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Dense chitosan surgical membranes produced by a coincident compression-dehydration process

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Abstract

High density chitosan membranes were produced via a novel manufacturing process for use as implantable resorbable surgical membranes. The innovative method utilizes the following three sequential steps: (1) casting an acidic chitosan solution within a silicon mold, followed by freezing; (2) neutralizing the frozen acidic chitosan solution in alkaline solution to facilitate polymerization; and (3) applying coincident compression-dehydration under a vacuum. Resulting membranes of 0.2 – 0.5 mm thickness have densities as high as 1.6 g/cm^3. Inclusion of glycerol prior to the compression-dehydration step provides additional physical and clinical handling benefits. The biomaterials exhibit tensile strength with a maximum load as high as 10.9 N at ~ 2.5 mm width and clinically-relevant resistance to suture pull-out with a maximum load as high as 2.2 N. These physical properties were superior to those of a commercial reconstituted collagen membrane. The dense chitosan membranes have excellent clinical handling characteristics, such as pliability and "memory" when wet. They are semi-permeable to small molecules, biodegradable *in vitro* in lysozyme solution, and the rates of degradation are inversely correlated to the degree of deacetylation. Furthermore, the dense chitosan membranes are biocompatible and resorbable *in vivo* as demonstrated in a rat oral wound healing model. The unique combination of physical, *in vitro*, *in vivo*, and clinical handling properties demonstrate the high utility of dense chitosan membranes produced by this new method. The materials may be useful as surgical barrier membranes, scaffolds for tissue engineering, wound dressings, and as delivery devices for active ingredients.

Keywords

chitosan; membrane; wound healing; biodegradation; resorbable; mechanical properties; suture

1. Introduction

The goal of this work was to develop a biocompatible, suture-able, and resorbable membrane material that exhibited excellent physical, *in vitro*, and *in vivo* properties indicative of high clinical utility as a barrier membrane. In order to develop a new implantable surgical membrane, one should first be aware of the routine indications of use and features of commercial materials used for these indications. Implantable surgical membranes typically have the following uses and properties: (a) to separate tissue layers; (b) to augment tissue defects or deficiencies; (c) to complement bone graft substitutes; (d) to prevent adhesions; (e) to provide strength; (f) to repair or prevent hernias; and (g) to promote blood clotting. Therefore, an ideal new biomaterial intended for use as a suture-able surgical membrane should proficiently address some or all of these *in vivo* uses and properties.

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Several biocompatible resorbable source materials have been employed in US FDA-cleared medical devices as commercial surgical membranes, most notably reconstituted mammalian collagen. Other biomaterials include polymers or co-polymers of lactic acid and glycolic acid (e.g., PLGA), and processed xenograft skin or gastrointestinal tissue. Of these the most common implantable surgical barrier membranes in use today by physicians and dentists are reconstituted bovine or porcine collagens. However, the physical and clinical handling characteristics of commercial collagen membranes at times display “weaknesses” (literal and figurative) and can fail to meet the needs of clinicians. For instance, once wetted collagen membranes tend to lose sufficient strength and contour “memory”. In some cases the materials are not sufficiently strong enough to support or maintain sutures or tacks to anchor the membrane into the tissues. Furthermore, the collagen material often absorbs blood rapidly during placement making it difficult to distinguish between the implant material and the surgical site.

Note that there are other types of resorbable biomedical membranes in the scientific literature, for instance electrospun/nanofibrous, laminated, wound dressing, and/or drug-eluting membranes. However, unlike those materials our primary focus is upon surgical barrier membranes intentionally designed with clinical strength and suture-ability in mind. Thus, our objective was to develop a biocompatible, suture-able, and resorbable material for use in medical devices that overcomes the inadequacies or deficiencies of the clinical *standard-of-care* collagen and other commercial resorbable barrier membranes.

In order to overcome some of the “weaknesses” in commercially-available surgical membranes, we have turned our attention to another biocompatible non-toxic material, purified chitosan. This material has well-established physical characteristics [1, 2] and a history of safety and efficacy in human subjects [3]. Chitosan is a linear polysaccharide consisting of beta(1,4) linked N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) monosaccharides [1]. Chitin, from which chitosan is derived, is nature's second most abundant biopolymer. Chitosan is routinely derived from the exoskeleton of marine crustaceans by a multi-step process of depolymerization/decalcification in acid, deproteinization, and N-deacetylation in alkali.

Chitosan degrades *in vivo* by enzymatic processes into harmless glucosamine and related oligosaccharide units. Chitosan is subject to enzymatic degradation within mammals by lysozyme, NAGases, chitinases [4–7], and possibly alpha-amylase [8]. Some of these enzymes are implicated in anti-bacterial innate immunity, in which the enzymes (for instance produced by macrophages) attack the polysaccharides of bacterial cell walls. Chitosan is coincidentally subject to the same degradative processes. In addition, *in vivo* resorption might also include the process of phagocytosis/endocytosis of chitosan nano-particulates by host cells, such as macrophages. The rates of enzymatic biodegradation *in vitro* and resorption *in vivo* vary based upon the percentage of deacetylation (% DD). Fully deacetylated chitosan does not serve as a substrate for enzymatic degradation by lysozyme. Thus, the rate of degradation is inversely proportional to the % DD. This feature can be manipulated to control the rates of biodegradation *in vivo*, and without the requirement for a crosslinking agent.

With regard to biocompatibility *per se*, purified chitosan has been demonstrated to be safe (and effective) in humans and other mammals [3, 9], as exemplified by seven broad contexts:

1. US FDA-Cleared Medical Devices for Humans: Purified chitosan is a component in multiple US FDA-cleared Class I medical devices. In most cases, chitosan is the principal component in the hemostatic wound dressings [10], and in some forms also serves as a physical barrier [11].

2. *GRAS* Food Additive for Humans: Chitosan is considered as *Generally Recognized as Safe* (GRAS) as a food additive at the level of "self-affirmed" by various manufacturers (e.g., Primex). To the best of our knowledge, a GRAS designation at the higher level of "no comment" following a full US FDA review has not yet occurred.

3. Cosmetic & Consumer Skincare Products for Humans: Chitosan is listed among the International Nomenclature of Cosmetic Ingredients (INCI). Chitosan and its various salt forms (e.g., lactate, glycolate, ascorbate, formate, & salicylate) and other organic derivatives are listed as ingredients for use in cosmetics and consumer skincare products. To the best of our knowledge, chitosan has not warranted consideration by the Cosmetics Ingredients Review (CIR) expert safety panel.

4. Purified Chitosan is Non-Allergenic in Humans: A very small portion of the human population exhibits allergies to marine crustaceans, with estimates at approximately 0.2 – 0.3 percent [12, 13]. This has led to the question of whether individuals with pre-existing allergies to marine crustaceans should avoid using medical devices composed of crustacean-derived purified chitosan. A respected authority on chitosan, Dr. Riccardo Muzzarelli reported, "The major shrimp allergen has been identified as the muscle protein tropomyosin … Shrimp-derived glucosamine is safe even for individuals hypersensitive to tropomyosin … Gray et al. clearly state that `shellfish allergy is caused by IgE antibodies to antigens in the flesh of the shellfish and not the shell; therefore it should be safe for patients with shellfish allergy to take glucosamine supplement'". ([14] p. 300). Furthermore, "In experimental and pre-clinical surgical trials, the use of chitin/chitosan and their derivatives has never led to allergies or other diseases." ([14] p. 304). In addition, according to a clinical study report from the University of Kuopio (provided to us by Primex ehf), a total of 221 humans were subjected to cutaneous allergy prick tests using purified chitosan. None of the subjects yielded a positive response to chitosan, even among the subset of individuals who tested positive for shrimp allergic reactions and/or shrimp-specific IgE. Thus, purified chitosan is non-allergenic.

5. Wound Healing in Humans: Beyond US FDA Class I wound dressings, chitosan has also been used as a biocompatible material with success *in vivo* in a variety of human wound-healing investigations. As examples, in a periodontal study a chitosan gel produced a radiographic benefit to bone relative to the control treatment, thus demonstrating a guided bone regeneration (GBR) benefit [15]. In another periodontal study, chitosan ascorbate gel provided an efficacious result for periodontitis [16]. Furthermore, chitosan membranes were used with benefit to promote the healing of cutaneous wounds [17] and nasal sinuses [18].

6. Wound Healing in Mammals: It has been well documented that chitosan-containing devices have been used with success *in vivo* in veterinary care, in wound healing, and in tissue engineering studies in a vast array of non-human mammalian species and organ sites [19, 20].

7. Delivery of Active Ingredients: Chitosan has been used successfully *in vivo* as controlled-release matrices for the delivery of drugs, biologics, peptides, nucleic acids, and vaccines [3, 21–44].

The historic challenge before us was to develop a process whereby purified chitosan could be formed into high density membranes that coincidentally retained appropriate physical and clinical handling properties suitable for use as a surgical membrane. None of the chitosan materials to date in the prior art had this unique desirable blend of attributes. Other chitosan

biomaterials have been developed and, in some cases, commercialized. However, none of these methods of manufacture delivered appropriate properties for use as surgical membranes, most notably: (a) high density; (b) pliability without fragility; (c) tensile strength; and (d) resistance to suture pull-out. For instance, a common commercial material is the low density chitosan "sponge" that is routinely used as a wound dressing for hemostasis. This product lacks sufficient density and strength to serve as a barrier membrane. In addition, it is too thick (ca. 4.5+ mm) and lacks adequate suture-ability for use as an implantable barrier membrane, which should be preferably about 0.2 − 0.5 mm in thickness, based upon commercially available resorbable collagen membranes. Therefore, herein we describe an innovative method for producing high density chitosan membranes by a coincident compression-dehydration process, and demonstrate for the first time a resulting suture-able chitosan-based barrier membrane with multiple beneficial properties of direct relevance to clinical utility.

2. Methods

2.1 Materials

Purified chitosan materials were obtained commercially from Heppe Medical Chitosan GmbH (Germany) and Primex ehf (Iceland). The batches with degrees of deacetylation from 70 – 95 percent and viscosities ranging from 416 – 621 mPas (both properties were determined by the vendors) are listed in Table 1. For comparison to a commercially available surgical barrier membrane, a common reconstituted bovine Type I collagen material was obtained, the *BioMend Absorbable Collagen Membrane®* (Zimmer). For comparison to a non-membrane material composed of low density chitosan, the *HemCon Dental Dressing®* (HemCon Medical Technologies) was obtained.

2.2 "Standard Method" of Manufacturing Dense Chitosan Membranes

Although various permutations were used during the development of the method, the standard method of manufacture of dense chitosan membranes involved the following steps:

(1) 4% w/v purified chitosan base was prepared at room temperature in 2% v/v acetic acid for the Heppe chitosan solution (or 1% acetic acid v/v for the Primex chitosan solution) and allowed to stand for a period of ~ 7 days. For moderate thickness membranes, 0.5 g (wet weight) of acidic chitosan solution was cast per cm^2 of silicon mold surface area. To do so, 116 g (wet weight) of acidic chitosan solution was poured into a 6" × 6" (15.2 cm ×15.2 cm = 231.04 cm^2) silicone mold, yielding 0.502 g (wet weight)/cm^2. This material's surface area will shrink in subsequent steps to ~ 5.5" × 5.5" (~ 14 cm × 14 cm = 196 cm^2) yielding the equivalent of ~ 0.592 g (wet weight)/cm^2, which at 4% chitosan in the acidic solution is equivalent to ~ 23.67 mg (dry weight of chitosan)/cm^2. For a thinner material, casting 0.3 g (wet weight of 4% chitosan solution) per cm^2 of silicon mold surface area would yield ~ 14.2 mg (dry weight of chitosan)/cm^2. As desired, one may vary the amount of cast material and/or the percentage of acidic chitosan solution.

(2) The viscous acidic chitosan solution within the mold was subjected to mechanical vibration on a horizontal platform shaker for 20 minutes in order to remove air bubbles and to settle the gel into a uniform thickness.

(3) The mold was subjected to ultra-freezing at −80C for one hour.

(4) The frozen acidic chitosan solution was removed from the silicon mold and immersed in 2 M NaOH solution at RT for 24 hours with gentle agitation, in order to displace the acid within the composition and to promote polymerization.

(5) The alkali-treated polymerized material was rinsed in slow-flowing deionized water for 24 hours to remove all of the alkali. This results in a material with a neutral pH. Then, the rinsed chitosan polymer was immersed in 10% glycerol in water (v/v) for 15 minutes. The inclusion of glycerol prior to coincident compression-dehydration (Step 6) has benefit to the resulting material, such as flexibility and ease of cutting to size while dry.

(6) The polymerized chitosan material was heat-sealed on all four sides between a pair of Cellophane® semi-permeable membranes (Innovia Films, NE coated). The seals should be in close proximity to the edges of the chitosan polymer, in order to minimize the void that could permit lateral expansion upon compression. The polymerized chitosan within the sealed Cellophane layers was soaked in deionized water for 15 minutes to wet the Cellophane, and then placed on a coincident compression-dehydration gel dryer (Labconco). Within this device a "sandwich" was prepared consisting of an uppermost flexible rubber seal (to maintain a vacuum), a solid smooth planar material (e.g., a metal plate), a filter paper (Whatman), the sealed and wetted Cellophane "envelope" containing the polymerized chitosan composition, another Whatman filter paper, and the gel dryer's mesh surface underneath from which the vacuum is applied. The vacuum pressure (preferably with a minimal pressure of 25 inches of Hg) was applied for 4 hours in conjunction with coincident heating of the gel dryer at 80C.

(7) The vacuum-dried dense chitosan membrane material was separated from the Cellophane films. The dehydrated chitosan membranes made according to these parameters are typically 0.2 – 0.5 mm in thickness and have densities > 0.8 g/cm^3 and can reach as high as 1.6 g/cm^3. The membranes may be stored at room temperature.

2.3 "Alternative Method" of Manufacturing Dense Chitosan Membranes

The alternative method lacks glycerol after rinsing (Step 5), and this aspect is referred to as the alternative "Step 5b". Whenever Step 5b was performed the subsequent process of soaking in deionized water to wet the Cellophane (Step 6) was increased from 15 minutes to 1 hour duration. However, it is uncertain whether the increase in time is necessary or beneficial. Membranes produced without glycerol (i.e., Step 5b) have the tendency to be brittle (when dry) and can inadvertently crack while cutting to the desired size. In view of this observation, the resulting dry membranes lacking glycerol were hydrated in deionized water followed by lyophilization (freezing followed by vacuum without compression), prior to cutting to size. This hydration/lyophilization modification is referred to as the additional "Step 8". In summary, the alternative method of manufacturing includes the hydration/lyophilization steps in lieu of glycerol.

2.4 Scanning Electron Microscopy

The surfaces of the dense chitosan membranes were sputter coated with gold-palladium and then examined using an FEI Quanta FEG 650 model scanning electron microscope with secondary electron (SE) imaging and 10 KV accelerating voltage at magnifications of 100 ×, 250 ×, and in some instances higher magnifications. An internal reference size bar of 500 um (0.5 mm) was included in the photographs. Dense chitosan membranes were manufactured by the standard method (with 10% glycerol) using 75% and 80% DD (Heppe) chitosan at 4% and 0.3 g/cm^2 (amount cast). The low density commercial acidic chitosan "sponge" material, *HemCon Dental Dressing*®, was also examined for comparison. The opposite planar surfaces of the membranes were annotated according to differences during the manufacturing: (a) frozen up, dehydrated up; and (b) frozen down, dehydrated down. In

other words, “frozen down” refers to the orientation of the acidic chitosan solution in contact with the latex mold surface during casting, and “dried down” refers to the orientation of the neutralized/polymerized chitosan gel on the side from which the vacuum was drawn.

2.5 Tensile Strength

The membrane materials made with Heppe chitosan were tested using an Instron 5565 machine with ASTM standard methodologies and minor modifications (e.g., semi-circular rather than semi-oval template cut-outs for tensile testing according to ASTM D 1708-06a) [45, 46]. The barbell-shaped dense chitosan membrane strips with ~ 2.5 mm minimum width were hydrated in Phosphate Buffered Saline (PBS) for 5 minutes prior to testing on the Instron machine. The test strips were anchored by pneumatic clamps and fine wet-or-dry sandpaper. Replicates (two or three samples per experiment) were analyzed at a crosshead speed of 1 mm/minute. The following parameters were determined: thickness while dry (by digital caliper in mm), minimum width while dry (by digital caliper in mm), maximum load (kgf and N), tensile extension at maximum load (mm), and maximum tensile stress (kgf/cm^2 and MPa). The conversion factor for kgf to N (Newtons) is 1 kgf = 9.80665 N. Averages of the replicates within each experiment were calculated for each parameter. A commercial reconstituted collagen membrane control, the *BioMend Absorbable Collagen Membrane®*, was included for comparison.

2.6 Resistance to Suture Pull-Out

The membrane materials made with Heppe chitosan were tested using an Instron 5565 machine to determine the resistance to suture pull-out. Strips of ~ 5.0 mm in width were hydrated in Phosphate Buffered Saline (PBS) for 5 minutes prior to testing on the Instron machine. The strips were subjected to suture pull-out tests, with a single suture loop created using 4.0 silk suture and FS-2 needle. The suture needle was inserted at the midline of the strip and several millimeters from the terminus. One end of the test strip was anchored by a pneumatic clamp and fine wet-or-dry sandpaper, and the suture loop was attached to a mechanical hook. Replicates (two or three samples per experiment; with an exception in experiment # 24 consisting of only one sample) were analyzed at a crosshead speed of 1 mm/minute. The following parameters were determined: thickness (by digital caliper in mm), minimum width (by digital caliper in mm), maximum load (kgf and N), tensile extension at maximum load (mm), and maximum tensile stress (kgf/cm^2 and MPa). Averages of the replicates within each experiment were calculated for each parameter. As a non-chitosan control the *BioMend Absorbable Collagen Membrane®* was included.

2.7 *In Vitro* Permeability

Dense chitosan membranes were subjected to Franz cell permeability testing with low molecular weight dye compounds, Methylene Blue (Mw 285; Fisher Scientific) and Crystal Violet (Mw 373; Sigma) [21]. Membrane discs of ~ 2 cm were hydrated overnight in 0.5× PBS + sodium azide, then mounted between the upper and lower chambers of 9 mm (inside diameter) Franz cells (PermeGear). The stock solution consisted of 0.1% (w/v) dye in 0.5× Phosphate Buffered Saline (PBS) + sodium azide followed by 0.45 micron filtration. 1.0 ml of the filtered dye concentrates (equal to or less than 0.1%) were placed on the top chamber and 5.0 ml of 0.5× PBS + sodium azide on the lower receiver chamber. The water-jacketed chambers were maintained at 30C using a recirculating water bath. Passive diffusion across the membranes was monitored by serial sampling of 100 ul aliquots from the lower 5 ml chamber at 1, 2, and 4 hours, and overnight (e.g., 24 hours). The samples taken from the lower chamber and the controls (buffer alone and a dilution series of the starting dye stocks) were loaded into 96-well plates and subjected to spectrophotometry at 668 and 590 nm for Methylene Blue and Crystal Violet, respectively. The pairs of samples at each time point

were averaged and then subjected to scatter plot regression analyses to determine the initial linear rates of diffusion during the time interval of 1–4 hours.

2.8 *In Vitro* Degradation

In order to assess the biodegradability of the dense chitosan materials, 12 mm discs of membranes (Heppe chitosans at various % DD) were placed in multi-well plates and immersed in 15 mg/ml lysozyme solution in PBS + sodium azide for up to 3 weeks at 37C (with daily exchange of the solution). Photographs of disc translucency were obtained at various time points and were analyzed by *Image J* imaging software as a measure of loss of chitosan.

2.9 *In Vitro* Cell Culture

Cellular adhesion and proliferation were investigated on the surfaces of the dense chitosan membranes (Heppe 80% DD, 0.3 g/cm^2 cast amount). Membranes were precisely cut with 8 mm biopsy punches. Membranes were wetted for 10 minutes with a low volume of appropriate medium for each cell line. Membranes were then temporarily “glued” to the bottom of a sterile culture plate well with 10 μl of 10% gelatin, warmed to 42C. After the gelatin hardened to adhere the membrane discs to the plastic, 1×10^5cells were applied per 8 mm disc. Cells were triturated (mono-dispersed) 3 times, allowed to settle for 20 minutes, and then incubated at 37C with 5% CO_2. Three human cell types were used: human primary keratinocytes (Lifeline Cell Technologies) cultured in DermaLife (Lifeline Cell Technologies); human primary dermal foreskin fibroblasts (ATCC) cultured in DMEM-high glucose, 5% Fetal Calf Serum, 1% penicillin-streptomycin; and human MC3T3 pre-osteoblasts subclone E4 (ATCC) cultured in Alpha MEM without aspartic acid (Invitrogen). Cell attachment and growth was analyzed during the next seven days using Cell Tracker Red live cell fluorescent stain (Invitrogen) and fluorescence microscopy.

2.10 *In Vivo* Biocompatibility & Resorption

A rat oral palate surgical model was used to assess *in vivo* biocompatibility, wound healing, and resorption. All animal studies were performed with IACUC approval at the University of Alabama at Birmingham. NIH guidelines for the care and use of laboratory animals (NIH Publication #85–23 Rev. 1985) have been observed. Bilateral full-thickness 1.5 mm punch wounds were made on the palate of male Lewis rats and dense chitosan membranes, control membranes, or no membranes were placed at the base of the wounds. To create the wounds and insert the membranes, a 4–5 mm linear palatal incision perpendicular to the ridge was made 7 mm mesial to the first molar across the maxillary arch from which a full thickness tunnel flap was raised along the ridge distally to the first molar. A precise 1.5 mm diameter circular biopsy punch (Robbins Instruments, Chatham, NJ) was used to create a full-thickness window in the flap. A 4 mm diameter membrane was slid under the flap so the membrane was exposed at the punch site toward the oral cavity. The membranes were manufactured by the alternative method (Primex 79–95% DD, 0.5 g/cm^2 cast amount). The flap was closed along the ridge at the mesial incision with surgical cyanoacrylate and photographed. The animals were maintained on a soft diet with acetaminophen analgesic in the drinking water for one week after surgery. Membranes were placed bilaterally with this procedure, each side requiring approximately 5–10 minutes operating time. Photographs were taken of the initial exposed membranes on the maxilla, then again at days 1, 2, and 3 in all animals, then at 1, 3, 6, and 12 weeks at the time of euthanasia of groups of animals and assessed for wound closure. Histologic assessments included re-epithelialization, membrane biodegradation, and collagen synthesis at the site of membrane resorption using Masson's trichrome stain.

3. Results

Dense chitosan membranes were produced using an innovative manufacturing process, and two variants of the general method were used to produce a dry and pliable dense material. The "standard method" included glycerol prior to coincident compression-dehydration. The "alternative method" lacked glycerol, but included an additional final step of hydration-lyophilization. The resulting physical, *in vitro*, and *in vivo* properties of the new materials are described below.

3.1 Physical Properties

3.1.1 Density—Membrane densities were calculated based upon the weights of 7 mm punched discs and digital micrometer measurements of thickness. Depending on the manufacturing variables (e.g., standard method v. alternative method, cast amount of acidic chitosan solution, and percentage of DD), the membranes had dry densities of ~ 1.0 – 1.6 g/cm^3. The results of representative experiments (18 experimental batches of chitosan membranes) are summarized in Figure 1a. The dry membranes that were characterized for this study were typically 0.2 – 0.5 mm in thickness (with one exception out of 18 batches). The standard method (with glycerol) produces a slightly thinner and denser material than the alternative method. During hydration the dense chitosan membranes of both methods expanded, more than doubling in thickness within 1 h (Figure 1b) with no significant increase in thickness beyond 1h (linear regression, $R^2 = 0.98$). This is comparable to the degree of hydration expansion of *BioMend Absorbable Collagen Membrane*®, and especially so for the standard method (Figure 1b).

The high density chitosan membranes displayed various properties unlike any chitosan materials disclosed in the prior art or commercially available. For example, the density of lyophilized acidic chitosan "sponge", *HemCon Dental Dressing*® was ~ 0.08 g/cm^3 (Figure 1a). Once wet, the HemCon material produced a gelatinous amorphous material, so it was not possible to determine the degree of hydration expansion, if any. In view of its low density and behavior when wet, this material is not suitable or intended for use as an implantable suture-able barrier membrane. Rather, it is a wound dressing intended primarily to control hemorrhage.

3.1.2 Scanning Electron Microscopy—Both surfaces of the dense chitosan membranes have been examined by scanning electron microscopy (SEM). Photographs of a dense chitosan membrane manufactured by the standard method (Heppe 80% DD, 0.3 g/cm^2 cast amount) are shown in Figure 2a & b. The images of both planar surfaces provided evidence of smooth surfaces at lower magnifications and some fine detail porosity at higher magnification. A minor asymmetry was noted between the two surfaces with regard to porosity.

By contrast the low density commercial chitosan "sponge", *HemCon Dental Dressing*®, displayed higher porosity, with very large pores on one surface (Figure 2c), some of which are nearly 0.5 mm in breadth. The opposite surface resembled a sponge with considerably smaller pores (Figure 2d). This material, produced by lyophilization of an acidic chitosan solution, displayed profound asymmetry and it is likely that the pores or cavities represent the areas formerly occupied by ice crystals. Note that both surfaces of the HemCon material are remarkably less dense than our chitosan membranes. Our chitosan membranes were almost entirely solid polymeric material with minimal (and small) pores, whereas the HemCon "sponge" material was the opposite with the majority of the space consisting of air.

3.1.3 Tensile Strength—The tensile strength results are shown in Table 2. An Instron 5565 machine was used to characterize the dense chitosan membranes as barbell-shaped

tensile testing strips with a minimum width of ~ 2.5 mm. The standard method of manufacturing (i.e., 8 experimental batches, with glycerol) was compared to the alternative method (10 experimental batches, with hydration/lyophilization and lacking glycerol). Other variables included % DD ranging from 70% to 95%, the amount of acidic chitosan solution cast into the mold ranging from 0.25 – 0.5 g/cm^2, and the dry membrane thicknesses typically ranging from ~ 0.2 – 0.5 mm. Note that 0.3 – 0.35 g/cm^2 of acidic chitosan solution at casting produced "thin" membranes with an average membrane thickness of 0.23 mm for the standard method (with 10% glycerol). As expected, the thicknesses of the various membrane batches positively correlated with the amount of acidic chitosan solution cast into the silicon molds (i.e., R^2 values of 0.68 for the standard method and 0.70 for the alternative method of manufacturing).

The barbell-shaped samples with ~ 2.5 mm in minimum width were hydrated for 5 minutes immediately prior to testing on the Instron machine. This step was performed to mirror how the barrier membranes are intended to be used in a clinical setting by a physician or dentist. An array of experimental variables (in Table 2) yielded the following ranges of physical properties: (a) maximum loads of 3.2 – 10.9 N for the standard method and 2.4 – 5.4 N for the alternative method of manufacturing; and (b) maximum stresses of 61.7 – 118.3 mPa for the standard method and 33.1 – 68.1 mPa for the alternative method of manufacturing. The maximum stress (accounting for cross-sectional area at the narrowest point) yielded a group average of 85.8 mPa for the 8 experimental batches of the standard method of manufacturing, whereas the alternative method (including post-manufacturing steps of hydration/lyophilization and lacking glycerol) yielded a group average of 45.2 mPa for the 10 experimental batches.

Correlation analyses were conducted probing the variables of input composition and empirically-derived results, and analyzed separately for each of the two methods of manufacturing, i.e., the standard method and the alternative method. In other words, the standard method materials were compared among themselves (8 experiments) as a group, and the alternative method materials were compared among themselves (10 experiments) as another group. As expected, the dense chitosan membranes of the standard method of manufacturing (with glycerol) displayed a strong positive correlation ($R^2 = 0.74$) for thickness v. tensile strength expressed as maximum load. The alternative method of manufacturing (hydration/lyophilization) also yielded a positive, albeit moderate, correlation ($R^2 = 0.43$) for thickness v. tensile strength expressed as maximum load.

Note that an additional variable (other than thickness or the amount cast) was the % DD, and it might account for reducing the anticipated R^2 values. In fact, this was demonstrated in part using six samples produced with 0.25 g/cm^2 cast amount (and the alternative method) that enabled us to assess the effect of the degree of deacetylation (ranging from 70 – 95%) on the strength. The maximum loads for this set of six matched samples were negatively correlated with increasing % DD ($R^2 = 0.65$). In other words, higher deacetylation makes the polymeric material weaker.

The new chitosan materials were compared to a commercial surgical barrier membrane, the reconstituted bovine Type I collagen membrane, *BioMend Absorbable Collagen Membrane*® (Table 2). The "thin" dense chitosan membranes of the standard method cast using 0.3 – 0.35 g/cm^2 of 4% acidic chitosan solution exhibit more than double the maximum load compared to this reference reconstituted collagen membrane (i.e., average of 4.74 N v. 2.24 N). In this comparison the thicknesses of the two types of materials were similar (i.e., average of 0.23 mm for chitosan v. 0.17 mm for the collagen).

3.1.4 Resistance to Suture Pull-Out—The resistance to suture pull-out results are shown in Table 2. Using an Instron 5565 machine, ~ 5.0 mm strips of the membrane were subjected to suture pull-out tests. In an array of experimental variables the membranes exhibited resistance to suture pull-out with maximum loads as high as 2.2 N. Note that all samples were hydrated for 5 minutes immediately prior to testing on the Instron machine. This step was performed to mirror how the materials are intended to be used in a clinical setting by a physician or dentist.

Using either our standard method or the alternative method with varying cast amounts (0.25 – 0.5 g/cm^2) the maximum load for resistance to suture pull-out exhibited similar ranges (both group averages were 1.4 N). In aggregate the two groups ranged from 0.92 – 2.2 N, and demonstrated by linear regression analysis a positive, albeit moderate, correlation between thickness and max load ($R^2 = 0.396$).

Suture pull-out test results show the dense chitosan membranes exhibit nearly double the suture pull-out strength of a commercial reconstituted collagen membrane, *BioMend Absorbable Collagen Membrane*® (0.75 N).

3.2 *In Vitro* Properties

3.2.1 Permeability—The chitosan membranes were subjected to Franz cell permeability testing with small molecule dye compounds [21]. The dense chitosan membranes permitted the passive diffusion of small molecules, such as Methylene Blue and Crystal Violet dyes, with molecular weights of 285 and 373, respectively. Duplicate membrane samples were tested at 30C, and the pairs of data points were averaged. Two separate experiments are demonstrated in Table 3.

The initial linear rates of diffusion across the membranes (at 1 – 4 hours) were calculated as a function of the amount of starting dye material v. time in hours. The linear regression functions were robust in all cases with R^2 values ranging from 0.93 – 1.0. As expected, in the first experiment using 80% DD chitosan (Heppe) the initial rate of flux of Crystal Violet was higher for the 0.3 g/cm^2 amount cast than for the 0.5 g/cm^2 amount cast. Similarly, the rate of flux for Methylene Blue was higher for 0.3 g/cm^2 amount cast than for the 0.5 g/cm^2 amount cast. As anticipated the rate of diffusion is inversely proportional to the thickness (expressed as a function of the amount cast of acidic chitosan solution). When comparing the two dyes Methylene Blue transferred more rapidly than Crystal Violet.

In another experiment the permeability with regard to Methylene Blue was compared between 75% and 80% DD chitosans (Heppe) and relative to a reference bovine collagen membrane - *BioMend Absorbable Collagen Membrane*®. The flux rate was ~ 2.5 × greater for the collagen membrane relative to the dense chitosan materials. In addition to having different chemical compositions between chitosan(s) and collagen with inherent physical/chemical properties (e.g., potential affinity for the dyes), the chitosan membranes were somewhat thicker (especially so for the 0.5 g/cm^2 amount cast in the first experiment, above) than the collagen material.

Similar attempts to demonstrate passive diffusion with two other dyes, Alcian Blue (Mw 1299) and Janus Green B (Mw 476), were not successful with the dense chitosan membranes (data not shown).

In summary, the dense chitosan membranes were permeable in an aqueous environment to two dissimilar low molecular weight compounds. In addition, permeability was demonstrated for different amounts of material cast (related to thickness) and for different percentages of deacetylation (i.e., 80 v. 75% DD). This indicated that at least some small

molecules could pass through the dense chitosan membranes. In other words, the chitosan membranes were semi-permeable.

3.2.2 Enzymatic Biodegradation—It is desirable for resorbable membranes to provide a mechanical barrier for weeks-to-months in duration during wound healing *in vivo*, yet be resorbable by enzymatic and/or non-enzymatic hydrolysis. Chitosan is subject to enzymatic biodegradation within mammals by lysozyme and other catalytic enzymes. *In vitro* degradation tests using lysozyme alone showed the dense chitosan membranes were susceptible to degradation, just as reconstituted collagen membranes are known to be susceptible to proteases. Compositions with varying % DD were tested, with the expectation that high deacetylation would have little or no enzymatic degradation. *In vitro* results indicated that 75% DD membranes (Figure 3) and 70% DD membranes (data not shown) commenced lysozymecatalyzed biodegradation relatively quickly within ~ 4 – 7 days. However, 80% DD membranes (Figure 3) exhibited minimal degradation *in vitro* in lysozyme solution, as did 85, 90, and 95% DD membranes (not shown).

3.2.3 Non-enzymatic Dissolution—At neutral pH the dense chitosan membranes remain a solid in aqueous solutions. However, discs of the membranes dissolved in weak acidic environments, similar to the purified chitosan starting material during manufacturing (i.e., in acetic acid solution). A range of pH levels was tested below neutrality. Dissolution of 75% DD membranes (Heppe, 0.3 g/cm^2 cast amount, standard method) occurred within 6 hours either at pH 2.5 in 1% lactic acid or at pH 3.0 in sodium phosphate buffer, but not at or above pH 3.5. Dissolution of 80% DD membranes (Heppe, 0.3 g/cm^2 cast amount, standard method) occurred within 6 hours at pH 2.5 in 1% lactic acid, but not at pH 3.0 or higher in sodium phosphate buffer. This indicated that the membrane's carbohydrate polymer was not irreversibly covalently cross-linked during manufacturing. Furthermore, one may feasibly speculate that if the dense chitosan materials were to be ingested, the harsh acidic pH of the stomach would likely dissolve (depolymerize) the material; it would no longer remain in a solid form within the stomach.

3.2.4 Cell Culture—Chitosan films have been shown to be biocompatible with human neonatal primary keratinocytes and fibroblasts grown *in vitro*, and regardless of the degree of deacetylation ranging from 53 – 97.5% DD [47]. Therefore, we anticipated adherence and biocompatibility with our dense chitosan membranes using keratinocytes and fibroblasts. Also included was a third cell type that is relevant to bone. Human primary keratinocytes and a preosteoblastic cell line adhered to either side of the dense chitosan membranes and remained viable for the duration of the experiment (Figure 4). Similar results were seen with primary human fibroblasts, each cell line proliferating and remaining viable for up to 14 days (not shown). The cellular adhesion and viability were demonstrated on both planar surfaces of membranes produced by the standard method (Figure 4) or the alternative method (data not shown). These results confirmed the *in vitro* biocompatibility and cellular adhesion for three distinct types of human cells, namely keratinocytes of the epidermis (ectoderm), fibroblasts of the dermis (mesenchyme), and osteoblasts (bone).

3.3 *In Vivo* Properties

3.3.1 Biocompatibility, Resorption, and Wound Healing—An *in vivo* study in a rodent animal model showed that implanted dense chitosan membranes (Primex 79–95% DD by the alternative method) are biocompatible, do not impede healing of the surgically-induced defects, and do not produce adverse tissue reactions. Bilateral full-thickness 1.5 mm punches were made on the palate of male Lewis rats over the maxillary ridge and a single dense chitosan membrane was placed at the base of the wound. Responses to the dense chitosan membranes were determined by clinical observation during the initial 3-day healing

period, then by histological analysis of groups of animals at various time points (1, 3, 6, and 12 weeks).

Clinical re-epithelialization was estimated each day by digital visual inspection as [1-(exposed wound area/original wound area) × 100%]. Wounds with the dense chitosan membrane implanted at their base achieved 80% ± 20% wound closure in the first 3 days of healing. Histologically after 1 week, new epithelium atop the implanted chitosan membrane demonstrated initial rete peg formation (not shown). These data demonstrated the *in vivo* biocompatibility of the dense chitosan membranes.

With regard to resorption, most of the implants (5 of 7) were substantially degraded (i.e, at least half of the material missing) between 3 and 12 weeks, an example of which is shown histologically in Figure 5 (i.e., at 6 weeks). None of the implants (0 of 2) were substantially degraded at 1 week post-implantation (Figure 5); they remained intact as a barrier membrane. The number of implants that were substantially degraded at the subsequent sampling times were 2 of 2 at 3 weeks, 1 of 2 at 6 weeks, and 2 of 3 at 12 weeks. These results indicated that the dense chitosan membranes were in fact serving as temporary barrier membranes separating tissue layers for at least one week, and were substantially resorbed *in vivo* within 3 to 12 weeks.

4. Discussion

We were aware of several physical and clinical handling “weaknesses” of resorbable collagen membranes. Therefore, we desired to develop a biomaterial that demonstrated superiority to them. Our attention was drawn to purified chitosan as a source material. In the prior art, chitosan biomaterials were produced by essentially two types of methods: (a) lyophilization of an acidic chitosan solution, producing a low density “sponge” [48, 49]; and (b) heated drying of a chitosan solution without coincident compression, that produces an “asymmetric air dried” high density material [50, 51]. The former lacks sufficient density and strength for use as a barrier membrane. Although dense, the latter asymmetric air dried material lacks pliability, cut-ability, uniformity, and adequate suture-ability for use as a barrier membrane. Therefore, it would require a new method of manufacturing to produce a dense chitosan barrier membrane with uniformity and having high clinical utility.

Through a process of reiterative progressive modifications we have developed an innovative manufacturing process to produce high density chitosan membranes that overcome the weaknesses of both commercial collagen membranes and chitosan materials of the prior art. The major distinction of this new method was the use of coincident compression-dehydration of a neutralized polymer of chitosan. To accomplish this we used a heated vacuum “gel dryer” to generate uniform pressure and desiccation of the polymeric chitosan inside of a semi-permeable membrane. Additional refinements to the general method included two approaches to obtaining a dry pliable material that could be easily cut without breaking. The “standard method” employed the inclusion of glycerol prior to compression-dehydration. The “alternative method” lacked the glycerol step, but included an additional final step of hydration-lyophilization of the dry membrane.

In general, the maximum tensile stress was nearly double in glycerol-containing samples (standard method) relative to those lacking it and which had undergone the additional step of hydration/lyophilization. We can speculate as to the reason(s). Glycerol might enhance binding of polymeric units (e.g., ionic, hydrogen, or Vanderwaal interactions) and/or the alternative method's post-manufacturing steps might disrupt binding of polymeric units, perhaps during the lyophilization step. Some portion of this ~ 2× differential can be attributed to an increased average thickness in the hydration/lyophilization samples (i.e.,

from 0.27 mm to 0.38 mm average; Table 2). This differential is also consistent with regard to the averages for the maximum loads (i.e., 6.2 N with glycerol v. 3.7 N with hydration/ lyophilization and lacking glycerol; Table 2). Regardless, a persuasive case can be made for inclusion of glycerol prior to compression-dehydration. There are multiple benefits of including glycerol during manufacturing, such as ease of cutting, pliability, higher density, lower thickness, and tensile strength (both max load and max stress).

The dense chitosan membranes produced by the new coincident compression-dehydration method have sufficient to superior (and in some instances unique) physical, *in vitro*, and *in vivo* properties for use in clinical indications. With regard to physical properties these new dense chitosan membranes, even when relatively thin (~ 0.2 – 0.5 mm), have sufficient flexibility, tensile strength, elasticity, and resistance to suture pull-out for surgical placement by a clinician. In addition, the tensile strength and suture-ability are correlated with the material's thickness. The material is semi-permeable to small molecules, biodegradable using lysozyme, and biocompatible with human cells *in vitro*. Furthermore, the chitosan membranes are biocompatible with tissues and resorbable *in vivo*. We anticipate that the rate of resorption can be “controlled” based upon the percentage of deacetylation and/or thickness of the membranes, and without requiring a chemical cross-linking agent. Furthermore, the source material, chitosan, displays inherent antibacterial and antifungal activities. Therefore, our chitosan-based membranes have this feature as a coincident benefit. This aspect is unique relative to all other resorbable and non-resorbable commercial surgical membranes.

Our chitosan-based material is fundamentally distinct from the prior art in density coupled to excellent clinical utility as a surgical barrier membrane. These membranes are entirely unlike the low density chitosan sponge materials used commercially as hemostatic wound dressings. It should be noted that chitosan materials have been produced by other processes that can result in low-to-high density chitosan membranes or films. The two most common alternatives are compression of a lyophilized acidic chitosan sponge or air-dried asymmetric chitosan membranes. The former does not produce the superlative densities of the coincident compression-dehydration method disclosed here. In fact, a commercial lyophilized material is only about one tenth of the density of our membranes, is too thick, and transforms rapidly into a gelatinous form when wet. Thus, this wound dressing lacks the appropriate physical properties needed for an implantable suture-able membrane. The air-dried asymmetric chitosan membranes can be engineered to have sufficient density. However, they have inferior properties, such as the lack of pliability, cut-ability, uniformity, and suture-ability. In essence the air-dried membranes are brittle and not at all suitable for clinical use as a barrier membrane.

The semi-permeable nature of the chitosan membranes was demonstrated using Methylene Blue and Crystal Violet. Similar attempts with two other dyes of higher molecular weights, Alcian Blue and Janus Green B, were not successful (data not shown). Lack of permeability might be due to multiple factors, such as increased molecular weights (1299 and 476, respectively), and/or due to affinity for chitosan *per se* in view of its chemical properties. This might be due to ionic, hydrophobic, and/or covalent interactions between the dyes and the chitosan membrane. Regarding ionic interaction, the amino groups of glucosamine (i.e., the deacetylated moieties) can be protonated and thus have a positive charge. Regarding hydrophobic interaction, both dyes and the acetylated moieties of chitosan have hydrophobic domains for association while in an aqueous environment. Therefore, one can speculate on several feasible explanations for lack of diffusion by these latter two dyes through the membrane into the aqueous solution of the collection chamber. Regardless, the positive results with Methylene Blue and Crystal violet demonstrate the material is semi-permeable.

The *in vitro* results indicated that lysozyme as a single enzyme could contribute to the hydrolysis of high density chitosan membranes, albeit dependent on the % DD. This result also suggested that one might design different membranes with "controlled" rates of degradation. A pair of membranes manufactured identically with the sole exception of a low versus high % DD would be expected to have differential rates of resorption *in vivo*. For instance, one can speculate that a 70–80% DD dense chitosan membrane might resorb rapidly (e.g., in weeks) in a specific surgical implant environment, whereas a 90–95% DD version might resorb slowly (e.g., in months or longer) in the same environment. Note that *in vivo* lysozyme would be only one of several possible degradative pathways toward resorption, and alternative pathways might synergize (e.g., lysozyme plus NAGase). The rate of resorption could be selected for the type of surgical indication, based upon the desired thickness and longevity of the implant. Furthermore, although we have not addressed it in this work, one could possibly use a chemical cross-linking agent during or following manufacturing to reduce the rate of resorption of dense chitosan membranes.

We anticipate that the dense chitosan membranes produced by this new method will have excellent utility as implantable surgical membranes in oral, orthopedic, general, plastic, and dermatologic surgeries, among other uses. Examples of oral clinical indications that could benefit from this new biodegradable material are periodontal ridge augmentation, as well as a barrier for bone graft substitutes in the mandible or maxilla, and in site preparation prior to metallic dental implants. We have already demonstrated that the chitosan material is biocompatible and resorbable within weeks to months in a rat oral palate model. In orthopedics, one can envision membrane barriers between bone and soft tissues, for instance as complements to long bone fractures (especially open fractures) being repaired with hardware and/or bone graft substitutes. In these cases the dense chitosan membranes could be sutured, tacked, and/or stapled in place to prevent in-growth by soft tissue (e.g., fibroblasts). In general surgery, the membranes could be sutured *in situ* to provide strength to repair hernias, or to prevent post-surgical tissue adhesion of visceral organs. In plastic surgery, the dense chitosan membranes could be used to augment soft tissue defects (e.g., facial reconstruction). In dermatologic surgery the membranes could be used as either intact sheets or in mesh form with holes to promote cutaneous healing following heat or chemical burns, diabetic ulcers, or traumatic abrasions. In this dermatologic context the material could serve as either a wound dressing or a tissue engineering film to facilitate the autologous grafting of normal skin-derived cells to debrided skin. In conclusion, the surgical indications of use for this new non-toxic resorbable biomaterial are myriad.

At present there are multiple types of resorbable membranes that are commercially available as US FDA 510(k) cleared Class II devices. The most common are reconstituted bovine and porcine collagens, although other biomaterials are available commercially (e.g., synthetic polymers of lactate and/or glycolate, and processed xenograft tissues). Our results indicate in head-to-head comparisons that the dense chitosan membranes have properties meeting and often exceeding those of commercial collagen membranes. Reconstituted collagen membranes represent the current *standard-of-care* in many, if not most, surgical indications. Compared to the new dense chitosan membranes, the *BioMend Absorbable Collagen Membrane®* exhibited lower tensile strength and resistance to suture pull-out. In addition, the clinical handling characteristics of the dense chitosan membranes are superior to that collagen membrane with regard to "memory" while wet and the ability to discriminate between the material and surrounding tissues. This is due to collagen rapidly absorbing blood and becoming red in color. The discrimination between host tissue and the membrane helps to guide placement of the material and sutures.

Our polysaccharide materials compare favorably to commercial collagen membranes, and do not have two additional down-side "weaknesses" of collagen membranes. First, some

collagen products require a chemical cross-linker to provide sufficient strength or resistance to suture pull-out, although it is formally possible that one could use a cross-linker to enhance our chitosan technology to an even higher level of strength and suture-ability. Second, there is a perceived concern, especially within the UK and Europe, about the possibility of inadvertently causing bovine spongiform encephalopathy (BSE) or "mad cow disease" using bovine collagen materials, which would not be the case for our chitosan-based membranes.

Numerous other types of resorbable membranes have been reported, mostly at the pre-commercial research stage (e.g., electrospun/nanofibrous, laminated, wound dressing, and/or drug-eluting membranes) [52-55]. In general, although these other types of composite materials can exhibit some physical and biological benefits, they typically lack sufficient resistance to suture pull-out and strength, unlike the properties exhibited by our dense chitosan membranes (i.e., ~ 1.4 N for suture pull-out and ~ 86 MPa in maximum stress) [53].

Beyond their potential use in US FDA Class II medical device indications, the dense chitosan films and membranes could also be used in at least three other broad areas, namely as wound dressings, delivery devices, and tissue engineering matrices: (1) FDA Class I wound dressings are used in external and some oral applications. Chitosan "sponge" at low density is already used for wound dressings, with a mechanistic emphasis on controlling hemorrhage; (2) Dense chitosan materials could be used as delivery devices for bioactive molecules. For instance the chitosan membranes (or post-manufacturing processed granular materials thereof) are capable of ionic attraction due to protonated amino groups of glucosamine residues. It is feasible that the dense chitosan materials could "carry" nucleic acids, protein and peptide biologics, pharmaceuticals, vaccines, among other bioactive molecules to promote a physiologic response *in vivo* either in or on an animal or human. These controlled delivery devices as combination products could provide beneficial wound healing, anti-inflammatory, angiogenic, antibiotic, and other desirable properties; and (3) Chitosan films could be used as *in vitro* scaffolds for tissue engineering purposes. We have already demonstrated that the materials facilitate the adherence and viability of human keratinocytes, fibroblasts, and osteoblasts. The material could serve in personalized medicine as an *ex vivo* bioengineering surface onto which autologous stem cells or adult cells could be adhered prior to placement on or within the host tissues(s). Variations on this theme could be used to develop artificial skin or other epithelia (e.g., oral, vaginal, gastrointestinal) prior to surgical attachment at a site of need. The materials should have sufficient strength, suture-ability, and durability to permit the material to "take" to the host environment. This type of autologous grafting with a patient's own cells already adherent to dense chitosan could provide a more rapid means to wound healing than could be accomplished in the absence of these therapeutic devices (e.g., in a cutaneous burn or diabetic ulcer environment).

5. Conclusions

The broad array of physical, *in vitro*, and *in vivo* testing results indicates that the chitosan membranes prepared by this method are unlike those produced by any prior art manufacturing methodology using chitosan as the source material. The dense chitosan membranes have superior, and in many cases unique, physical and clinical handling characteristics to those of a commercial lyophilized acidic chitosan sponge, air-dried asymmetric chitosan membranes, and a commercial reconstituted collagen membrane. The dense chitosan membranes should have excellent clinical utility as implantable, resorbable, suture-able surgical membranes that one anticipates to have inherent anti-infective

properties. The suture-able membranes should provide benefits in wound healing and surgical repair, and can also be designed as coincident carriers of active ingredients.

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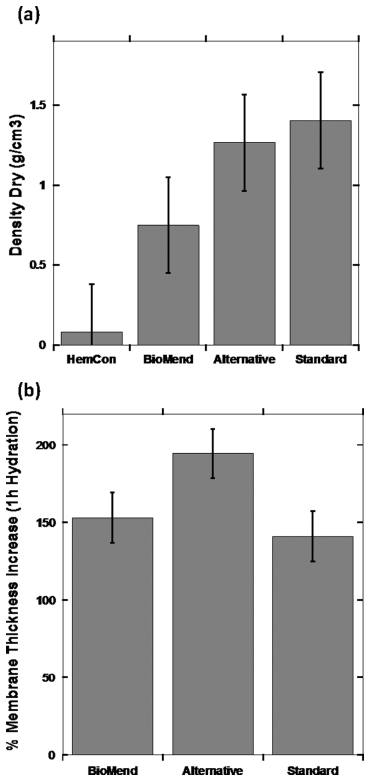

Figure 1.

Densities and increases in membrane thicknesse upon hydration: (a) The dry (pre-hydration) densities of four materials (i.e., *HemCon Dental Dressing*®, *BioMend AbsorbableCollagen Membrane*®, dense chitosan membranes produced by the alternative and standard methods) measured directly as a function of mass and volume; (b) The increases in material thickness after hydrating the membranes for 1h. Note that it is not possible to measure the re-hydrated *HemCon Dental Dressing*®, as it is gelatinous and amorphous lacking sufficient strength. The error bars represent the Standard Error of the Mean.

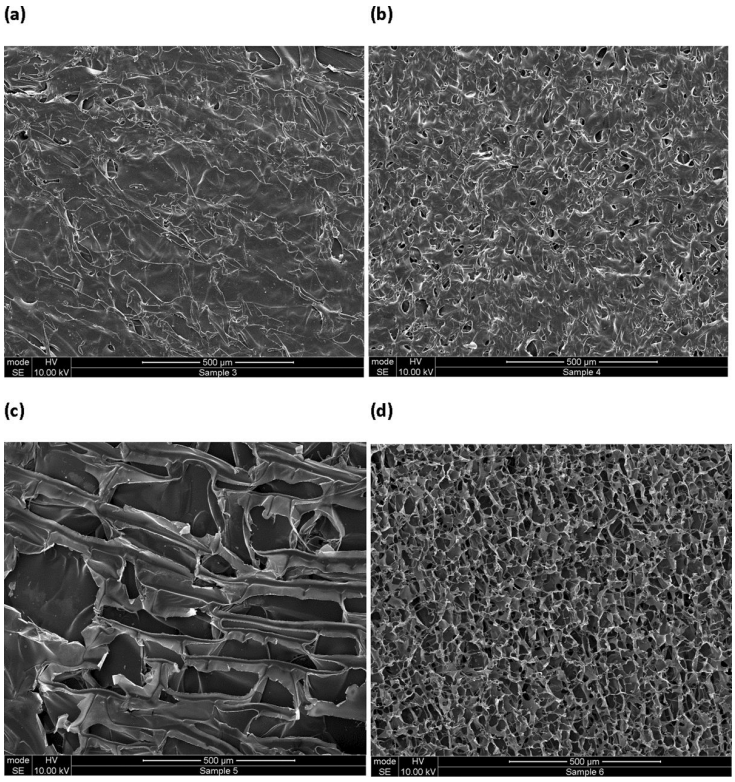

Figure 2.
Scanning Electron Microscopy at 250 × magnification: (a) A planar surface of a dense chitosan membrane (Heppe 80% DD, 0.3 g/cm^2 cast amount, standard method); (b) Same material as “a”, but the opposite planar surface; (c) *HemCon Dental Dressing*®, surface with very large pores; (d) Same material as “c”, but the opposite surface with smaller pores. Scale bars = 500 microns.

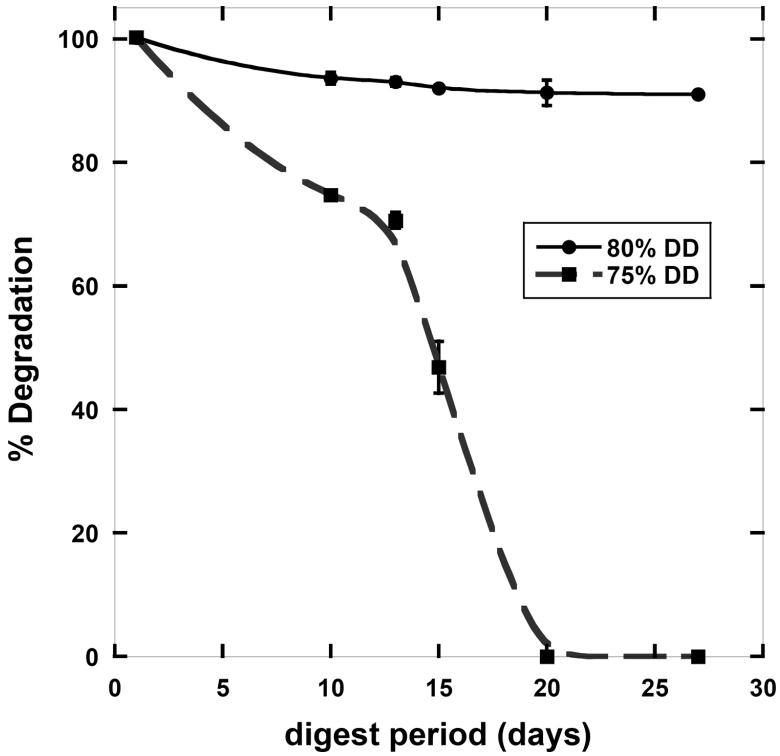

Figure 3.

Lysozyme-mediated degradation of dense chitosan membranes. Dense chitosan membranes (Heppe 75 & 80% DD, 0.3 g/cm^2 cast amount, the standard method) were incubated in lysozyme solution at 37C with daily (M–F) changes of buffer and enzyme. The membranes were imaged at various times over a period of 3 weeks and densitometry of the membrane translucence was used to calculate % loss of membrane (75% DD dashed line & squares; 80% DD solid line & circles).

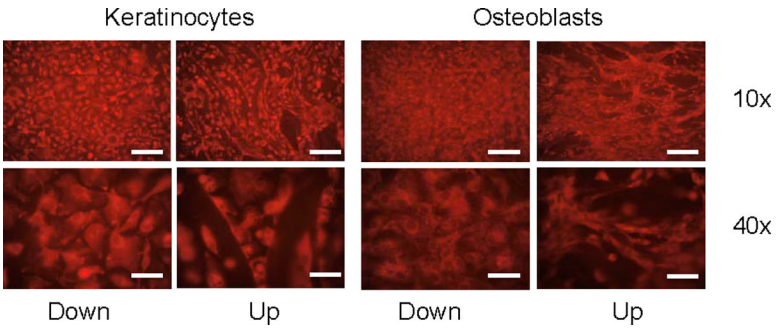

Figure 4.

Dense chitosan membranes (Heppe 80% DD, 0.3 g/cm^2 cast amount, the standard method) were seeded directly with primary human keratinocytes or pre-osteoblasts and cultured in serum-containing medium for 3 days at 37C. Live cells on the surface of the chitosan membranes were stained with Cell Tracker Red and imaged by fluorescence microscopy. Duplicate representatives of each condition are provided. Scale bars: at 10 × magnification = 200 microns and at 40 × magnification = 50 microns.

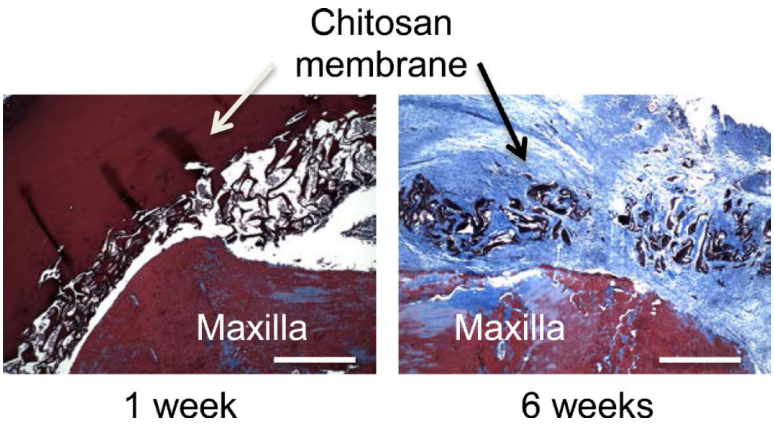

Figure 5.
Masson's trichrome stain of rat maxilla after 1 week (left) or 6 weeks (right) of healing with implanted dense chitosan membranes (Primex 79–95% DD, 0.5 g/cm^2 cast amount, the alternative method). Membranes were placed within full-thickness ulcerative wounds adjacent to the maxilla and allowed to heal. Note that during resorption the degraded membrane is replaced by a collagenous interstitial matrix (stained blue). Scale bars = 500 microns.

Table 1

Chitosan source materials

Vendor - Brand	Label	% DD	Viscosity
Heppe - *Chitoceuticals*	70/500	72.1%	416 mPas
Heppe - *Chitoceuticals*	75/500	73.8%	458 mPas
Heppe - *Chitoceuticals*	80/500	80.1%	621 mPas
Heppe - *Chitoceuticals*	85/500	86.5%	473 mPas
Heppe - *Chitoceuticals*	90/500	90.8%	418 mPas
Heppe - *Chitoceuticals*	95/500	95.2%	449 mPas
Primex - *ChitoClear*	cg400	75%	453 mPas
Primex - *ChitoClear*	cg400	79–95% *	463 mPas

* viscosities determined by alternative methods

Table 2

Tensile strength & resistance to suture pull-out

Material (Exp.#)			**Tensile Strength**			**Suture Pull-Out**	
	DD %	**Amount Cast 4% Chitosan**	**Thickness**	**Max Load**	**Max Stress**	**Thickness**	**Max Load**
		(g/cm^2)	**(mm)**	**(N)**	**(MPa)**	**(mm)**	**(N)**
Standard Method:							
10%Glycerol (43)	75	0.30	0.24	4.28	65.1	0.22	1.08
50% Glycerol (33)	75	0.50	0.40	8.91	98.0	0.47	2.20
10%Glycerol (41)	80	0.30	0.20	3.23	64.3	0.20	1.21
10% Glycerol (45)	80	0.30	0.23	5.90	90.5	0.26	1.31
10% Glycerol (44)	80	0.35	0.20	5.62	102.8	0.25	1.39
10% Glycerol (46)	80	0.35	0.26	4.67	61.7	0.26	1.48
10% Glycerol (34)	80	0.50	0.36	10.92	118.3	0.37	0.94
10% Glycerol (42)	80	0.50	0.27	6.18	86.2	0.27	1.67
Group Average			*0.27*	*6.21*	*85.84*	*0.29*	*1.41*
Alternative Method:							
Hydration/Lyo (20)	70	0.25	0.25	3.86	68.1	0.26	0.92
Hydration/Lyo (21)	75	0.25	0.27	4.18	68.0	0.31	1.38
Hydration/Lyo (22)	80	0.25	0.33	2.74	33.1	0.28	0.75
Hydration/Lyo (23)	85	0.25	0.35	3.24	38.9	0.35	0.73
Hydration/Lyo (24)	90	0.25	0.30	2.38	35.5	0.27	1.60
Hydration/Lyo (25)	95	0.25	0.23	2.68	49.7	0.28	1.19
Hydration/Lyo (29)	75	0.40	0.52	4.15	36.9	0.58	2.06
Hydration/Lyo (30)	75	0.50	0.72	4.91	33.1	0.74	1.99
Hydration/Lyo (31)	80	0.40	0.47	5.37	49.7	0.49	1.40
Hydration/Lyo (32)	80	0.50	0.41	3.72	38.8	0.52	1.91
Group Average			*0.38*	*3.72*	*45.17*	*0.41*	*1.39*
Collagen Membrane:							
BioMend Absorbable			0.17	2.24	45.3	0.18	0.75

Table 3

Diffusion rates using Franz cells

Membrane Samples	Dye	Rate (%/hr)	R^2
Experiment 1			
0.3 g/cm^2 – 80 DD	CV	0.63	0.974
0.5 g/cm^2 – 80 DD	CV	0.37	0.931
0.3 g/cm^2 – 80 DD	MB	0.92	0.998
0.5 g/cm^2 – 80 DD	MB	0.52	0.972
Experiment 2			
0.3 g/cm^2 - 75 DD	MB	1.12	1.000
0.3 g/cm^2 - 80 DD	MB	1.02	0.999
BioMend Absorbable	MB	2.53	1.000

Rates are expressed as the percentage of the starting dye material in the upper chamber that diffused into the lower receptor chamber per hour during the time interval of 1 to 4 hours. CV = Crystal Violet; MB = Methylene Blue.