly deacetylated type 2 chitosans.

Any of the drug delivery systems of the present invention may be used for delivery of a wide variety of drugs, including, but not limited to, analgesics, anesthetics, antiacne agents, antiaging agents, antibacterials, antibiotics, antiburn agents, antidepressants, antidermatitis agents, antiedemics, antihistamines, antihelminths, antihyperkeratolyte agents, antiinflammatory agents, antiirritants, antilipemics, antimicrobials, antimycotics, antioxidants, antipruritics, antipsoriatic agents, antirosacea agents antiseborrheic agents, antiseptics, antiswelling agents, antiviral agents, antiyeast agents, cardiovascular agents, chemotherapeutic agents, corticosteroids, fungicides, hormones, hydroxyacids, keratolytic agents, lactams, mitocides, non-steroidal anti-inflammatory agents, pediculicides, progestins, sanatives, scabicides, and vasodilators.

According to another aspect, the present invention provides a method for the production of a stable hydrogel, wherein the method comprises dissolving in an acidic aqueous solution at least one highly acetylated chitosan having a degree of acetylation in the range of from about 40 to about 60%, and at least one highly deacetylated chitosan having a degree of acetylation of no greater than about 20%, so as to form a composite solution; adjusting the pH of the composite solution to a value of 6.5 to 7.2; and increasing the temperature of the composite solution to 37 °C while raising the pH to a value of 7.0 to 7.6; so as to produce a hydrogel which at 25 °C has a viscosity of at least 100 mPa·s at a shear rate of 50 rpm.

In some embodiments, dissolving of the highly acetylated chitosan and the highly deacetylated chitosan is performed simultaneously in the same vessel.

Optionally, dissolving the highly acetylated chitosan and the highly deacetylated chitosan is performed in separate vessels to form two solutions. In such embodiment, the process further comprises mixing these two solutions to form the composite solution.

Further according to some embodiments of the present invention there is provided a pH-dependent and temperature-dependent hydrogel formed by the process described herein.

The present inventors have further found that a mixture of a highly deacetylated chitosan and various saccharide oligomers including chitosan oligomers, also forms a hydrogel having the desired properties, as described hereinabove.

Thus, according to a further aspect of the present invention, there is provided a chitosan composition comprising at least one highly deacetylated chitosan having a molecular weight in the range of from about 100 to about 4000 kDa, such as from about 100 kDa to about 2000 kDa (a chitosan polymer) and a degree of acetylation of no greater than about 20%, and at least one chitosan oligomer having a molecular weight in the range of from about 200 to about 20000 Da, the composition being in a form of an aqueous solution. The chitosan oligomer may be a highly deacetylated chitosan oligomer having a degree of acetylation of no greater than about 20%, or alternately, it may be a highly acetylated chitosan oligomer having a degree of acetylation of in the range from 40% to 60%, for example, about 45% to about 55%. In a particular embodiment, the highly deacetylated chitosan oligomer has a degree of acetylation of about 15% or less. According to a particular embodiment, the saccharide oligomer is a D-glucosamine oligomer having between 3 and about 100 units. In a particular embodiment, the D-glucosamine oligomer has between 3 and about 50 units. In a particular embodiment, the saccharide oligomer is an N-acetyl-D-glucosamine oligomer having up to about 7 units. In some embodiments, the ratio between the saccharide oligomer and the highly deacetylated chitosan polymer is greater than 1:1, and can be in the range of from 2:1 to 20:1, depending, inter alia, on the MW of the highly acetylated chitosan and the chitosan oligomers.

Accordingly, the concentration of the highly acetylated chitosan polymer can be, for example, 1%, 2%, 4%, 10% and any concentration is the range of 1-20% (w/w).

The concentration of the oligomers is selected according to the desired ratio.

The saccharide chitosan oligomers are water-soluble at a pH of 6.5 and higher and thus can also serve as "protectors" of the highly deacetylated chitosan polymer, in a manner analogous to that effected by a highly acetylated chitosan polymer, as discussed herein.

In a particular embodiment, at 25 °C and at pH in the neutral range the composition has a viscosity of at least 100 mPa·s at a shear rate of 50 rpm. In a particular embodiment, at 25 °C and at pH in the neutral range the composition has a viscosity of no greater than 70 mPa·s at a shear rate of 200 rpm.

Hydrogels comprising a combination of a chitosan polymer and a chitosan oligomer can be utilized in any of the applications described herein and may be prepared as described in Example 8 herein.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various

embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

EXAMPLE 1

Preparation of chitosan hydrogel

Chitosan with a degree of acetylation of 15% and molecular weight of 65 kDa (Koyo, Japan) was dissolved by mixing with 0.9 % HCl for 24 hours, forming a type 1 chitosan solution having a chitosan concentration of 3 % (w/v).

Homogenously deacetylated chitosan with 51% deacetylation and molecular weight of 220 kDa (Koyo, Japan) was dissolved by mixing with 0.9% HCl for 24 hours, forming a type 2 chitosan solution having a chitosan concentration of 3% (w/v).

The type 1 and type 2 chitosan solutions were mixed according to the following ratios of type-1 to type-2: 1:1, 1:2 and 1:3, titrated to pH 6.8 and left for 24 hours at 4 °C, followed by further titration to pH 7.2 at 4 °C with sodium hydroxide.

The resulting composition was liquid at room temperature. Upon increasing the temperature to 37 °C and raising the pH to 7.4, the liquid solution formed a stable semi-solid hydrogel, as illustrated in Figure 1.

EXAMPLE 2

Degradation profiles of chitosan hydrogels

Pseudo-thermosetting hydrogel (3% w/v) was prepared using homogenously acetylated type 2 chitosan and non-homogenously deacetylated type 1 chitosan, as described in Example 1 above (hydrogel A). Hydrogel A was prepared at ratios of 1:1, 1:2 and 1:3. A different hydrogel (hydrogel B) was prepared using the same type 2 chitosan as used for hydrogel A, and a type 1 non-homogenous chitosan of molecular weight 100 kDa.

One gram of each gel was placed in 50 ml plastic tubes, in triplicate.

Aliquots of 3 ml of 10% bovine serum media were added to each tube for predefined times intervals (1, 2, 3, 4, 5, 6, and 7 days). At the end of each time interval,

the gel was washed 3 times over a period of 24 hours by repeatedly adding 50 ml of distilled water, leaving at room temperature for a few hours and removing the water. The washing process removed all soluble materials from the gel.

The gel was then frozen, lyophilized and weighed. Weight degradation was calculated from the change in weight of the samples, as a function of time interval.

The degradation of hydrogel A by serum enzymes is shown in Figure 2A.

Two distinct types of degradation kinetics are exhibited by each of the compositions shown in Figure 2A: an initial fast phase that terminates within 3-6 days and a slower one that exhibits only partial degradation after 14 days. The rate of degradation of the gel is more rapid in compositions having a higher ratio of type 2 chitosan to type 1 chitosan i.e. a 1:1 composition degrades more rapidly than a 1:3 composition. It is believed that the fast phase reflects degradation of type 2 chitosan, which is highly soluble and readily recognized by serum enzymes. The slow kinetic phase is related to chitosan type 1 chitosan, which is not readily recognized and digested by serum enzymes.

Controlling the reacetylation of glucosamine polymer is a very important tool for manipulating the extent of recognition of the chitosan by lysozyme and consequently for manipulating the rate of hydrogel degradation. The main factor that controls the activity of the enzyme is the percentage of N-acetyl glucosamine (GlcNAc) in the polymer (Ran et al., 2005). For this reason decreasing the reacetylation degree from 50% to 35% in chitosan type 2 should allow the rate of degradation to be significantly decreased, resulting in a much shallower slope. On the other hand, increasing the degree of acetylation of type 1 chitosan should result in faster degradation of the polymer. Selection of the appropriate combination of the two types of chitosans is expected to result in a single, linear degradation curve over time, instead of the two slopes shown in Figure 2A. In addition, increasing the molecular weight of the type 1 chitosan to at least 100 kDa decreases the rate of degradation of the composition and results in a linear rate of degradation, as shown in Figure 2B.

EXAMPLE 3

Slow release of proteins by chitosan hydrogels

In order to study the potential of the chitosan pseudo thermosetting hydrogel presented herein as a slow release vehicle, hemoglobin and bovine serum albumin (BSA) were used as solutes. These compounds are well accepted as protein standards.

To one ml solution containing a chitosan mixture as described in Example 1 above, in which the final concentration of both chitosans is 3.5%, a 25 µl aliquot of BSA or 40 µl of hemoglobin were added, resulting in a final concentration of 4 mg/ml and 1 mg/ml protein in the hydrogel, respectively. The protein-containing hydrogels were incubated in 3 ml PBS for one week at 37 °C. The media was replaced daily, and the amounts of the released protein from the hydrogel was measured, as shown in Figures 3-7.

As shown in Figure 3, in all the tested series, high amounts of hemoglobin were initially released and the rate of release decreased with time. No initial burst was shown.

BSA showed the same profile as hemoglobin (Figures 4 and 5). A near linear slope was obtained (Figure 4). Mixing of the BSA with the gel improved the gel stability, providing a decreased degradation rate compared to that of the chitosan mixture alone (Figure 6).

Comparison of the amounts of the released BSA versus the amounts of the degraded gel (Figure 7) showed that the rate of release of the protein was faster than the rate of gel degradation.

The data shown in Figure 7 relate to a single degree of acetylation of type 1 chitosan and a single molecular weight, which resulted in a protein release profile having a rate of release which decreased each day. However, appropriate selection of additional variables such as degree of reacetylation and molecular weights of the two types of chitosans allows the characteristics of the gel to be determined, and enables affinity of the protein drug for the chitosan structure to be improved. Such specific combinations would be expected to provide a fixed rate of release of a specific drug, reflecting a combined diffusion and matrix degradation rate.

EXAMPLE 4

In vivo study of chitosan hydrogel as wound dressing

Psammomys obesus strain rats, which are known to develop diabetic symptoms when raised in captivity on a high fat diet, were used as a model of type II Diabetes mellitus. These animals are considered to be an excellent model for simulating chronic skin ulcers of diabetics, and study of skin wound healing, due to their tendency to develop profound infections, gangrene and sepsis, leading to morbidity and even mortality.

Common parameters for assessing skin and wound healing include

1. Timing of neovascularization appearance in the reparative tissue.
2. Reduction in neutrophil activity.
3. Accelerated macrophage activity
4. Timing of scar wound closure by a complete re-epithelialization of the wound.
5. Formation of keratinocytes monolayers.
6. Binding of the epidermis and the dermis layers by activation of the fibroblast - depositing extracellular matrix network.

A chitosan-based hydrogel was used as a biological dressing, avoiding the need for bandaging or suturing, and providing a direct coating of the wound bed. The rate at which various healing stages occurred, especially the wound contraction-scar shrinkage and closure stage, was studied over a period of eight days.

The composition of the chitosan mixture includes: a highly deacetylated chitosan (660 kDa, DA 15), a highly acetylated chitosan (220 kDa, DA 50) and a mixture of N-acetyl-D-glucosamine oligomers from 1-7 units (Koyo, Osaka, Japan). The formulation includes the three components in a ratio of 1:0.8:0.2, respectively.

Twenty five female Psammomys obesus rats of mature age, each weighing 150-160 grams, were used.

Thirteen animals were found to have developed diabetes following administration of a high fat diet, starting from 4 to 6 weeks prior to day zero.

Six animals having normal euglycemia (normoglycemia), indicating resistance to development of diabetes upon feeding with a high fat diet, were used as a first control, and six animals with normoglycemia when fed on a normal low fat, low energy diet, were used as a second control.

At day zero, a round full depth punch biopsy of 6 mm diameter was made through the epidermis, dermis and hypodermis to the muscles, at the shaved skin of the neck, using a Metricoconventer-production device.

The injuries of seven diabetic animals were treated by administration of the chitosan based-gel of Example 1 to the wound area, while a further six animals were left untreated. The gel was reapplied to the wound area of the treatment group every day. A conventional plaster bandage was applied over the gel in the treatment group. In half of the animals in the control group, a plaster bandage was applied over the wound, whereas in the other half the wound area was left uncovered.

For a period of seven days all the animals were macro-photographed, and the dimensions of the wound measured every 3 days. Weight and blood glucose levels were

measured once a week, using digital glucometer, (Ascensia Elite of Bayer), by absorbing a blood drop from a cut created at the tail of the rat.

After 7 days, the animals were sacrificed and full depth biopsies were performed. The skin was collected and placed in fixation solution. Skin samples were further processed for histological and immuno-histochemical staining procedures, to evaluate the differences between treated and untreated wounds.

The results obtained indicate that rats treated with the gel showed a statistically significant increase in wound healing as assessed by the wound surface area (Figure 9). A representative histological sample showing evidence of wound healing, as indicated by smooth surfaces is shown in Figure 8A, whereas a control untreated sample (Figure 8B) shows no evidence of wound healing.

EXAMPLE 5

Rotator cuff repair

Rotator cuff tears are a common source of shoulder pain. The incidence of rotator cuff damage increases with age and is most frequently caused by degeneration of the tendon, rather than injury from sports or trauma. Treatment recommendations vary from rehabilitation to surgical repair of the torn tendon(s). The best method of treatment is different for every patient and indeed many patients do not achieve satisfactory repair of their injuries.

The present invention, in some embodiments, overcomes these drawbacks of the background art by providing an injectable product allowing delivery of autologous cells into rotator cuff tears under ultrasonographic control. In other embodiments, the injectable product allows the incorporation of bone marrow cells as well, for example for tissue healing.

Preferably, the procedure is performed as an outpatient procedure or an ambulatory procedure requiring local anesthesia.

The initial liquid property of the gel allows full adherence to the tendon tear area.

In vivo experiments were performed on rats for tendon damage and repair (using surgically damaged tendons). The damaged tendons were sutured and were treated with a chitosan hydrogel as presented herein with bone marrow cells; the control animals only received sutures. Twenty animals were studied for 3 months. Histologically proven tendon repair and prevention of muscle atrophy were both achieved.

Further in vivo experiments were performed on rats with surgically induced rotator cuff damage. This damage was treated as above. Histological slices of tissue, 6 weeks post surgery, show that endogenous cells were trapped from the neighboring tissues, improving the status of the injured site, compared to non treated control defects in the contra lateral shoulder. Exemplary results are shown in Figure 10A (treated) and Figure 10B (non-treated).

EXAMPLE 6

A chitosan mixture was designed so as to serve as a reservoir of negatively charged substances such as proteoglycans (e.g. chondroitin sulfate), hyaluronate, and/or phospholipids (e.g., phosphatidyl choline). This mixture has rheological properties simulating normal synovial fluid, allowing for cartilage regeneration and correction of joint mechanics, and for use for example, in treatment of osteoarthritis.

In order to evaluate the properties of these compositions, the static coefficient of friction was measured in vitro between two flat cartilage surfaces, to which various test compositions were applied. Following application of the compositions, the cartilage surfaces were placed one on top of the other, and one edge was slowly lifted manually. The exact angle at which motion started was measured with a digital inclinometer. Determinations were carried out in triplicate and the mean static coefficient of friction μ was calculated according to the equation $\mu \leq$ tan(sliding angle) as disclosed in http://www.pa.uky.edu/~phy211/Friction_book.html and illustrated in Figure 11.

Figure 12 presents the static friction coefficient between two layers of normal cartilage, as measured for: a control system with no applied hydrogel (denoted as "Saline"); a combination chitosan hydrogel containing a chitosan type 1 (MW of 660 kDa; DA 15), and a chitosan type 2 (MW of 220 kDa; DA50) (denoted as "Combination"); the aforementioned combination chitosan hydrogel and further comprising 0.1 % chondroitin sulfate (denoted as +CS), the aforementioned combination chitosan hydrogel further comprising 0.1% chondroitin sulfate and 0.1% phosphatidyl choline (denoted as +CS/PC), and a 1% solution of hyaluronic acid (denoted as HA).

The obtained data clearly show that combining a hydrogel composition comprising type 1 and type 2 chitosans in combination with chondroitin sulfate and phosphatidyl choline results in a synovial fluid-like friction coefficient.

EXAMPLE 7

Controlling the conditions for formation of a chitosan hydrogel

A chitosan polymer (or oligomer) is defined by its molecular weight, its degree of deacetylation, its crystallinity and the mode of distribution of its acetyl groups.

The solubility of deacetylated chitosan in aqueous solutions is limited. For example, using HCl 0.15N, a chitosan having a MW above 200 kDa provides solutions having concentrations less than about 10% (w/v), and as the molecular weight increase, the maximal solution concentration decreases. HCl 0.15N is a concentration which when fully titrated becomes the physiological NaCl concentration. Higher concentrations of HCl (or other acids such as acetic acid) allows higher concentrations of chitosan solutions.

The most common commercially available chitosans have a low DA (degree of acetylation) in the range of 5-30%, and is referred to herein as type 1 chitosan. Type 1 chitosan precipitates from solution when at a pH above 6.5. Type 1 chitosan solutions at physiological environments therefore do not exist, and most of the currently practiced applications involving implementation of chitosan utilize various types of solid chitosan.

Chitosans homogenously deacetylated to 50% or homogenously reacetylated to 30-60 % are referred to as type 2 chitosans. Such a chitosan has a superior solubility in aqueous solutions, as compared to highly deacetylated chitosans (type 1) and typically remains soluble at neutral and physiological pH, depending on its concentration. An exemplary such chitosan having a MW of 220 KDa and a concentration of 3% (w/v) or more, forms a gel at pH higher than 7.5.

Hydrogel Formation using a mixture of chitosan polymers

When mixing type 1 chitosan and type 2 chitosan at a physiological pH and under certain conditions, no precipitation of the polymers is observed and instead, the mixture forms a hydrogel. Hydrogel formation involves "coating" (or "protection") of the type 1 chitosan by the type 2 chitosan , and is effected by the affinity between the two chitosan types, which leads to interactions therebetween (e.g., hydrogen bonds, hydrophobic interactions and/or Van der Waals interactions).

The gelation process may be as short as several minutes or as long as many days and is demonstrated by a gradual yet continuous increase in the viscosity of the system.

In the conducted (ex vivo) experiments, hydrogel formation is determin by turning a glass tube that contains the initial solution on its side and assessing whether

the solution flows, or alternately whether a semi-solid gel is formed which remains stuck to the bottom of the glass tube. Gel formation depends on the type, shape and parameters (e.g., diameter) of tube, as well as the assay time frame.

In this study, hydrogel formation was defined as follows: a one ml solution was placed in a 14 mm (in diameter) round-bottomed glass tube and was incubated at 37 °C overnight. Thereafter, the glass tube was turned into a horizontal position and the presence or absence of liquid flow was determined. Absence of liquid flow indicated that a hydrogel was formed. The hydrogel contains the whole amount of water and remained rigid in a semi solid state. Presence of flow but in a "well distinguished" structure also indicated that a hydrogel was formed. The presence of liquid flow and/or the formation of two separate phases, solid and liquid, indicated that a hydrogel was not formed.

Controlling hydrogel formation

Materials

Two commercially available highly deacetylated chitosan polymers (chitosan type 1) were used in this study:

FM™80 (MW = 660 kDa; 85 % DDA (degree of deacetylation); and

FM™80S (MW = 420 kDa; 91.3% DDA), both obtained from Koyo, Osaka, Japan.

As a highly acetylated chitosan (chitosan type 2), DAC™50 (MW = 220 kDa; 50% DDA), also obtained from Koyo, Osaka, Japan, was used.

Assay Protocol

Preparation of stock solutions. A chitosan polymer (as a powder) was mixed with HCl 0.15N and the solution was agitated during 24 hours at room temperature. The following stock solutions were made:

FM™80-2 2% (w/v) solution

FM™80S-2 2% (w/v) DAC™50-3 3% (w/v).

Mixture formation. Using the above-described stock solutions, mixtures having a defined final concentration (w/v) of each chitosan and a defined ratio thereof, were prepared. An exemplary mixture is FM™80:DAC™50 1.2:1.2, in which each of the chitosans are at a final concentrations of 1.2% (w/v), and the ratio therebetween is 1:1.

Titration The above-described mixtures were slowly titrated, while being cooled in ice water (0 °C), with NaOH (at 2N, 1N and 0.5N concentrations), until a pH of about 7.3 was achieved. One ml samples were then continuously taken from the mixture

during the titration and each sample was placed in a 14mm glass tube. The samples were sealed and placed in an incubator at 37 °C overnight.

Gel formation testing Each glass tube was placed in a horizontal position and gel formation was determined as described hereinabove.

Results

Table 1 below presents the results obtained for various FM™80:DAC™50 mixtures:

Table 1

Mixture			Mix Ratio	Hydrogel Formation at various pH values*				
FM80-2	50-3	HCl .15N	FM80:DAC50					
3	2	0	1.2 : 1.2	7.32	**7.42**	**7.5**	**7.66**	
				N	**Y**	**Y**	**Y**	
2.5	1.667	0.833	1 : 1	7.35	7.43	**7.6**	**7.7**	
				N	N	**Y**	**Y**	
2	1.333	1.667	0.8 : 0.8	7.27	7.4	7.48	7.54	7.62
				N	N	N	N	N
1.5	1	2.5	0.6 : 0.6	7.32	7.43	7.52	7.6	
				N	N	N	N	
2.5	1	1.5	1 : 0.6	7.4	7.47	7.54	7.61	
				N	N	N	N	

* Y = yes, a gel is formed; N = no gel formed

The results show that a mixture of a 660 kDa chitosan type 1, with DA of 15% (and similar crystallinity as in FM™80) and DAC™50 at equal (1:1) w/w ratios forms gel at near pH 7.5, when a final concentration of each chitosan is higher than 0.8% (w/v).

Such a mixture, at final concentrations of 1.2% (w/v) of each chitosan, forms a hydrogelat a wider pH range (7.42-7.66), as compared to a mixture at a final concentration of 1% (w/v) of each chitosan (7.6-7.7), thus indicating that at higher final concentrations the pH range for hydrogel formation is increased (as discussed herein).

At a FM80:DAC50 ratio higher than 1:1 (e.g., 1:0.6), no hydrogel is formed.

Table 2 below presents the results obtained for various FM™80S:DAC™50 mixtures:

Table 2

Mix formula			Mix Ratio	Gel Formation at various pH values*				
FM80S-2	50-3	HCl .15N	FM80S:DAC50					
3	2	0	1.2 : 1.2	7.37	**7.40**	**7.44**	**7.50**	
				N	**Y**	**Y**	**Y**	
2.5	1.667	0.833	1 : 1		7.44	**7.53**	**7.63**	
					N	**Y**	**Y**	
2	1.333	1.667	0.8 : 0.8		7.42	7.51	7.61	7.7
					N	N	N	N
1.5	1	2.5	0.6 : 0.6		7.44	7.55	7.63	
					N	N	N	

* Y = yes, a gel is formed; N = no gel formed

These results further support the findings that a minimal concentration of about 1% of each polymer is required in order to achieve hydrogel formation at near pH 7.5 at the indicated conditions.

This study has shown that parameters influencing hydrogel formation in the tested systems include pH, the relative ratio (w/w) of the chitosan type 1 and type 2 polymers, the molecular weight (MW) of each chitosan polymer, the final concentration of each chitosan polymer and the temperature.

pH. Gel formation is pH-dependent, such that solution mixtures form a hydrogel only within a certain range of pH. This pH range is increased as the final concentrations of the chitosans are increased. The absolute pH values increase as the final concentrations of the chitosans decrease.

For example, for a chitosan type 1 chitosan having MW of 420 kDa and a type 2 chitosan having MW of 220 kDa, each at a concentration of 1%-1.2% and at a 1:1 w/w ratio, the pH range in which a gel is formed at 4 °C is 7.4-7.7. At higher pH values, precipitation is observed.

It is noted that pH values at 4 °C correlate to pH values lower by 0.5 units at 25 °C. Thus, pH 7.4 at 4 °C is found to be pH 6.9 at 25 °C.

Final concentration of type 1 chitosan. The type 1 chitosan is considered the backbone of the hydrogel. Hence, hydrogel formation depends on the final concentration of type 1 chitosan. As the MW of the type 1 chitosan is increased, its final concentration in the composition should be decreased, and vice versa, i.e. as the MW decreases its final concentration in the composition should be increased.

Molecular Weight of type 1 chitosan. Use of type 1 chitosans of high MW, decreases the pH working range i.e. the range that allows hydrogel formation. For example, a composition comprising a type 1 chitosan of MW 660 kDa forms a hydrogel in the pH range 7.4-7.7, while a composition comprising a type 1 chitosan of MW 2,000 kDa forms a hydrogel in the pH range 7.0-7.3. Furthermore, as the MW of the type 1 chitosan is increased, the relative concentration of the type 2 chitosan required is increased. Furthermore, as the concentration of the type 1 chitosan is decreased (e.g., to 0.5 %), the pH range at which hydrogel formation occurs shifts down to 7.2-7.4 (at 4 °C).

Furthermore, as the MW of the type 1 chitosan is decreased (e.g., from 420 kDa to 200 kDa), the concentration of that chitosan required for hydrogel formation is increased. Exemplary concentrations required for type 1 chitosans of MW of 200 kDa or less are in the range 1.2-1.5% (w/v) or higher. Under such conditions pH values for hydrogel formation may shift to 7.5-7.8 (at 4 °C).

Concentration of type 2 chitosan. A minimal relative concentration of the type 1 chitosan and the type 2 chitosan is required for hydrogel formation (e.g., a 1:1 ratio). In addition, keeping the type 1 chitosan at constant final concentration and increasing the concentration of the type 2 chitosan extends the range of other parameters for hydrogel formation (for example, increases the pH working range). Increasing the concentration of the type 2 chitosan further decreases the pH at which a hydrogel is formed.

Molecular Weight of type 2 chitosan. Using a type 2 chitosan of MW higher than 220 kDa enables use of a reduced relative concentration of type 2 chitosan in the mixture. A type 2 chitosan of higher MW results in high protection and improved stability of the chitosan type 1 (over a wide range of MW of type 1 chitosan). At such conditions, the pH range for hydrogel formation may increase.

For example, using a type 2 chitosan of MW 2000 kDa and a type 1 chitosanof MW 660 kDa , the pH for hydrogel formation should be about 7.8, whereas using a type 2 chitosan of MW 2000 kDa and a type 1 chitosan of MW 2000 kDa, the pH for hydrogel formation should be about 7.6.

Temperature. The temperature affects the rate of hydrogel formation linearly. Thus, at 37 °C, the hydrogel will form faster than at room temperature or at 4 °C.

Relative ratio of type 1 chitosan and type 2 chitosan. The ratio required for hydrogel formation depends on the MW of each chitosan. For example, as the MW of type 1 chitosan increases (e.g., to 2000 kDa), its required concentration can be reduced possibly to about 0.5%. However, it is assumed that the ratio between type 2 and type 1 would increase to, for example, 2:1, 3:1 and even 4:1.

Increasing the MW of type 2 chitosan from 220 kDa to e.g., 2000 kDa, the minimal concentration thereof required for hydrogel formation decreases to e.g., 0.5% (instead of 1%), such that when high MW type 1 chitosan is used, the ratio would be about 1:1.

EXAMPLE 8

Chitosan hydrogels formed from highly acetylated and highly deacetylated chitosan oligomers and a highly deacetylated chitosan polymer

Oligomers of highly deacetylated chitosan are soluble at pH higher than 6.5, in contrast to highly deacetylated chitosan polymers having a similar degree of deacetylation. Thus, the formation of a hydrogel from a mixture of highly deacetylated chitosan polymers (e.g., MW of 200-2000 kDa), in combination with chitosan oligomers (e.g., MW of 200-20000 Da) was tested.

Solutions of a type 1 chitosan polymer (MW 660) were each mixed with a solution of a highly deacetylated oligomer (MW 200-1500 Da) for assessing hydrogel formation. The highly deacetylated oligomer was present in the solutions tested at final concentrations of 1%, 2%, 4% and 10%, and the highly deacetylated polymer was present at a final concentration of 1%. The tested ratios (oligomer to polymer) were 1:1; 2:1; 4:1; and 10:1.

All tested mixtures, except for the 1:1 solution, formed a chitosan hydrogel, surprisingly indicating that mixtures of highly deacetylated chitosan polymer and highly deacetylated chitosan oligomers of highly deacetylated chitosan can form hydrogels.

Similarly, hydrogel formation was assessed using combinations of a type 1 chitosan polymer (MW 420; DA 9) with highly deacetylated chitosan oligomers (MW 200-2000 Da), at various ratios. In addition, combinations using different saccharides in place of chitosan oligomers were examined. The results, as shown in Table 3, indicate that chitosan hydrogels are formed with: monosaccharides and oligomers of N-acetyl-

D-glucosamine; monosaccharides and oligomers of D-glucosamine; fructose; methylcellulose and hydroxyethyl cellulose. In contrast, hydrogel formation was not observed using oligomeric starch, laminarin, or inulin.

Table 3

Saccharide	Ratio of Type 1 polymer:saccharide	Concentration of Type 1 polymer (%)	Hydrogel formation
Oligo-chitosan	1:2	1.5	+
Oligo-chitin	1:2	1.5	+
Methylcellulose	1:2	1.5	+
Hydroxyethyl cellulose	1:2	1.5	+
D-glucosamine (monomeric)	1:3	1	+
N-acetyl-D-glucosamine (monomeric)	1:3	1.5	+
Fructose	1:3	1.5	+
Laminarin	1:2	1.5	-
Inulin	1:2	1.5	-

Similar results, namely hydrogel formation, are obtained for mixtures of highly deacetylated chitosan polymers (e.g., MW 200-2000 kDa) and highly acetylated chitosan oligomers (MW of 1000-20000 Da).

EXAMPLE 9

Viscosity studies of chitosan hydrogels formed from highly acetylated and highly deacetylated chitosan polymers

The principles discussed in Example 7 were validated in viscosity studies of various hydrogels formed from chitosan polymers.

Materials and Methods

The chitosan polymers, all obtained from Koyo (Osaka, Japan) used in this study are described in Table 4.

Table 4

Chitosan Trade name	Degree of acetylation (%)	Molecular weight (kDa)
Type 1 chitosan		
FVL™	15	65
SK™10	8.9	100
FM™80S	8.7	420
FM™80	15	660
Type 2 chitosan		
DAC™50	50	220

Combination chitosan solutions were prepared in either of two ways. In one method, individual stock solutions of a type 1 and a type 2 chitosan were prepared at the desired concentrations, as described in Example 7. Slow drop wise titration with NaOH at either 1 °C or 25 °C was carried out until the desired pH was achieved. In an alternate method, pre-weighed amounts of type 1 and a type 2 chitosans in powder form were combined in an acid solution e.g. HCl (0.15N) or acetic acid (0.25N). Following solubilization the solutions were slowly titrated with NaOH at either 1 °C or 25 °C until the desired pH was achieved.

Solutions comprising mixtures of either FVL™, SK™10 or FM™80 in combination with DAC™50 were prepared, each at a ratio of 1:1 (respectively denoted as FVL:50 1:1; SK10:50 1:1, and FM80:50 1:1). Samples (0.5 ml) were analyzed in a Brookfield RVDV-II+ Pro viscometer, fitted with cone spindle CP-51 with a range coefficient of 51780. Dividing the range coefficient by the rotation speed (in RPM) determines the maximal viscosity that can be measured in centipoises (cP). Thus, for example, in this system at 200 RPM, viscosity of up to 250cP may be measured.

Results

Figure 13 illustrates results obtained with compositions containing DAC™50 on its own at a concentration of 1% (diamond symbols) or 2% (square symbols); or FM™80S on its own at a concentration of 1% (triangle symbols); or FM™80S on its own at a concentration of 1% (asterisk symbols); or a combination of FM™80 and DAC™50 at a ratio of 1:1 (oval symbols).

As shown in Figure 13, 1% solutions of individual type 1 chitosans (FM™80S or FM™80) have low viscosity at 25 °C in the tested pH range from about 4 to 6.5. When titrated to higher pH values, the viscosity of these compositions cannot be determined since the chitosans precipitate, apparently due to pH conditions exceeding that of the pKa of the chitosan. These results confirm that solutions containing a single highly deacetylated chitosan are not suitable for in vivo use. A 1% solution of the type 2 chitosan DAC™50 remains soluble at 25 °C in the tested pH range from about 5.4 to about 7.6, suggesting that it is compatible for in vivo use, but it displays low viscosity and does not form a hydrogel. In contrast, the combination solution FM80:50 1:1 transforms from a liquid to a semi-solid gel at a pH of about 6.5 at 25 °C, as indicated in Figure 13. In particular, at this pH, the composition starts to display non-Newtonian behavior, and a further increase in pH significantly increases its viscosity i.e. to about 175 mPa·s at pH 6.9. These results indicate that a 1:1 mixture of a 15% acetylated (i.e. highly deacetylated) chitosan of molecular weight 660 kDa and a 50% acetylated (i.e. highly acetylated) chitosan of molecular weight 220 kDa is suitable for hydrogel formation at physiological conditions.

Figure 14 illustrates results obtained with compositions containing a combination of the highly deacetylated chitosan FVL™ and the highly acetylated chitosan DAC™50 at a ratio of 1:1 (diamond symbols), or a combination of the highly deacetylated chitosan SK™10 and DAC™50 at a ratio of 1:1 (square symbols). Figure 14 shows that each of the mixtures FVL:50 1:1 and SK10:50 1:1 display a significant increase in viscosity at pH values approaching neutrality, leading to the formation of a semi-solid hydrogel. These results confirm that combination of a highly acetylated chitosan with a highly deacetylated chitosan prevents precipitation of the latter at neutral pH and enables hydrogel formation. In addition, the viscosity of SK10:50 1:1 is significantly higher than that of FVL:50 1:1 over a range of pH values. In particular, at pH of about 7, these mixtures display viscosities of about 55 and 42 mPas, respectively. Since these mixtures differ only with respect to the molecular weight of the highly deacetylated chitosan i.e. 100 and 65 kDa, respectively, it is evident that the molecular weight of the highly deacetylated chitosan is an important factor, together with pH, for determining the viscosity and non-Newtonian behavior of the hydrogel compositions of the invention.

These conclusions are further supported by the results shown in Figure 15, obtained with compositions containing a combination of SK™10 and DAC™50 at a

ratio of 1:1 (Figure 15A) at pH values of 5.13 (x symbols); 5.67 (empty square symbols); 6 (empty triangle symbols); 6.24 (empty diamond symbols); 6.51 (asterisk symbols); 6.65 (filled circle symbols); 6.75 (empty circle symbols); 6.87 (filled diamond symbols) and 7 (filled square symbols); or a combination of FVL™ and DAC™50 at a ratio of 1:1 (Figure 15B) at pH values of 5.45 (empty diamond symbols); 5.7 (filled square symbols); 6.04 (empty triangle symbols); 6.3 (x symbols); 6.56 (asterisk symbols); 6.68 (filled circle symbols); 6.82 (filled triangle symbols); 6.88 (filled diamond symbols) and 7.03 (empty square symbols).

Figure 15A indicates that the viscosity of SK10:50 1:1 increases dramatically upon a decrease in shear rate, when the pH value of the solution is in the neutral range i.e. 6.87 and 7, whereas the same compositions at lower pH values do not exhibit increased viscosity at decreasing shear rates. That is, SK10:50 1:1 exhibits liquid Newtonian behavior at pH values below the neutral range, and exhibits non-Newtonian behavior at pH values in the neutral range. FVL:50 1:1, in contrast, displays only a minor increase in viscosity upon a decrease in shear rate at neutral pH and remains in the liquid state (Fig. 15B), reaching a peak viscosity of about 35 mPas, as compared to 105 mPa·s in SK10:50 1:1.

Figure 16 shows that at low temperature i.e. 1 °C, the pH-induced increase in viscosity in compositions of the invention is diminished. The results in this figure were obtained with compositions containing a combination of SK™10 and DAC™50 (SK10:50) at a ratio of 1:1 at pH values of 5.35 (filled circle symbols); 5.92 (filled diamond symbols); 6.22 (asterisk symbols); 6.81 (filled square symbols); 6.95 (filled triangle symbols) and 7.01 (x symbols); or a combination of FVL and DAC™50 (FVL10:50) at a ratio of 1:1 at pH values of 5.85 (empty diamond symbols); 6.2 (gray square symbols); 6.44 (empty triangle symbols); 6.76 (gray triangle symbols); 6.94 (empty square symbols) and 7.00 (empty circle symbols).

At 1 °C SK10:50 1:1 exhibits only a moderate increase in viscosity, as compared to the dramatic increase observed at 25 °C. More specifically, at 1 °C and low shear rate, the viscosity of SK10:50 1:1 increases from 50 to 65 mPa·s (i.e. a 30% increase) upon increase in pH from about 5.3 to about 7 (Figure 16), whereas at 25 °C and low shear rate, the viscosity of the same composition increases from 20 to 110 mPa·s (i.e. a 550% increase) upon the same increase in pH (Figure 15A). This suggests that chitosan compositions according to the invention may be stored at low temperatures in order to

preserve them in the liquid or relatively non-gelled state. Notably, at neutral pH and low shear rate, FVL:50 1:1 exhibits a greater viscosity at 1 °C as compared to that at 25 °C (compare Figure 15B to Figure 16), suggesting the unsuitability of this composition for hydrogel formation in vivo, due to its tendency to have decreased viscosity upon an increase in temperature to that of room temperature and above.

Figure 17 shows results obtained with compositions comprising a combination of SK™10 and DAC™50 at a ratio of 1:1 when measured at varying shear rates and at 1 °C (Figure 17A), the compositions having pH values of 5.35 (vertical bar symbols); 5.92 (circle symbols); 6.22 (asterisk symbols); 6.45 (x symbols); 6.81 (triangle symbols); 6.95 (square symbols) and 7.01 (diamond symbols); or at 25 °C (Figure 17B), the compositions having pH values of 5.13 (x symbols); 5.67 (empty square symbols); 6 (empty triangle symbols); 6.24 (empty diamond symbols); 6.51 (asterisk symbols); 6.65 (filled circle symbols); 6.75 (empty circle symbols); 6.87 (filled diamond symbols) and 7 (filled square symbols). Both at 1 °C and at 25 °C, SK10:50 1:1 exhibits Newtonian behavior over a range of pH values from 5.35 to about 6.8, indicated by the observation that the viscosity remains relatively unchanged irrespective of the shear force i.e. there is no appreciable difference in viscosity between a shear force of 200 versus that of 50 rpm. However, at pH values greater than about 6.8, the composition exhibits increased viscosity upon decreased shear force, indicative of non-Newtonian behavior. This increase is particularly evident at 25 °C, wherein at pH 7 the viscosity increases from 65 to 110 mPa·s upon a decrease in shear force from 200 to 50 rpm (Figure 17, panel B). Under these conditions (pH 7, 25 °C) the composition continues to increase in viscosity and undergoes hydrogel formation over a period of time. In contrast, at 1 °C, the viscosity increases only from about 55 to about 65 mPa·s (Figure 17, panel A). Notably, the compositions having pH of 6.8 and less exhibit lower viscosity at 25 °C, as compared to the same compositions at 1 °C, suggesting that the combined conditions of physiological pH and temperature are optimal for hydrogel formation.

Figure 18 shows that a combination chitosan composition comprising a highly deacetylated chitosan of molecular weight of less than about 100 kDa is not suitable for gel formation under physiological conditions. The results in this figure were obtained with a combination of FVL™ and DAC™50 at a ratio of 1:1 when measured at varying shear rates and at 1 °C (Figure 18A), the compositions having pH values of 5.85 (circle symbols); 6.2 (asterisk symbols); 6.44 (x symbols); 6.76 (triangle symbols); 6.94 (square

symbols) and 7.00 (diamond symbols); or at 25 °C (Figure 18B), the compositions having pH values of 5.45 (open diamond symbols); 5.7 (open square symbols); 6.04 (open triangle symbols); 6.3 (x symbols); 6.56 (asterisk symbols); 6.68 (circle symbols); 6.82 (closed triangle symbols); 6.88 (closed diamond symbols) and 7.03 (closed square symbols).

As shown, FVL:50 1:1 exhibits an increase in viscosity as the pH approaches neutrality but does not undergo gel formation at either 1 °C (Figure 18A) or 25 °C (Figure 18B). In fact, as previously indicated, this composition exhibits a higher viscosity at 1 °C (neutral pH, low shear rate) as compared to that at 25 °C. This result indicates that molecular weight of the highly deacetylated chitosan is an important parameter for determining gel formation in the compositions of the invention.

Figure 19 shows that increasing the molecular weight of the highly deacetylated chitosan improves the viscosity thereof, as shown with a composition containing a combination of FM^{TM}80 and DAC^{TM}50 at a ratio of 1:1 at pH values of 4.81 (diamond symbols); 5.93 (square symbols); 6.24 (triangle symbols); 6.43 (x symbols); 6.64 (asterisk symbols) and 6.91 (circle symbols). At 25 °C, hydrogel formation of FM80-50 1:1 is initiated at a lower pH (between about 6.4 to about 6.6) as compared to that for SK10:50 1:1 (about 6.8 to about 6.9) (see Figure 17B. Furthermore, the viscosity achieved at neutral pH is significantly higher in the composition containing type 1 chitosan of higher molecular weight i.e. 420 mPa·s for FM80-50 1:1 versus about 110 mPa·s for SK10:50 1:1. These results indicate that the molecular weight of the type 1 chitosan can be varied for different applications, while still achieving hydrogel formation. For example, a more viscous hydrogel may be desirable for an implant requiring prolonged durability in vivo, whereas a hydrogel of lower viscosity may be sufficient to achieve the desired release properties of a slow release drug formulation.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

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AMENDED CLAIMS
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STATEMENT UNDER ARTICLE 19

1. A chitosan composition in the form of a hydrogel, the composition comprising a highly acetylated chitosan having a degree of acetylation in the range of from about 40% to about 60%, and a highly deacetylated chitosan having a degree of acetylation of no greater than about 20%, wherein at 25 °C and at pH 7.0 ± 0.2 the composition has a viscosity of at least about 100 mPa·s at a shear rate of 20 rpm, wherein the highly acetylated chitosan and the highly deacetylated chitosan are each independently present at a concentration of from 0.5% to 2% w/v of the total composition, wherein the highly deacetylated chitosan has a molecular weight of at least about 100 kDa, and the highly acetylated chitosan has a molecular weight of at least about 200 kDa, and wherein the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is in the range of 1:1 to 1:4.

2. The chitosan composition of claim 1, wherein the highly acetylated chitosan has a degree of acetylation in the range of from about 45% to about 55%, and the highly deacetylated chitosan has a degree of acetylation of about 15% or less.

3. The chitosan composition of claim 1, wherein the highly deacetylated chitosan is non-homogenously deacetylated, and the highly acetylated chitosan is homogenously reacetylated.

4. The chitosan composition of claim 1, wherein the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is selected from the group consisting of 1:1; 1:2; 1:3 and 1:4.

5. The chitosan composition of claim 1, wherein at 25 °C and at pH 7.0 ± 0.2, the composition has a viscosity of at least about 100 mPa·s at a shear rate of 50 rpm.

6. The chitosan composition of claim 1, wherein at 25 °C and at pH 7.0 ± 0.2, the composition has a viscosity of at least about 200 mPa·s at a shear rate of 50 rpm.

7. The chitosan composition of claim 1, wherein at 25 °C and at pH 7.0 ± 0.2, the composition has a viscosity of at least about 400 mPa·s at a shear rate of 20 rpm.

8. The chitosan composition of claim 1, wherein the highly deacetylated chitosan has a molecular weight in the range of from about 100 kDa to about 2000 kDa, and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 2000 kDa.

9. The chitosan composition of claim 8, wherein the highly deacetylated chitosan has a molecular weight in the range of from about 100 kDa to about 700 kDa, and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa.

10. The chitosan composition of claim 9, wherein the highly deacetylated chitosan has a molecular weight selected from the group consisting of about 100 kDa; about 400 kDa, and about 650 kDa; and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa.

11. The chitosan composition of claim 1, wherein the highly deacetylated chitosan has a molecular weight in the range of from about 100 kDa to about 700 kDa, and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa; wherein the highly deacetylated chitosan and the highly acetylated chitosan are each present at a concentration of about 1% to about 1.2% w/v of the total composition, and wherein the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is 1:1.

12. The chitosan composition of claim 1, wherein the highly deacetylated chitosan has a molecular weight of about 2000 kDa and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa, wherein the concentration of the highly deacetylated chitosan is 0.5% w/v of the total composition, and wherein the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is selected from the group consisting of 1:2; 1:3 and 1:4.

13. The chitosan composition of claim 1, further comprising tri-N-acetyl-glucosamine, wherein the tri-N-acetyl-glucosamine is bound to the highly acetylated chitosan.

14. The chitosan composition of claim 1, further comprising at least one negatively charged substance selected from the group consisting of a polysaccharide, a phospholipid and combinations thereof.

15. The chitosan composition of claim 14, wherein the negatively charged polysaccharide is selected from the group consisting of an animal-derived polysaccharide, a plant-derived polysaccharide, chondroitin sulfate, dermatan sulfate, dextran sulfate, heparan sulfate, heparin, hyaluronic acid, keratan sulfate and combinations thereof.

16. The chitosan composition of any of claims 1-15, further comprising at least one of a drug, a polypeptide, an animal cell and a plant cell.

17. An implantable device comprising the composition of any of claims 1-16.

18. A slow release drug delivery device, comprising the composition of any of claims 1-16 and a drug embedded therein.

19. A cell-loaded artificial matrix comprising the composition of any of claims 1-16, wherein the cells are selected from the group consisting of chondrocytes, fibrochondrocytes, ligament fibroblasts, skin fibroblasts, tenocytes, myofibroblasts, mesenchymal stem cells and keratinocytes.

20. Use of the chitosan composition of any of claims 1-16 for the preparation of a medicament for preventing or treating surgical adhesions.

21. Use of the chitosan composition of any of claims 1-16 for the preparation of a medicament for wound healing.

22. Use of the chitosan composition of any of claims 1-16 for the preparation of a medicament for tissue repair or tissue replacement.

23. A chitosan composition in the form of an injectable solution, the composition comprising a highly acetylated chitosan having a degree of acetylation in the range of from about 40% to about 60%, and a highly deacetylated chitosan having a degree of acetylation of no greater than about 20%, wherein at 25 °C and at pH below the neutral range the composition has a viscosity of no greater than about 150 mPa·s at a shear rate of 50 rpm, wherein the highly acetylated chitosan and the highly deacetylated chitosan are each independently present at a concentration of from 0.5% to 2% w/v of the total composition, wherein the highly deacetylated chitosan has a molecular weight of at least about 100 kDa, and the highly acetylated chitosan has a molecular weight of at least about 200 kDa, and wherein the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is in the range of 1:1 to 1:4.

24. The chitosan composition of claim 23, wherein the highly acetylated chitosan has a degree of acetylation in the range of from about 45% to about 55%, and the highly deacetylated chitosan has a degree of acetylation of about 15% or less.

25. The chitosan composition of claim 23, wherein the highly deacetylated chitosan is non-homogenously deacetylated, and the highly acetylated chitosan is homogenously reacetylated.

26. The chitosan composition of claim 23, wherein the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is selected from the group consisting of 1:1; 1:2; 1:3 and 1:4.

27. The chitosan composition of claim 23, wherein at 25 °C and at pH below the neutral range the composition has a viscosity of no greater than about 100 mPa·s at a shear rate of 50 rpm.

28. The chitosan composition of claim 23, wherein at 25 °C and at pH below the neutral range the composition has a viscosity of no greater than about 50 mPa·s at a shear rate of 50 rpm.

29. The chitosan composition of claim 23, wherein the highly deacetylated chitosan has a molecular weight in the range of from about 100 kDa to about 2000 kDa, and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 2000 kDa.

30. The chitosan composition of claim 29, wherein the highly deacetylated chitosan has a molecular weight in the range of from about 100 kDa to about 700 kDa, and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa.

31. The chitosan composition of claim 23, wherein the highly deacetylated chitosan has a molecular weight in the range of from about 100 kDa to about 700 kDa, and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa; wherein the highly deacetylated chitosan and the highly acetylated chitosan are each present at a concentration of about 1% to about 1.2% w/v of the total composition, and wherein the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is 1:1.

32 The chitosan composition of claim 23, wherein the highly deacetylated chitosan has a molecular weight of about 2000 kDa and the highly acetylated chitosan has a molecular weight in the range of from about 200 kDa to about 250 kDa, wherein the concentration of the highly deacetylated chitosan is 0.5% w/v of the total composition, and wherein the ratio of the highly deacetylated chitosan and the highly acetylated chitosan in the composition is selected from the group consisting of 1:2; 1:3 and 1:4.

33. The chitosan composition of claim 23, further comprising tri-N-acetyl-glucosamine, wherein the tri-N-acetyl-glucosamine is bound to the highly acetylated chitosan.

34. The chitosan composition of claim 23, further comprising at least one negatively charged substance selected from the group consisting of a polysaccharide, a phospholipid and combinations thereof.

35. The chitosan composition of claim 34, wherein the negatively charged polysaccharide is selected from the group consisting of an animal-derived polysaccharide, a plant-derived polysaccharide, chondroitin sulfate, dermatan sulfate, dextran sulfate, heparan sulfate, heparin, hyaluronic acid, keratan sulfate and combinations thereof.

36. The chitosan composition of claim 34, further comprising hyaluronic acid and phosphatidylcholine.

37. The chitosan composition of any of claims 23-35, further comprising at least one of a drug, a polypeptide, an animal cell and a plant cell.

38. Use of the composition of any of claims 34-36 for the preparation of a medicament for treating osteoarthritis.

39. Use of the chitosan composition of any of claims 23-37 for the preparation of a medicament for treating rotator cuff damage.

40. The use of claim 39, wherein the medicament further comprises autologous cells.

41. A chitosan composition comprising a highly deacetylated chitosan having a molecular weight in the range of from about 100 kDa to about 2000 kDa, and having a degree of acetylation of no greater than about 20%, and at least one saccharide oligomer having a molecular weight in the range of from about 200 to about 20000 Da, wherein the concentration of the highly deacetylated chitosan is in the range 1 to 1.5% w/v of the total composition, wherein the concentration of the saccharide oligomer is in the range 1 to 10% w/v of the total composition, wherein the ratio of the saccharide oligomer and the highly deacetylated chitosan is in the range from 2:1 to 10:1, and wherein the composition is in a form of an aqueous solution.

42. The composition of claim 41, wherein the saccharide oligomer is selected from the group consisting of a chitosan oligomer; a D-glucosamine oligomer, an N-acetyl-D-glucosamine oligomer and combinations thereof.

43. The composition of claim 42, wherein the chitosan oligomer is selected from the group consisting of a highly deacetylated chitosan oligomer having a degree of acetylation of no greater than about 20%, and a highly acetylated chitosan oligomer having a degree of acetylation in the range of from about 40% to about 60%.

44. The composition of claim 43, wherein the highly deacetylated chitosan oligomer has a degree of acetylation of about 15% or less.

45. The composition of claim 42, wherein the N-acetyl-D-glucosamine oligomer has up to about 7 units.

46. The composition of claim 42, wherein the D-glucosamine oligomer has between 3 and about 100 units.

47. The composition of claim 41, wherein at 25 °C and at pH below the neutral range the composition has a viscosity of no greater than about 150 mPa·s at a shear rate of 50 rpm.

Statement under Article 19(1)

Claims of International Application No. PCT/IL2009/000582 were amended to better define certain embodiments of the claimed invention as described in the specification as filed. The claims were merged to reduce the number of claims and no new matter has been added.

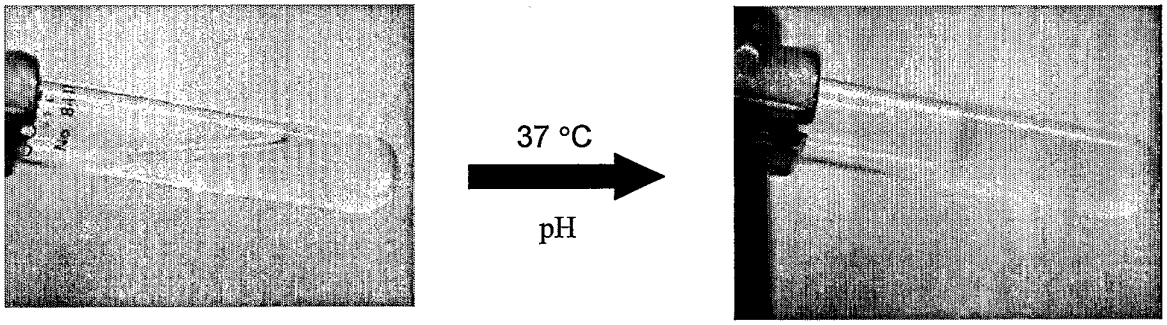

Figure 1

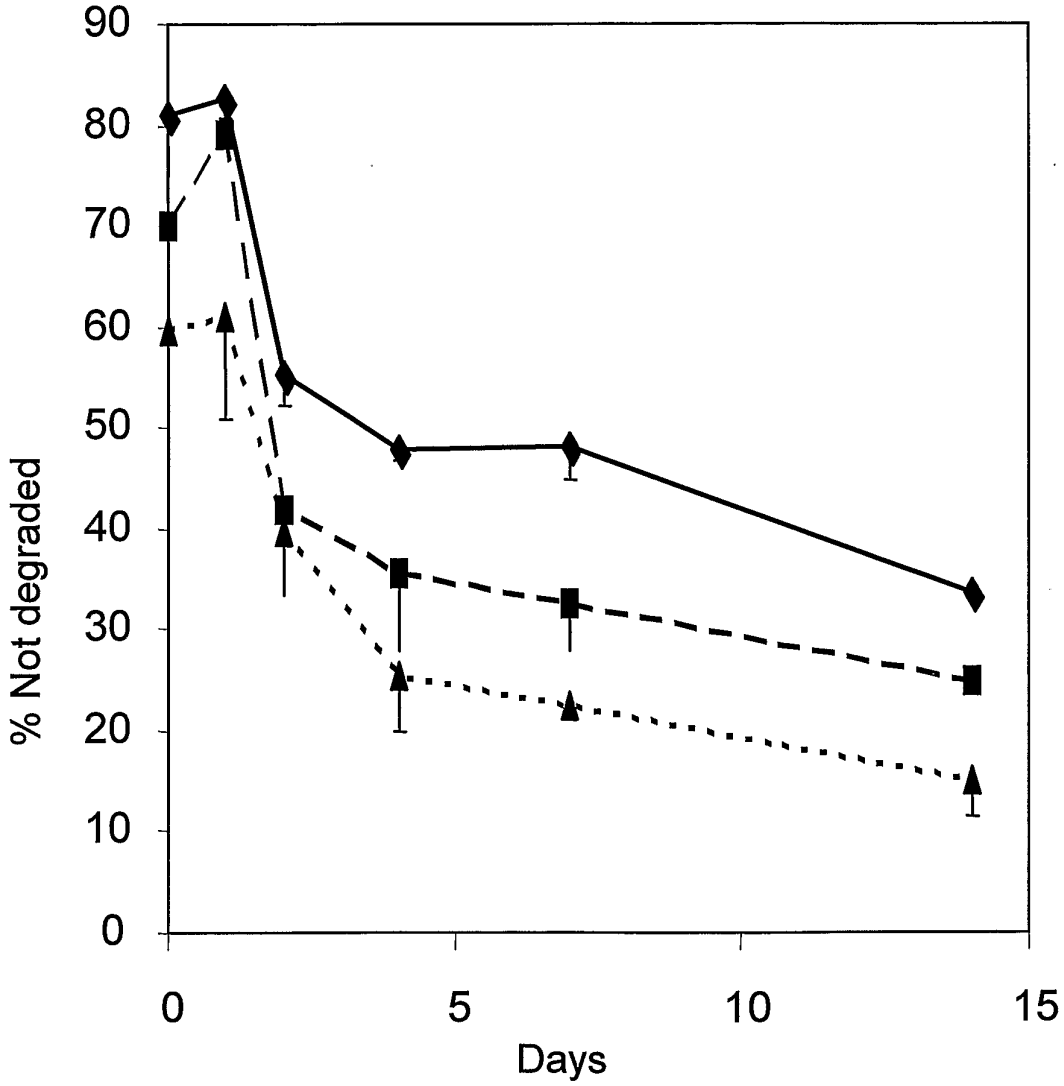

Figure 2A

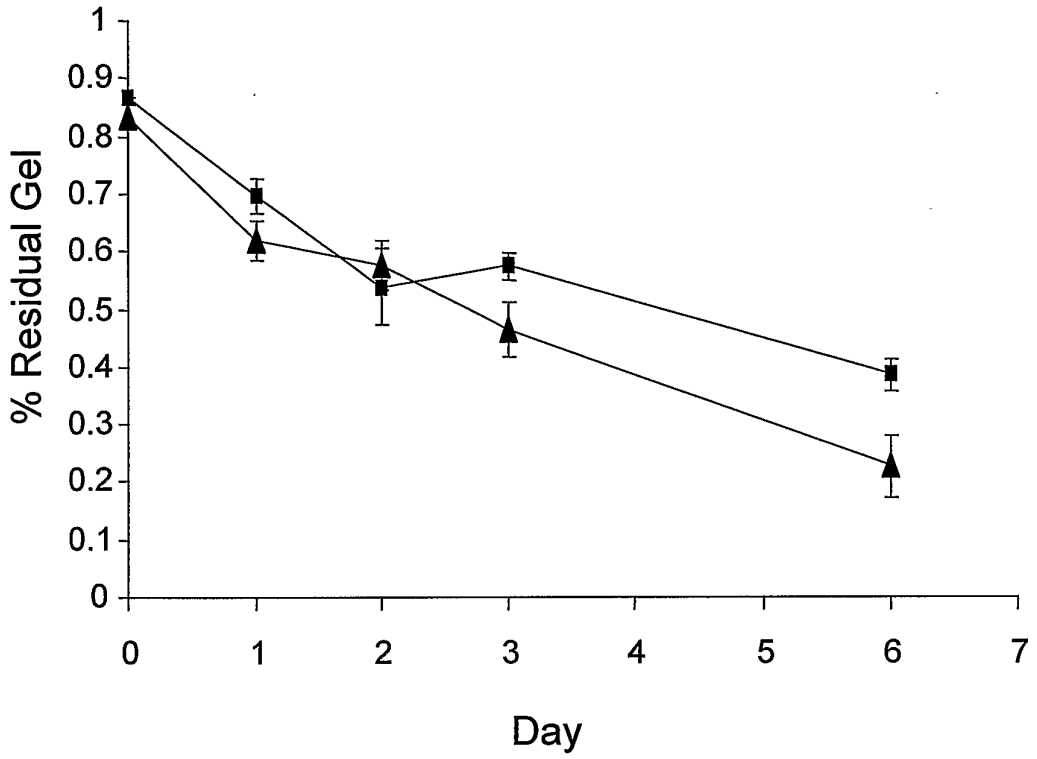

Figure 2B

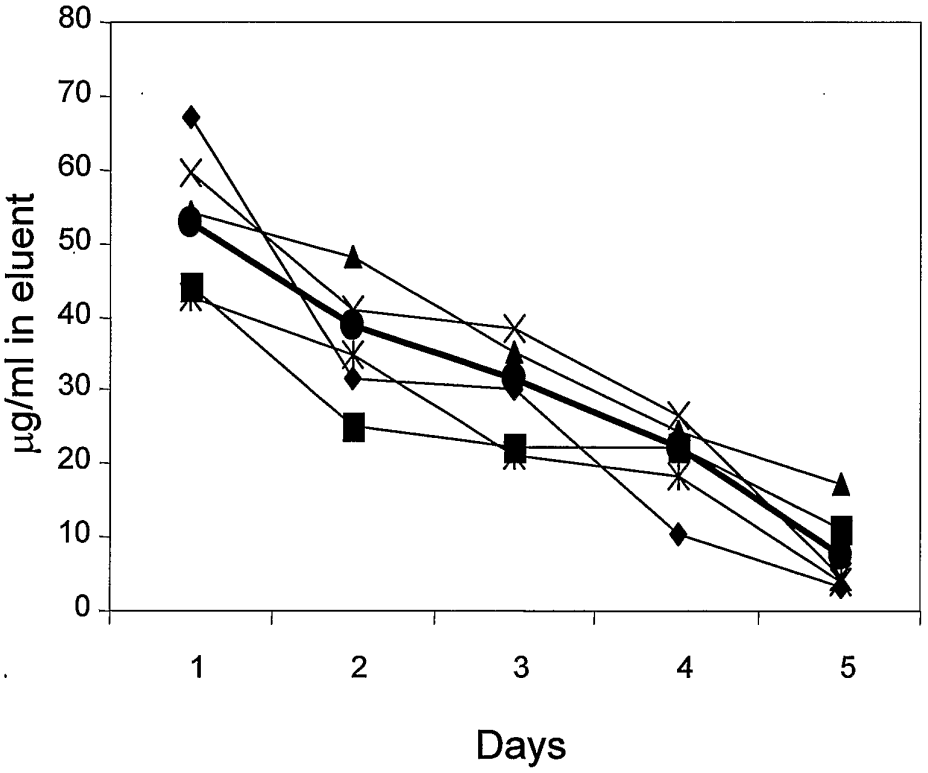

Figure 3

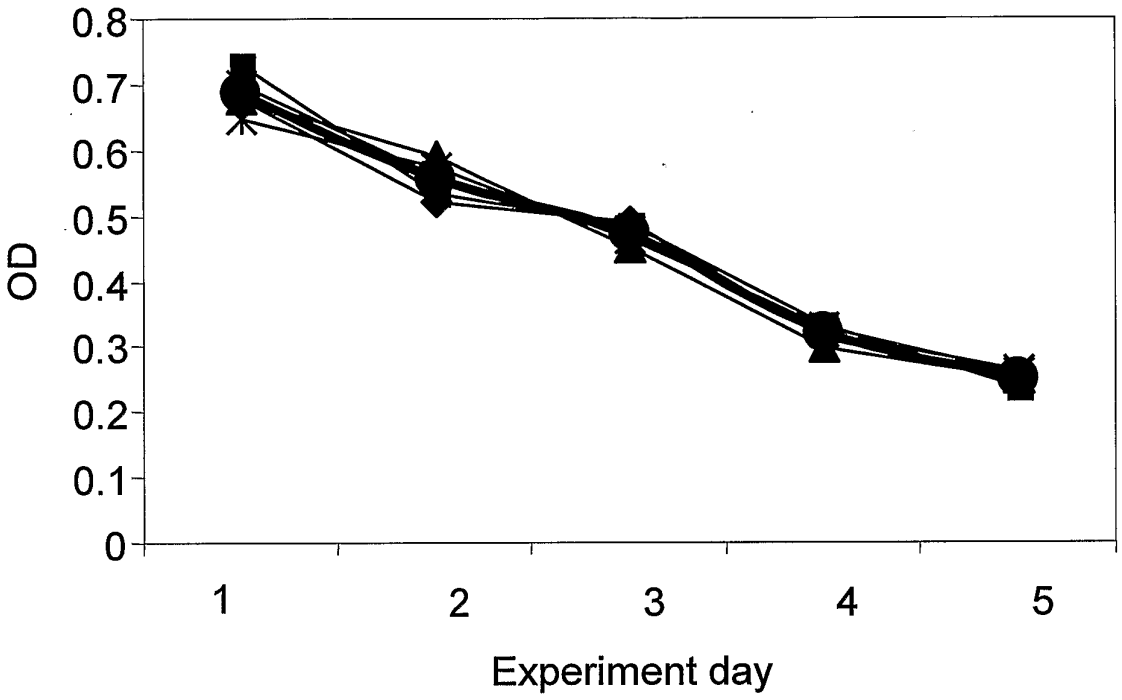

Figure 4

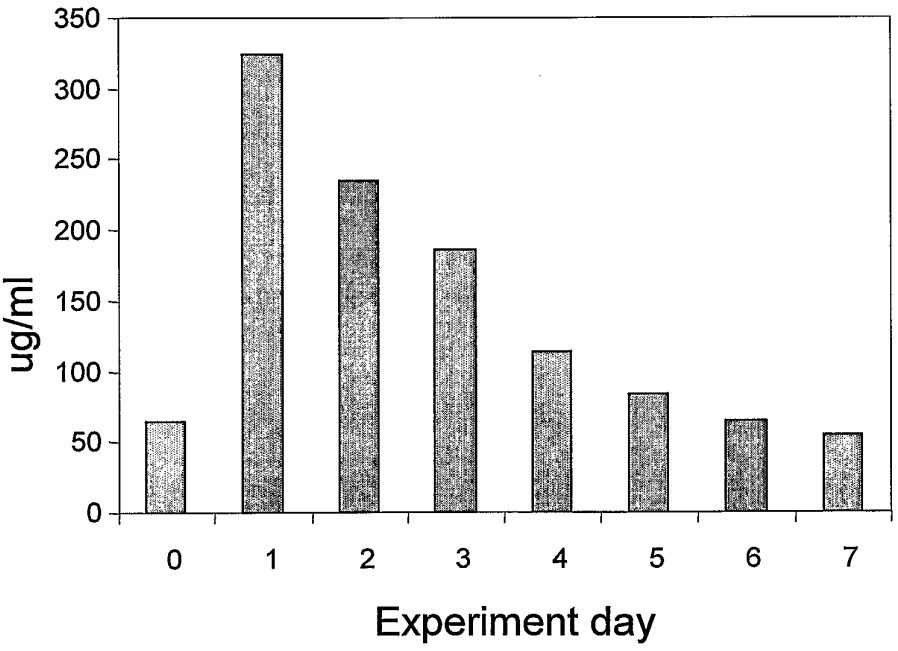

Figure 5

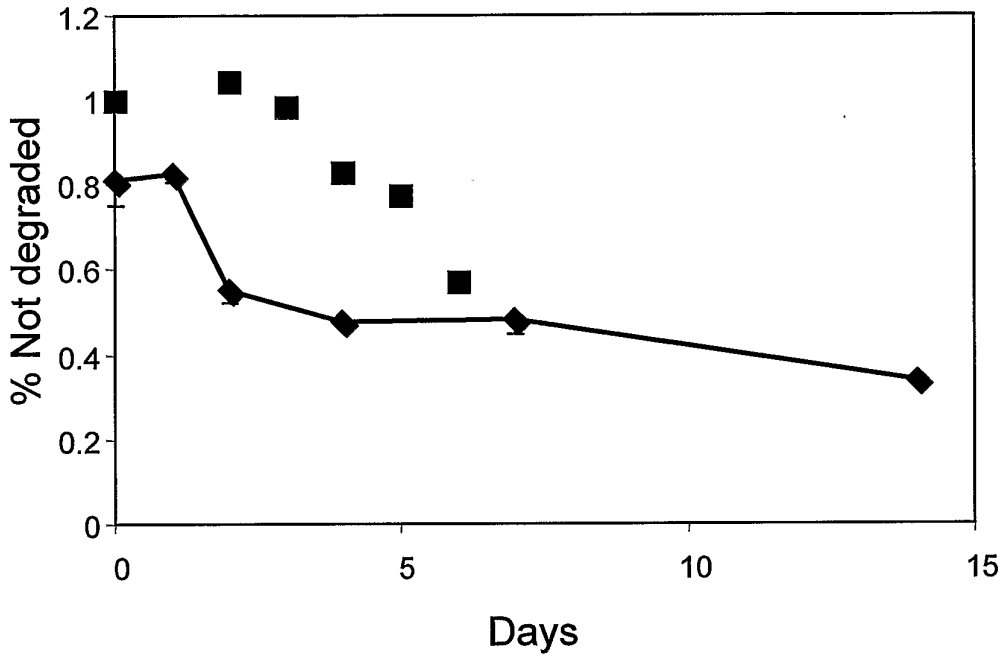

Figure 6

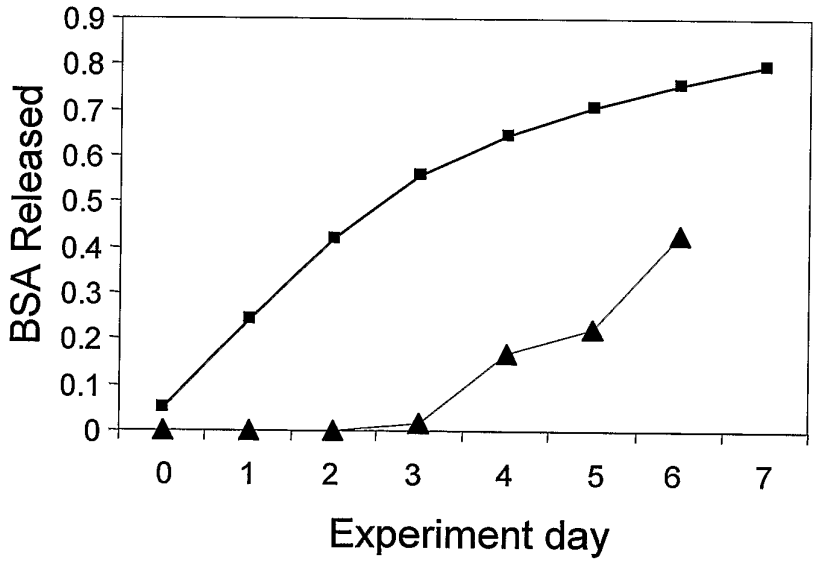
BSA Released
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7
Experiment day

Figure 7

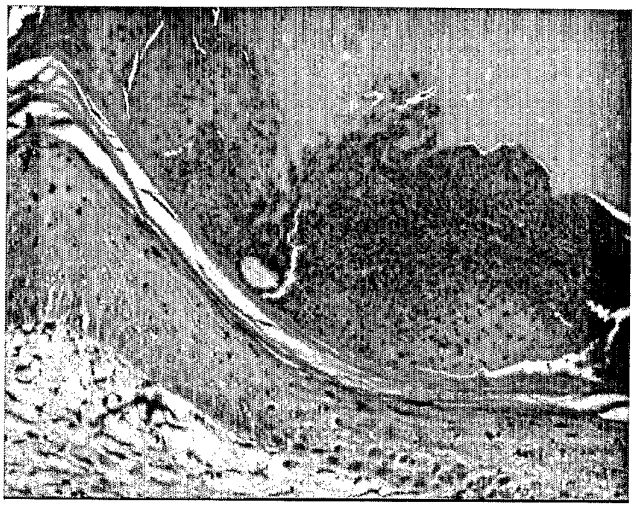

Figure 8A

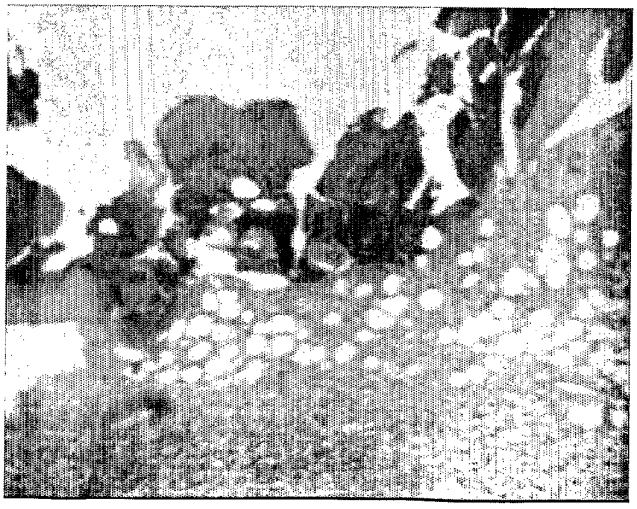

Figure 8B

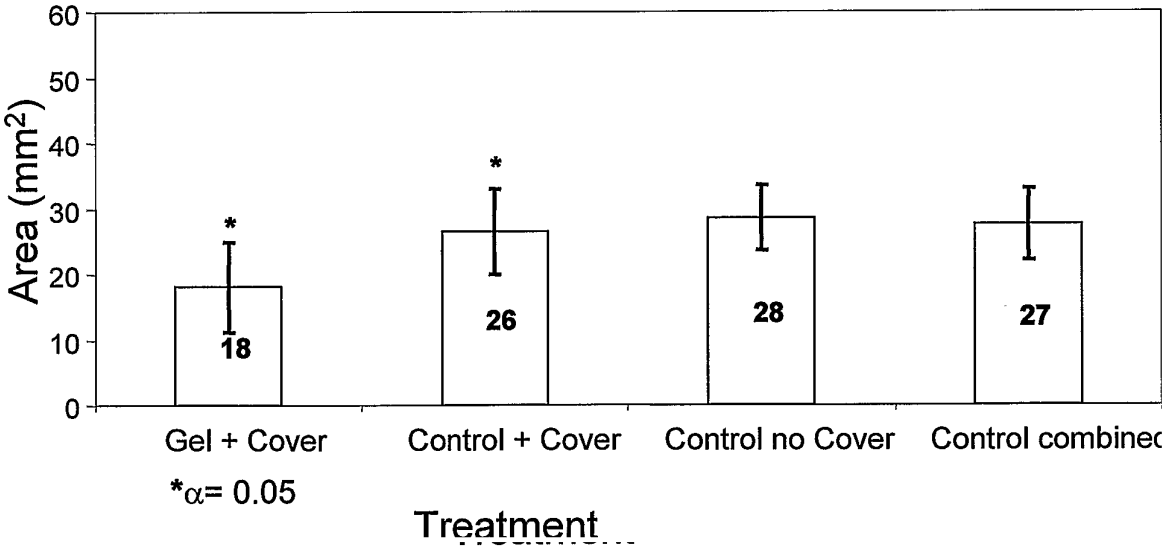
60
50
40
30
20
10
0
Area (mm2)
*
*
18
26
28
27
Gel + Cover
Control + Cover
Control no Cover
Control combinec
*α= 0.05
Treatment

Figure 9

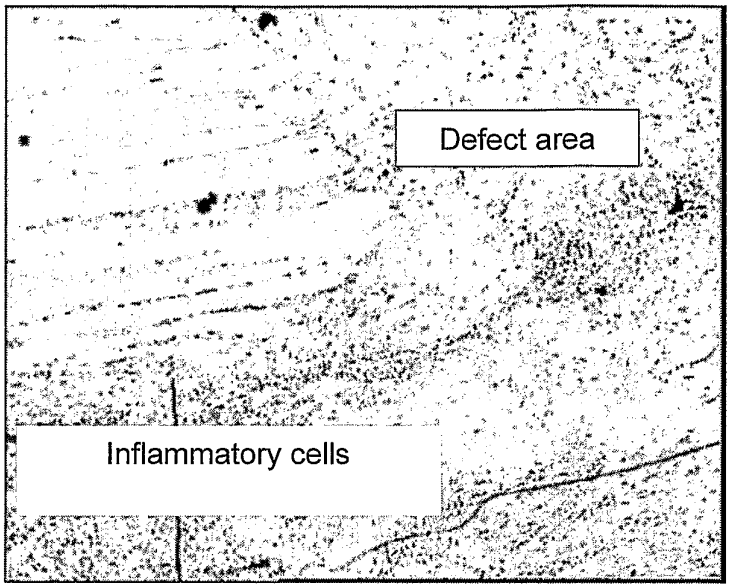

Figure 10A

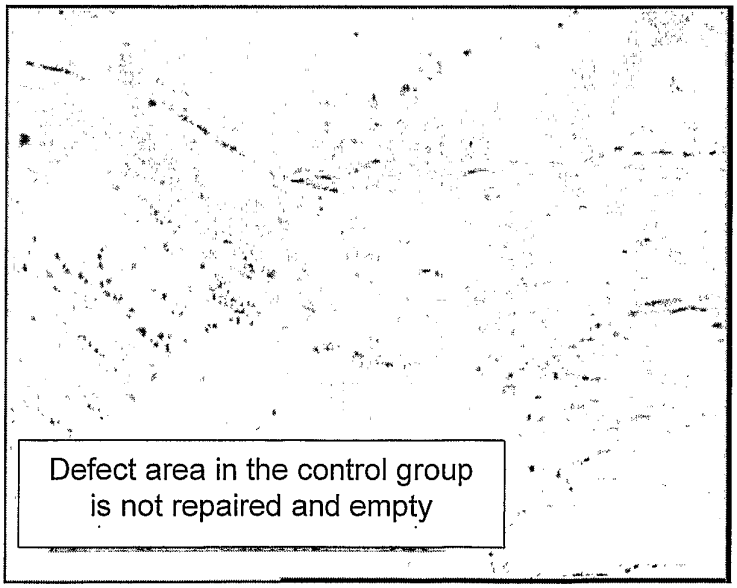

Figure 10B

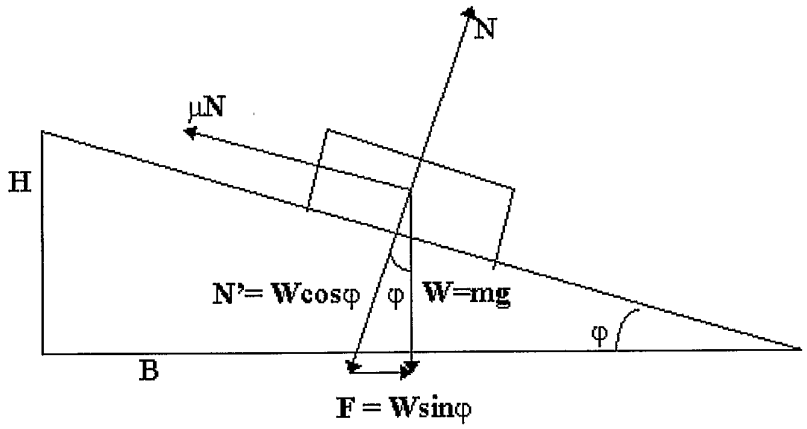

Figure 11

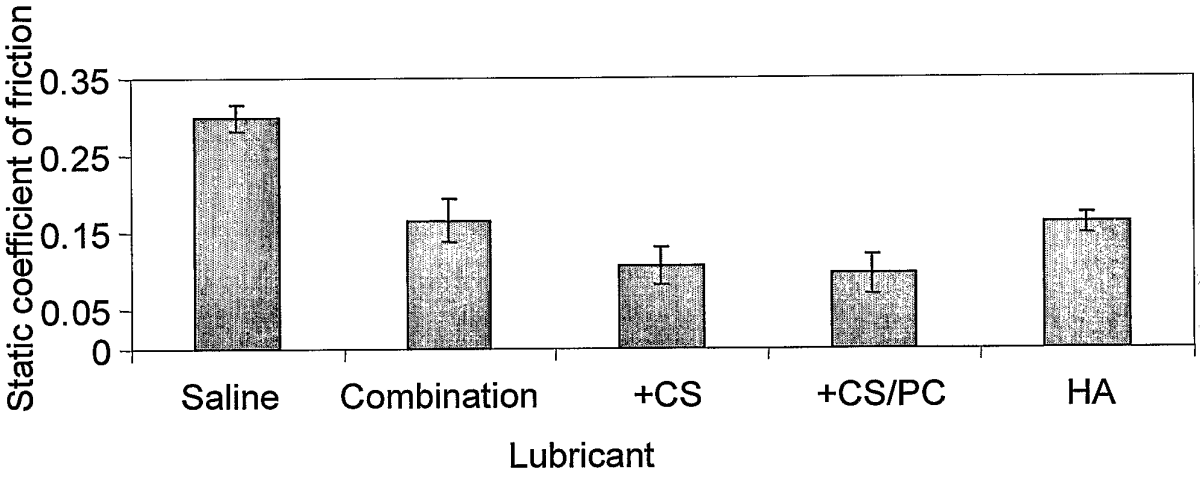
Static coefficient of friction
0.35
0.25
0.15
0.05
0
Saline
Combination
+CS
+CS/PC
HA
Lubricant

Figure 12

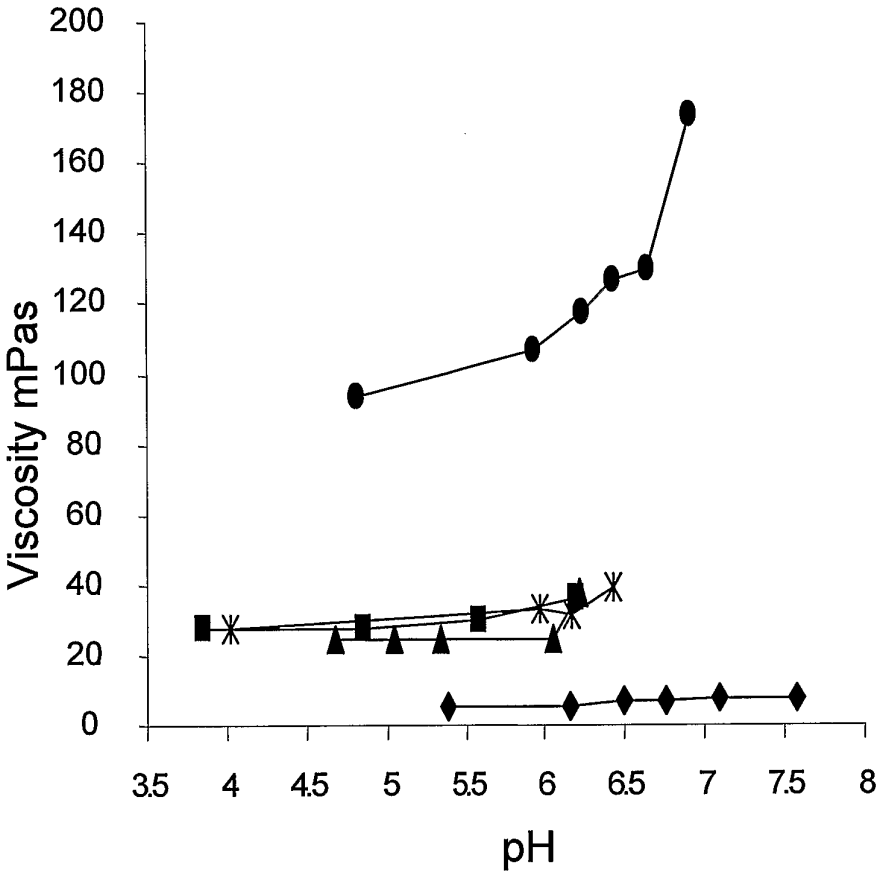
Viscosity mPas
200
180
160
140
120
100
80
60
40
20
0
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
pH

Figure 13

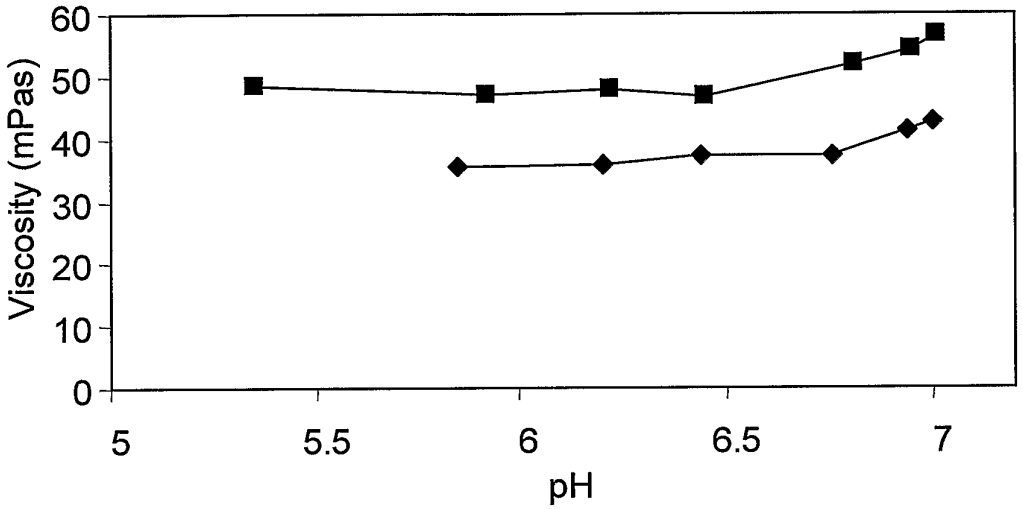

Figure 14

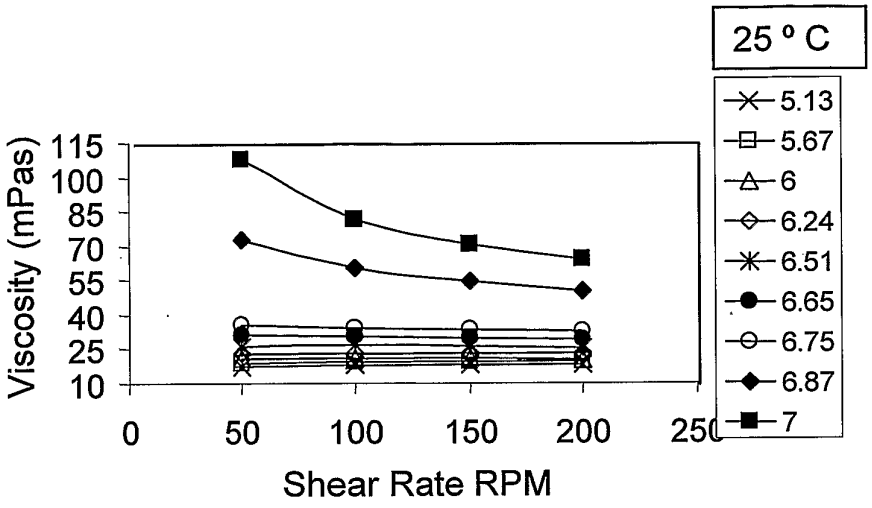

Figure 15A

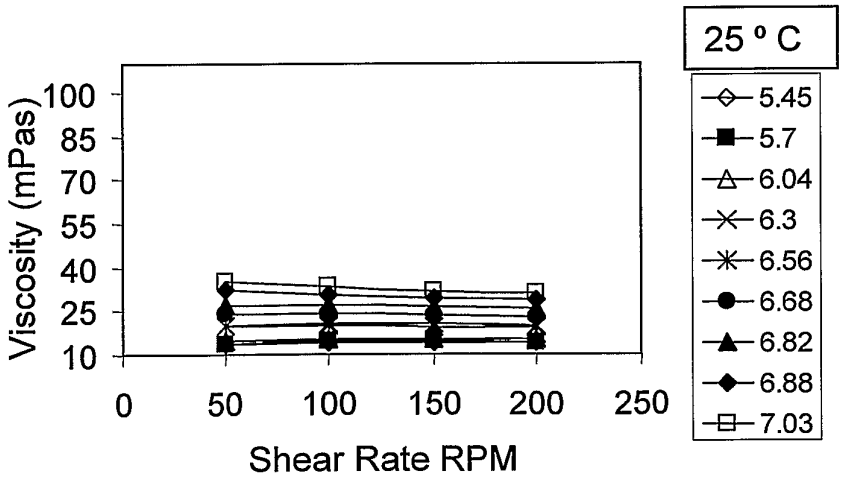

Figure 15B

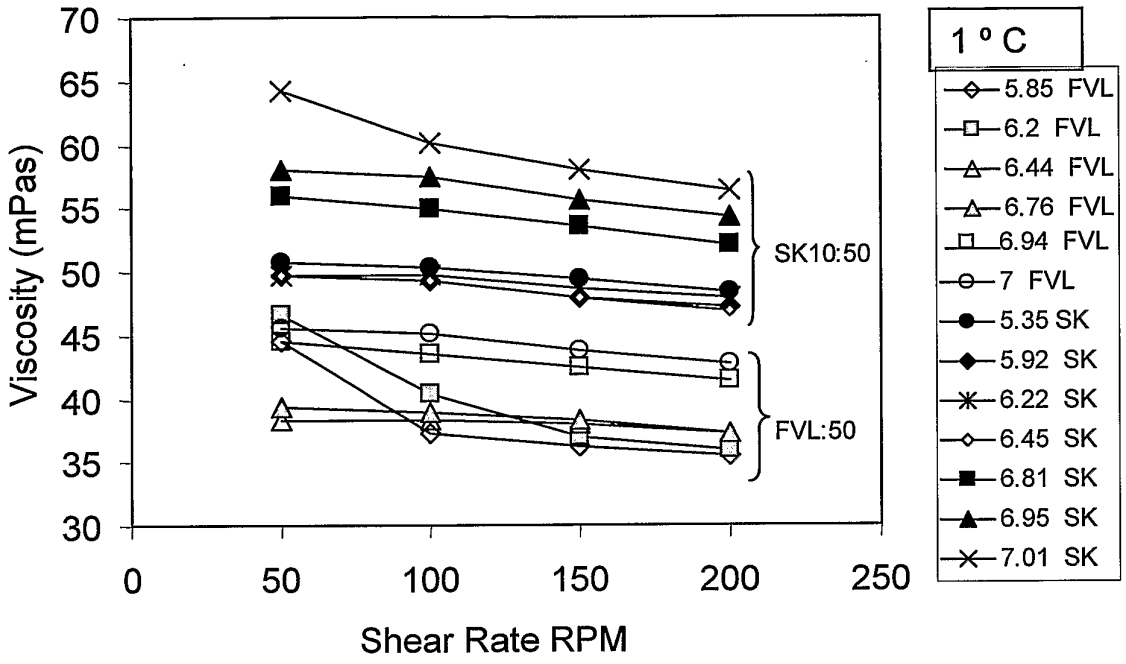
70
65
60
55
50
45
40
35
30
Viscosity (mPas)
0
50
100
150
200
250
Shear Rate RPM
SK10:50
FVL:50
1 ° C
5.85 FVL
6.2 FVL
6.44 FVL
6.76 FVL
6.94 FVL
7 FVL
5.35 SK
5.92 SK
6.22 SK
6.45 SK
6.81 SK
6.95 SK
7.01 SK

Figure 16

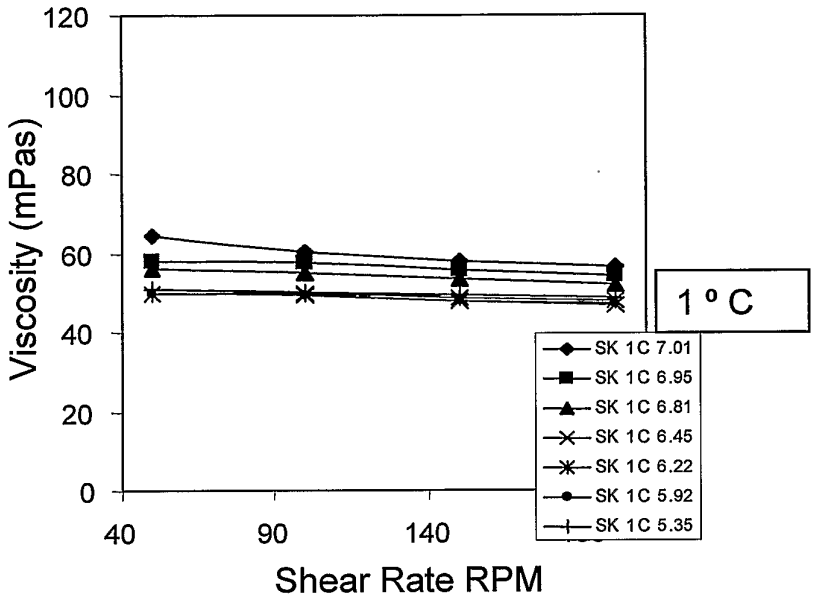

Figure 17A

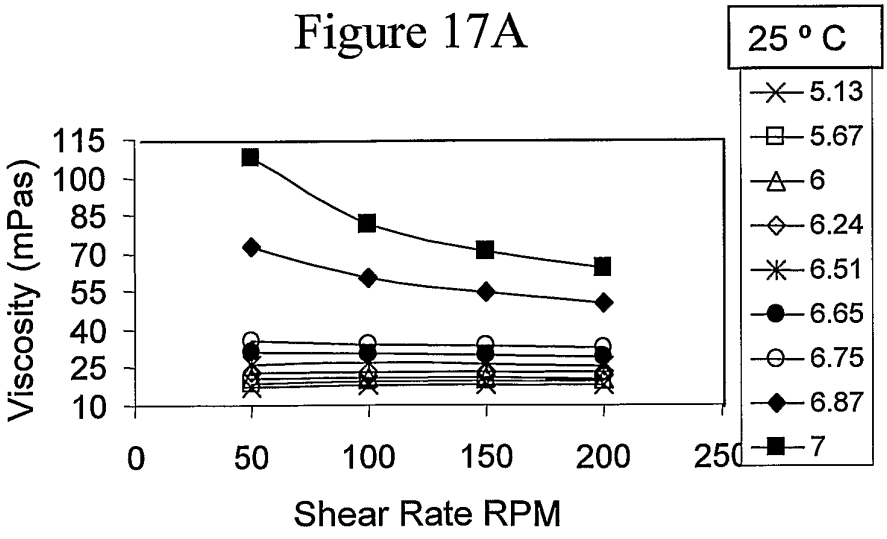

Figure 17B

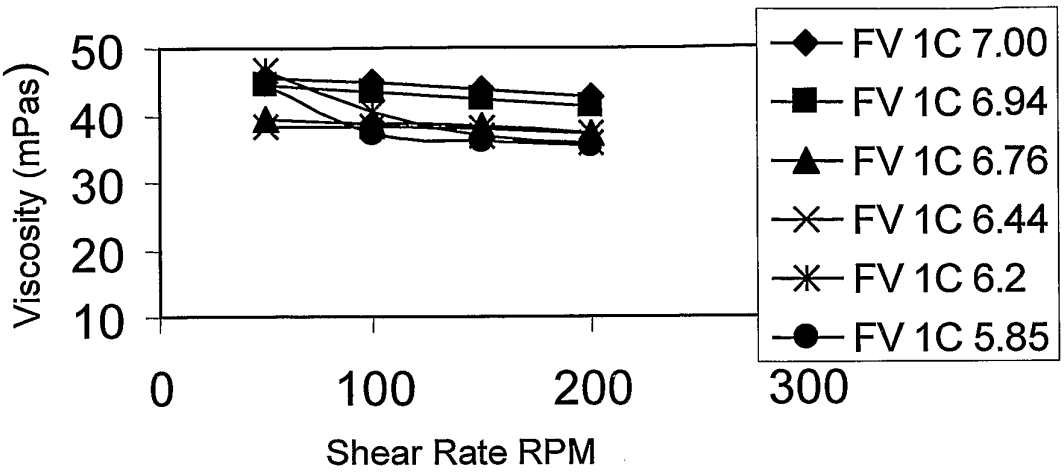

Figure 18A

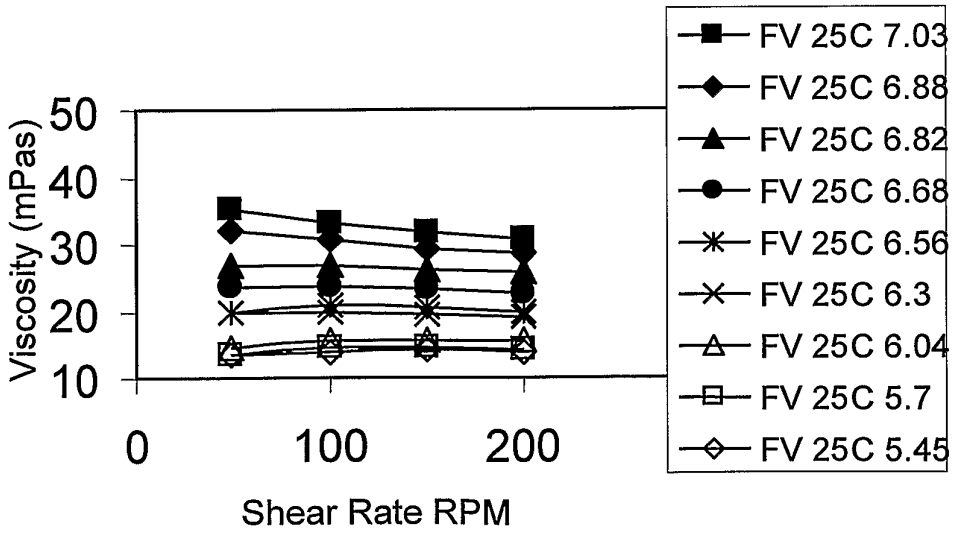

Figure 18B

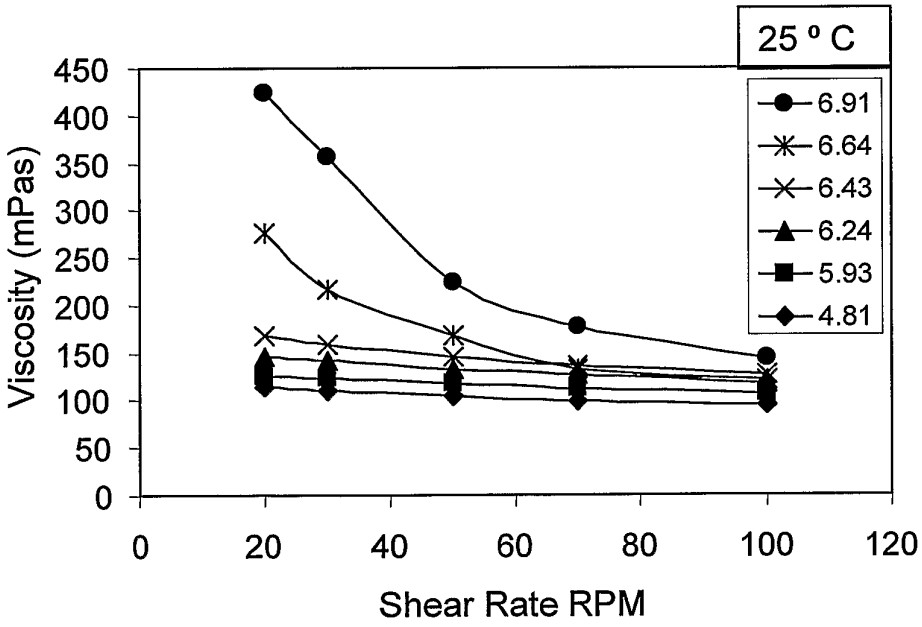
25 ° C
6.91
6.64
6.43
6.24
5.93
4.81
Viscosity (mPas)
450
400
350
300
250
200
150
100
50
0
0
20
40
60
80
100
120
Shear Rate RPM

Figure 19