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An In Vitro Evaluation Of Chitosan As A Biomaterial Focusing On The Effects Of The Degree Of Deacetylation

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AN *IN VITRO* EVALUATION OF CHITOSAN AS A BIOMATERIAL FOCUSING ON THE EFFECTS OF THE DEGREE OF DEACETYLATION

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The material characteristics play a role in the suitability of chitosan for biomedical applications. This is not surprising since the degree of deacetylation of chitosan influences antimicrobial activity, degradation rate, immune reaction and mechanical properties such as strength and elongation.

This study examines chitosans of variable material characteristics for wound and bone healing applications. Chitosan films of 76, 78, 80, 87, 91, 92, and 95% degree of deacetylation were tested *in vitro* for cellular responses by fibroblast and bone cell lines. The *in vitro* responses were compared to the material characteristics of molecular weight, degree of deacetylation, swelling index, and ash content.

DEDICATION

I would like to dedicate this research to my family and friends who have listened to hours of talk about this research and even edited part of the thesis: my family, John and Maxine Hamilton and Jesse Hamilton and my friends, Frank Zhan, Lori Norton, Grace Gao, Behnam Daghigh, Kerry Eley, June Giles, and Benson Chow.

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INTRODUCTION

Abstract

Chitosan has been widely investigated as a biomaterial. It has been shown to enhance wound healing rates (Biagini, *et al.*, 1991; Braye, *et al.*, 2001; Prudden, *et al.*, 1970; Ueno, *et al.*, 1999) and wound strength (Cho, *et al.*, 1999). Increased wound healing has been attributed in part to its similarity to extracellular matrix molecules like hyaluronic acid (Muzzarelli, *et al.*, 1999). Chitosan is also structurally similar to heparin (Biagini and Muzzarelli, 1992). Degradation products are simple oligosaccharides which may be processed by normal metabolic pathways down to CO_2 and water (Onishi and Machida, 1999). Furthermore, chitosan has demonstrated bacteriostatic and fungistatic properties which guard against infection (Mi, *et al.*, 2001; Seo, *et al.*, 1992; Tomihata, *et al.*, 1997). Its high cationic nature is reported to promote cell adhesion (Muzzarelli *et al.*, 1994; Muzzarelli *et al.*, 1988).

For these reasons, chitosan is a promising material for biomaterial applications. Yet, the widespread adaptation of chitosan as a biomaterial has not occurred, since scientific studies have reported conflicting and/or inconsistent results. For example, chitosan has been cited as inhibiting fibrosis (Bartone, *et al.*, 1988), while others have

noted fibrous tissue formation with the use of chitosan materials (Hidaka, *et al.*, 1999 and Lu, *et al.*, 1999). The differences in the biological responses observed could be attributed to differences in the degree of deacetylation, molecular weight, source, and preparation of the samples used in the different studies (Chatelet, *et al.*, 2001; Chung, *et al.*, 1994; Nunthanid, *et al.*, 2001). The aim of this study is to evaluate the effects of degree of deacetylation on surface and material properties of different chitosans. The surface and material characteristics will be compared to the *in vitro* cell response.

The hypotheses for this study is that macrophage, osteoblast and fibroblast attachment, growth and release of extracellular molecules will be increased on 95% deacetylated chitosan materials as compared to lower percent deacetylated chitosans. The surface energy will be measured by contact angle and may be used to relate adhesion of the cells to a biomaterial. The crystallinity will be examined through differential scanning calorimetry DSC (Billmeyer, 1984). Cell attachment and growth will be determined via spectrophotometric assays. The release of nitric oxide (NO) by macrophage cells, an indicator of macrophage activation and tissue inflammation, will be measured by the Griess method (Green, *et al.,* 1982).

The attachment, proliferation, and production of extracellular matrix components or release of pro-inflammatory compounds will be related to the surface and material characteristics of the chitosan films. The significance of these data will begin establishing a relationship between basic well characterized material properties and

known biological outcomes. This information may ultimately be used to identify particular chitosan materials and properties for specific biomaterial applications.

Background and Significance

History

Powdered cartilage from sharks and exoskeletons of crustaceans were employed to heal wounds in ancient Japan (Khor, 2001). This material was largely forgotten until the later half of the twentieth-century (Prudden, *et al.*, 1970), when a renewed interest in the medicinal use of natural materials began. The active biological component from powdered cartilage and exoskeleton is chitin. While the exact mechanism by which wound healing is promoted has not yet been determined, chitin and its derivatives have shown promise in multiple biomedical applications such as wound repair, tissue engineering, and drug delivery (Khor, 2001; Kumar, 2000 and Suh and Matthew, 2000).

The first reference to chitin in scientific literature was in 1881 when Braconnot isolated chitin from a fungus (Khor, 2001). In 1823, Odier isolated the same material from the exoskeletan of a beetle and named this substance chitin from the Greek word "chiton" meaning "coat of mail." Rouget boiled chitin in potassium hydroxide rendering a material that was soluble in organic acids in 1859. This substance was named chitosan by Hoppe-Seyler 35 years later. The structure of chitosan was not resolved until 1950 (Khor, 2001).

Structure and Manufacture

The structure of chitin and chitosan is shown in Figure 1.1. Either an acetamido group (-NH-$COCH_3$) or an amino group (-NH_2) is attached to the C-2 carbon of the glucopyran ring. When more than 50% of the C-2 attachment is an amino group, the material is termed chitosan. Ideally, chitin is a linear polysaccharide of β-(1-4)-2 acetamido-2-deoxy-D-glucopyranose where all residues are comprised entirely of the acetamido group -NH-COCH3. This is termed fully acetylated. Chitosan is a linear polymer of β-(1-4)-2 acetamido-2-deoxy-D-glucopyranose where all the residues are comprised entirely of the

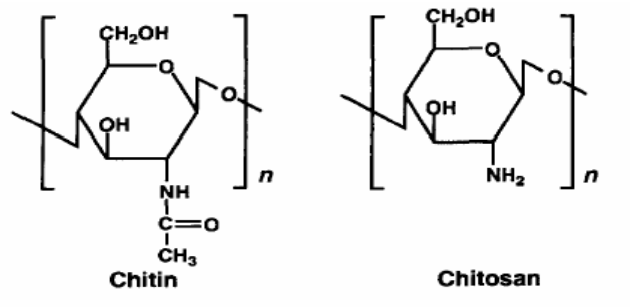

Figure 1.1. The chemical structure of Chitin and Chitosan.

amino group -NH_2. This is termed fully deacetylated. In reality, the range of deacetylation is commonly 70 to 99%.

Generally, chitin is refined from the shells of crustaceans. It is also found in the exoskeleton of marine zooplankton, the wings of butterflies and ladybugs, and cell walls of yeast, mushrooms, and other fungi. Chitin and its derivatives are chemically isolated

from natural sources by a few general steps (Figure 1.2). After crushing and washing, shells are demineralized by calcium carbonate. The extent of the removal of minerals is

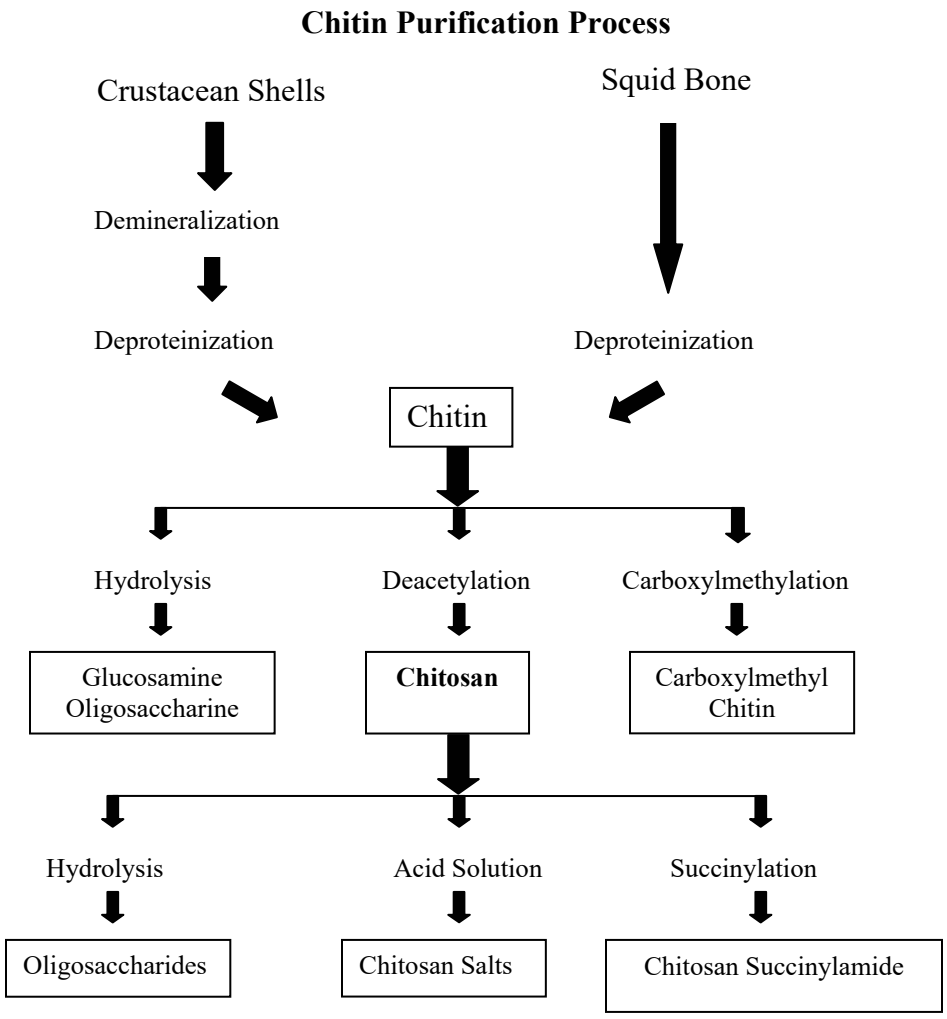

Figure 1.2. Manufacturing Methods of Chitin and its derivatives.
Modified from France-Chitine. Boxes indicate products.

expressed in the ash content. High ash content would indicate more impurities than low ash content. The next step is the removal of covalent bonds between the chitin and protein complex through deproteinazation. NaOH is generally used to deproteinize chitin down to a protein content of 1 %. To achieve chitosan, chitin is deacetylated with additional NaOH. Further treatments, such as hydrolysis or the application of an acid solution, can be used to produce varying forms of chitosans.

Applications

Chitin and its derivatives have many useful applications from health care to waste and water treatments (Table 1). It has shown promise as a drug delivery system because it cleaves at desired pHs (Kast, *et al.*, 2002; Risbud, *et al.*, 2000). Chitosan shows particular promise for the delivery of cancer treatments since a disproportionate amount of chitosan is taken into the growing cancer mass (Nsereko and Amiji, 2002; Chen, *et al.*, 2002). Its charge allows for delivery of DNA (Guang, *et al.*, 2002; Romoren, *et al.*, 2002) and of growth factors (Lee, *et al.*, 2002; Park, *et al.*, 2000). Chitosan's support of cell growth has produced positive results in tissue engineering applications (Cast, *et al.*, 2001; Risbud, *et al.*, 2001; Risbud, *et al.*, 2002; Nettles, 2001; Elder, 2004).

Table 1.1 Applications of Chitin and its derivatives.
(Khor, 2001; Majeti and Kumar, 2000 and Riccardo, *et al.*, 1994)

Application Areas	Specific Use
Health Care	Burn and Wound dressing Tissue Engineering Drug and gene delivery
Food and Beverages	Preservative agent Food additive and natural thickener Food processing (e.g. sugar)
Agriculture	Seed coating Fertilizer Antimicrobial agent
Waste and Water Treatment	Removal of metal ions Flocculating agent for polluted water Treating food waste
Cosmetic and Diet-aids	Oral health care Dietary aid (fat binding properties) Cosmetic component
Product Separation	Membrane separation Chromatographic columns Encapsulating adsorbents

Scientific Studies

Theories to Chitosan's Mechanisms

Scientific claims of accelerated and improved wound healing with the application of chitosan have been made since the 1960s. Yet, the mechanism by which this occurs has not been elucidated. Popular theories include: breakdown products providing building blocks for new tissue, an increase in growth factor and cytokine production

and/or stability, an increase in cellular migration and proliferation at the injury site, and a stimulation of the resident cells to form vessels and tissue. None of the proposed mechanisms are mutually exclusive. An examination of the *in vivo* studies which originated or support these theories follow.

The first *in vivo* chitosan paper credited the breakdown product of N-acetyl-glucosamine in accelerating wound healing was by Prudden, *et al.* (1970), who reported increased wound healing in diabetic ulcers treated with chitosan. Mori, *et al.* (1997) showed that N-acetyl-glucosamine, a degradation product of chitosan, increased the release of the growth factor interleukin-8, IL-8, from cultured rat dermal fibroblast. IL-8 is responsible for increased neutrophil and monocyte chemotaxis during wound healing events. An increase in mitotic cells has been noted in surgically created 2 cm by 2 cm canine wounds treated with 82 % deacetylated chitosan when compared to the untreated control (Ueno, *et al.*, 1999) supporting the increased proliferation theory. Increased blood vessels have been noted in wounds treated with chitosan and credited for the decreased scarring (Biagini, Bertani, *et al.*, 1991; Biagini, Pugnaloni, *et al.*, 1991; Muzzarelli, *et al.*, 1988).

The theory of chitosan inducing cellular migration and then stimulating these cells to differentiate into mature functioning dermis was introduced by Muzzarelli *et al.* (1987) in one of many studies examining the biological effects of chitosan. Using chitosan of 86% deacetylation and average molecular weight of 191,000 Daltons (Da), chitosan films

and chitosan ascorbate gels were made and placed into defects in the dura mater of 2 mongrel cats. Healing was followed for 60 days. Increased vascularization and organization were noted for the chitosan films and gels. Evidence pointed to the migration of stromal cells and their differentiation into structurally needed cell types as the fundamental cause of decreased wound healing times and improved wound tissue organization (Muzzarelli, *et al.*, 1987). Because of the great successes of chitosan in the Prudden and Muzzarelli's studies additional work has been directed toward developing chitosan as a wound treatment.

As a wound dressing, chitosan has shown improved and scarless healing, higher numbers of mitotic cells in the wound bed, greater macrophage infiltration into the site, faster re-epithelialization of the wound, increased angiogensesis, superior wound strength, and greater collagen deposition (Biagini, Bertani, *et al.*, 1991; Biagini, Pugnaloni, *et al.*, 1991; Braye, *et al.*, 2000; Cho, *et al.*, 1999; Mi *et al.*., 2000; Ueno, *et al.*, 1999). Anecdotal evidence even suggests chitosan may provide analgesic effects for serious burns (Kifune, 1992). Chitosan performance as a wound dressing has resulted in a commercial brand of chitosan dressing in Asia.

Perhaps the most amazing claim associated with chitosan is improved or scarless healing. This claim has been made by multiple groups using both human and animal models. A water soluble form of chitosan, N-carboxybutyl-chitosan, was placed on donor sites of humans undergoing surgical procedures and was shown to aid in wound repair more than the phystostimuline gauze control. The absence of scar formation and

contraction was also seen when using N-carboxybutyl-chitosan in both animal and human models (Biagini, Bertani, *et al.*, 1991; Biagini, Pugnaloni, *et al.*, 1991). When chitosan-based skin grafts resulting from *ex vivo* expansion of human cells were placed in a porcine model, scarless healing occurred in all cases (Braye, *et al.*, 2001). Since full-thickness skin grafts usually result in scaring, the results support earlier claims that chitosan positively influences wound healing through inducing true angiogenesis, preempting the generally avascular scar which retracts during remodeling (Biagini, Bertani, *et al.*, 1991; Biagini, Pugnaloni, *et al.*; 1991; Muzzarelli, *et al.*, 1988).

During the wound healing process, increased cell proliferation, angiogenesis, and collagen deposition have been associated with chitosan. Increased cell proliferation was found in canine wounds treated with 82% deacetylated cotton-type chitosan. On day 6 the granulation tissue of the wounds treated with 82% deacetylated chitosan contained significantly more mitotic cells than the untreated control. The accelerated growth of new capillaries throughout the experiment was also attributed to chitosan. (Ueno, *et al.*, 1999). A sponge-like chitosan membrane of 87 % deacetylated with a molecular weight of approximately 70,000 was evaluated in 3 month old Wistar rats by Mi, *et al.* (2000) as a wound dressing. Re-epithelialization rate for the chitosan dressing was histologically confirmed to be greater than the untreated control. In addition, the deposition of collagen in the dermis was well organized and orientated along the same axis paralleled to the skin's surface, suggesting a "perfect repair" of the damaged tissue (Mi, *et al.*, 2000).

Furthermore, treatment with 84% deacetylated chitosan improved wound healing strength in rats on days 3, 7, and 10 when compared with an untreated control (Cho, *et al.*, 1999).

Not all *in vivo* wound studies have yielded extremely positive results. Only slightly improved wound healing was observed in Sprague-Dawley rats treated with 100% deacetylated chitosan when compared with the untreated control (Denuziere *et al.*, 1998). When chitosan of greater than 80 % deacetylation was studied in canine wounds over the course of 28 days no statistical difference was observed in re-epithelialization, development of granulation tissue, number of fibroblasts, or extent of revascularization when compared to untreated controls (Okamoto, *et al.*, 1995). Even fibrous scar tissue formation has been observed when using chitosan of 94 and 100 % deacetylation (Hidaka, *et al.*, 1999) and a commercial chitosan based gel (Alini, *et al.*, 2002).

While the role of chitosan in skin repair has not yet been fully determined, research into other biomedical applications has begun. Bone, cartilage, and nerve cells have shown an affinity for chitosan in both *in vivo* and *in vitro* studies. Hidaka, *et al.*, 1999 studied osteogenesis in rat calvaria treated with chitosan membranes of 65, 70, 80, 94, and 100 % deacetylation. One-week post implantation, samples of 65, 70, and 80% deacetylation showed an induction of neutrophil accumulation and slight inflammation. Granulation tissue was immature. Ninety-four and 100 % deacetylation samples showed mild inflammation surrounded by granulated tissue. All samples showed signs of osteogenesis. The most prominent signs of osteogensis occurred in samples of 60, 70, and 80 % deacetylation. By week 2, neutrophil infiltration had decreased in samples of

60, 70, and 80 % deacetylation. Collagen fiber formation was noted in the granulation tissue as it matured. For samples of 90 and 94 % deacetylation epithelioid-like cells were observed at the surface of the implant which was surrounded by fibrous connective tissue. Fibrous connective tissue and mild to moderate immune reactions were observed in samples of 60 and 70% deacetylation on weeks 4 and 8. At the same time, components remained from 80 % deacetylation implant. Macrophages containing cell debris were still present. For 90 and 94 % deacetylated membranes, the membranes were encapsulated and remained in tact. The osteogenesis seen in the first two weeks of the study was theorized to be from the migration of osteoblasts from the calvarial surface to the granulation tissue. This coincides with Klokkevold, *et al.* (1996) who noted the similarities in structure between chitosan and hyaluronic acid which promotes migration and proliferation of progenitor cells (Hidaka, *et al.*, 1999). With osteogenesis being strongest in the samples that degraded over the course of the study, an argument could also be made for the influence of the degradation products on the healing process. Degradation is a function of the degree of deacetylation. Taking out other factors such as structure such as block or random, the higher the DDA, the slower the absorption or degradation of the chitosan.

In an attempt to increase the osteogenic effects of chitosan, Muzzarelli, *et al.* (1994) modified chitosan to enhance the cationicity. The modified chitosan was greater than 99% deacetylated with an average molecular weight of 200,000. An 8 mm defect was created in the femurs of sheep. The defect of one leg was filled with the modified

chitosan. The other leg was left untreated. Forty days after surgery the untreated control exhibited only a fibrous pad around the hole, absent of osteogenic activity. The surgical hole was occluded for the modified chitosan-treated leg. New trabeculae were observed; but the center of the hole had fibrotic aspects to the tissue. Modified chitosan clearly induced bone formation beyond the normal formation (Muzzarelli, *et al.*, 1994).

Increased activity of bone cell lines was also noted with *in vitro* studies. Normal human osteoblast proliferation was increased when exposed *in vitro* to chitosan films of greater than 90 % deacetylation. By day two the standard tissue culture plastic control had 40 to 60 cells per field at 100x magnification while cells grown on chitosan films had 60 to 70 cells per field at 100x magnification (Lahiji, *et al.*, 2000). Differences in cell morphology were also noted. Approximately 90% of the control cells assumed a spindle, fusiform appearance while less than 5% of the chitosan cells did. It is theorized that shape may indicate function. The non-spindle shaped cells are thought to retained a more progenitor cell phenotype (Lahiji, *et al.*, 2000). UMR 106 osteoblast cells, a rat osteosarcoma line, exhibited greater growth and attachment on titanium coupons bonded with chitosan of 91.2% deacetylated and 200,000 molecular weight than the control titanium coupons (Bumgarnder, *et al.*, 2003).

Chitosan has also been shown to have positive effects on cartilage regeneration (Alini, *et al.* 2002; Lu, *et al.*, 1999; Nettles, *et al.*, 2002). Since cartilage is avascular, repair and regeneration are generally not seen (Buckwalter, 1983; Huber *et al.*, 2000).

Nerve tissue, another difficult to repair tissue, has also shown to be stimulated by chitosan (Gingras, *et al.*, 2003; Haipeng, *et al.*, 2000; Muzzarelli, *et al.*, 1999).

Chitosan has great potential in many biomedical applications. However, variability in tissue responses to chitosan, e.g. fibrosis or complete healing, (Bartone, *et al.*, 1988; Hidaka, *et al.*, 1999; Lu, *et al.*, 1999; Muzzarelli, *et al.*, 1988) has been a major problem in the development of chitosan as a biomaterial. Unfortunately, not all studies report material properties such as degree of deacetylation. In particular, Bartone, *et al.* (1988) and Lu, *et al.* (1999), failed to note the degree of deacetylation, molecular weight, source, and other important material characteristics of their chitosan. Bartone, *et al.* (1988) claimed an inhibition of fibrosis; Lu, *et al.* (1999) noted an increase of fibrosis. Without material characteristics information, it is difficult to develop chitosan materials with known and predictable biological outcomes. Chitosan material characteristics such as the degree of deacetylation have been shown to influence material properties such as crystallinity and degradation as well as biological responses.

Degree of deacetylation

The degree of deacetylation has been shown to influence many physical and biological properties of chitosan such as the molecular weight and elongation at break (Blair, *et al.*, 1987), tensile strength (Blair, *et al.*, 1987; Tomihata, *et al.*, 1997), biodegradation by lysozymes (Tomihata, *et al.*, 1997; Varum, *et al.*, 1997), proliferation of

fibroblast (Howling, *et al.*, 2001), osteogenesis (Hikdaka, *et al.*, 1999), and wound healing (Sathirakul, *et al.*, 1995). The degree of deacetylation also affects mechanical strength, stress and elongation curves (Figure 1.3) and swelling index (Figure 1.4) (Tomihata, *et al.*, 1997; Nunthaid, *et al.*, 2001). Chitin elongation increased as the degree of deacetylation increased to approximately 50 % deacetylation. Then, elongation slowly decreased between the deacetylation levels of 50 to 70 %. A sharp decrease of approximately 50% in elongation was seen between the deacetylation levels of 70 to 100 % (Tomihata, *et al..,* 1997). The strength curve mirrored the elongation curve. It initially decreased and then slowly climbed for the levels of deacetylation from 0 to 70 %. An increase in strength of 200 g/mm^2 occurs from 70 to 100 % deacetylation. Water content of chitin increased until approximately 50 % deacetylation (Figure 1.4). The peak then fell from approximately 80 % water content to slightly more than 50 % water content at 100 % deacetylation.

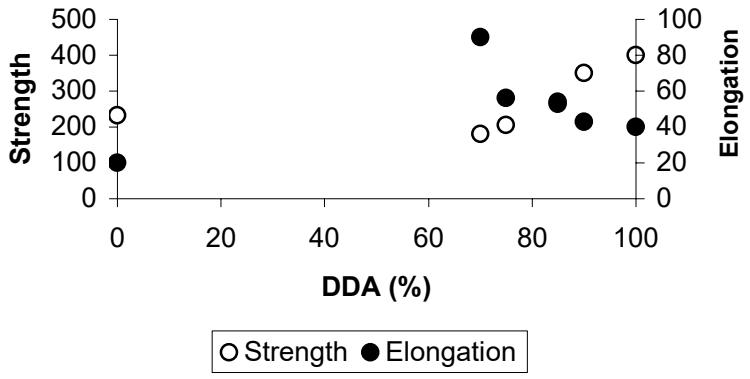

Figure 1.3. Mechanical properties of films of chitin and its deacetylated derivatives. Films swollen with phosphate-buffered saline at 25°C as a function of the degree of deacetylation: ○strength ●elongation. Modified from Tomihata *et al.*,1997.

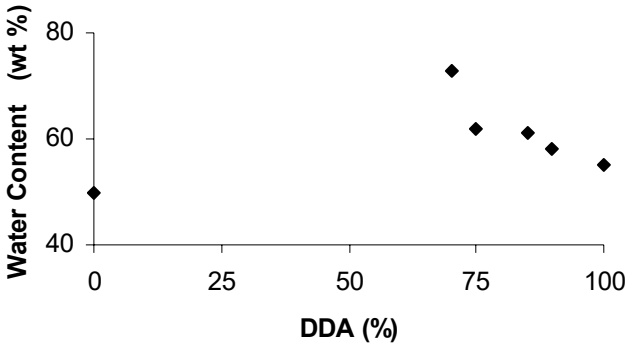

Figure 1.4. The water content of films of chitin and its deacetylated derivatives. Films swollen with phosphate-buffered saline at 37°C as a function of the degree of deacetylation. Modified from Tomihata *et al.* , 1997.

With clear and convincing data that the degree of deacetylation influences chitosan's material characteristics, the degree of deacetylation will be noted in the *in vitro* literature review which follows.

In vitro **studies examining theories of chitosan's biological responses**

Most *in vitro* studies have focused on investigating the theory of increased proliferation, cell migration, and cytokine and growth factor expression in addition to the material characteristics that elicit positive responses.

Proliferation

The reports on the effects of chitosan on the proliferation of cells are varied. For example, fibroblast proliferation has been shown to be stimulated (Chung, *et al.*, 1994) and inhibited (Mori, *et al.*, 1997) by chitosan. Keratinocyte proliferation has also been reported to be stimulated (Chatelet, *et al.*, 2001) and inhibited (Denuziere, *et al.*, 1998) by chitosan materials. In these studies, the chitosan materials were derived from fungal to crustacean sources, the degree of deacetylation ranged from less than 30 % deacetylated to practically 100% deacetylated, and the molecular weight of the material varied from 191 to 300 kDa. With the variability in the types and properties of the chitosan materials used in these studies, it is not surprising that widely different results were obtained.

Fibroblasts will be the initial focus of the review since they determine the extent of scar formation (Hunt, *et al.*, 2000). In wound healing, fibroblasts are the dominant active cell type producing the new matrix which serves as a scaffold for angiogenesis and reestablishing tissue continuity as well as producing a number of growth factors and cytokines such as IL-6 and IL-8 which increase cell proliferation and maturation (Goretsky, *et al.,* 1996; Hunt, *et al.*, 2000; Liechty, *et al.*, 1998).

The proliferation response of fibroblasts varies with the degree of deacetylation. Howling *et al.* (2001) examined chitin from 39 % to 89 % degree of deacetylation in culture with normal human fibroblast cells. A high and low molecular weight sample was used for each degree of deacetylated material studied. The cells were exposed to dose amounts of 2.5 µg/ml, 5 µg/ml, 50µg/ml, and 500 µg/ml. Only the 89% deacetylated

materials showed a convincing increase in proliferation. While the molecular weight and dose did in some cases produce differing responses, there was no clear trend. The results of Howling, *et al.* (2001) did show material characteristics influenced cellular response; yet, did not demonstrate a clear trend in the effect of the degree of deacetylation or the molecular weight of the chitosan on the proliferation of the fibroblast cells.

Chung, *et al.* (1994) also examined the proliferation of fibroblasts with exposure to chitosans of varying degrees of deacetylation. Chitosan of 91% deacetylated derived from *Phycomyces blakesleeanus,* a fungus, was shown to increase proliferation of F100 fibroblasts 42% above the untreated control (Chung, *et al.*, 1994). The increase was dose-related over the range of 0.005, 0.01, and 0.05 % chitosan powder weight per volume of media. Attachment, in addition to proliferation, showed a positive relationship with increasing levels of chitosan (Chung, *et al.*, 1994). However, chitosan of 52% deacetylation derived from *Mucor Mucedo,* a fungus, and chitosan of 32% deacetylation derived from *Aspergillus oryzae,* a fungus, had a positive effect on proliferation only at the 0.005 and 0.01 % chitosan respectively. The 0.05 % sample of both the 52 and 32 % deacetylated chitosan displayed an 80% decrease in proliferation in comparison to the untreated control. The authors concluded that the source was responsible for the varying responses observed (Chung, *et al.*, 1994). The variation in degree of deacetylation was not addressed and could equally be responsible for the difference in response seen.

Chitosan films with degrees of deacetylation ranging from 52.5 to 97.5 % were found to affect proliferation of fibroblasts and keratinocytes obtained from the foreskin of

children (Chatelet, *et al.*, 2001). Proliferation for the fibroblast was so poor that the group characterized chitosan substrates to be cytostatic (inhibitive of proliferation but not cytotoxic) toward fibroblast. Kerationcytes showed increased proliferation with an increase in the degree of deacetylation. Adhesion also increased with an increase in the degree of deacetylation. Lower levels of deacetylation corresponded with decreased cell adhesion for both fibroblast and keratinocytes (Fig 1.5). Fibroblast where thought to have a more negative cell membrane than keratinocytes resulting in extreme adhesion which limited proliferation. The group concluded that as the level of deacetylation

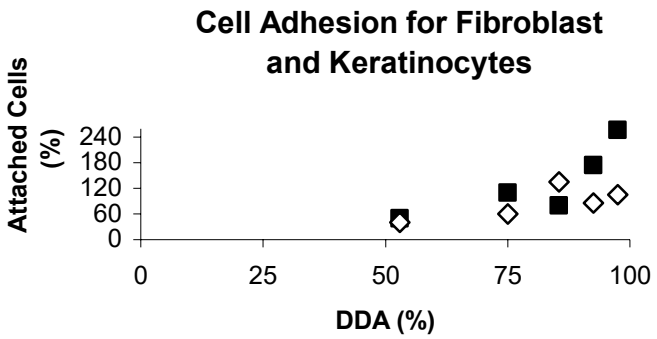

Figure 1.5. Percentage of cell adhesion versus the DA of chitosan films. Keratinocytes (◊), and Fibroblasts (■). Modified from Chatelet *et al.*, 2001.

increased, the charge density of chitosan increased causing greater cell to substrate adhesion (Chatelet, *et al.*, 2001). Conversely, treatment of 3T6 fibroblast cells derived from fetal mice with chitosan of 80 % deacetylation and 80 kDa molecular weight at 0.01 mg/ml, 0.1 mg/ml, and 1.0 mg/ml yielded no change in the proliferation rates (Okamoto,

et al., 2002). Differences in the results of these studies may be due in part to the source, degree of deacetlylation, and form of chitosan as well as the types of cells used.

With the data for the proliferation of fibroblasts mixed, cellular response may be tied to the degree of deacetylation. Two studies examining a range of deacetylation found proliferation was related to the degree of deacetylation (Chatelet, *et al.*, 2001; Chung, *et al.*, 1994). Unfortunately, these studies established opposite relationships of cell proliferation to the degree of deacetylation.

Migration

Filling the void in a wound or tissue engineering situation can be accomplished by more than proliferation. Migration of cells to chitosan may also play an important role. Chitosan may attract stem cells which are known to be present in muscle (Deasy, *et al.*, 2002; Simper, *et al.*, 2002) and adipose tissue (Bennett, *et al.*, 1991; Zuk, et al., 2002) to the wound site. When chitosan was tested in an adult rabbit critical-size-defect model, a marked increase in calcification and bone union was noted over the 12 weeks it was compared to the untreated control. Osteogenic activity was noted, not only in the periosteal, cortical, and marrow elements as expected, but also for the surrounding soft tissues such as tendon and muscle in chitosan treated models (Borah, *et al.*, 1992).

The attraction of inflammatory cells to an injured site can increase the production of growth factors and cytokines, ultimately increasing the growth of cells and production of collagen. Increased migration of canine neutrophils has been reported for both chitin

and chitosan *in vitro* (Usami, *et al.*, 1994*)*. Chitosan of more than 80 % deacetylated was more effective than chitin of less than 30% deacetylated at inducing the migration of canine neutrophils (Usami, *et al.*, 1994). In an attempt to overcome the suppression of the immune system caused by anesthesia used during surgical procedures, 5 mg/kg, 10 mg/kg, and 20 mg/kg cotton-type chitosan was administered to female beagles within 30 minutes of anesthesia. Increases in leukocyte and macrophage numbers were noted during the first three days. The number of leukocytes and macrophages continued to increase until day 4 (Kosaka, *et al.*, 1996). An increase in the infiltration of polymorphonuclear cell (PMN) and macrophage inflammation cells was also associated with 87 % deacetylated chitosan tested *in vitro* (Mi, *et al.*, 2000). A prolonged increased in inflammatory response has been correlated with poor healing (Hunt, *et al.*, 2000). While an initial increase in the number of inflammation cells is seen with chitosan, there is a significant decrease in the number of inflammatory cells at the wound site by day 28 in wounds treated with chitosan of greater than 80 % deacetylation when compared to untreated dog wounds (Okamoto, *et al.*, 1995).

While chitosan is known to increase macrophage, PMN, and leukocyte infiltration, it has also been theorized that it causes osteoblast, fibroblast, and Human Umbical Vein Endothelial Cells (HUVECS) to migrate as well. Chitin of deacetylation of less than 10% and a molecular weight of 300 kDa and chitosan of greater than 80% deacetylation and a molecular weight of 80kDa were tested on the migration of 3T6 fibrobast and HUVEC cells at the doses of 0.01 mg/ml, 0.1 mg/ml, and 1.0 mg/ml. Both

chitin and chitosan reduced the migratory effects of 3T6 cells when compared with controls. With exception to 1.0 mg/ml concentration, chitin and its oligomers and monomers reduced HUVECs migration. Chitosan showed a significant increase in migration of HUVEC cells. The chitosan oligomers induced migration rates were similar to control. The remaining levels produced results similar to control with the exception of the 0.01 mg/ml dose of chitosan oligomers, which inhibited HUVEC migration (Okamoto, *et al.*, 2002). Okamoto, *et al.* (2002) studies suggest the degree of deacetylation and possible the dose levels are very important in producing a migratory response. Using 82% deacetylated chitosan on dog wounds, Ueno, *et al.* (1999) found that increased effusion seen resulted in a thick fibrin line promoting the migration of fibroblast into the wound area. Osteoblasts have also been credited with moving into the wound in a rat model (Hidaka, *et al.*, 1999). In addition to immune cells, fibroblast and osteoblast likely are induced to migrate toward chitosan.

These studies have demonstrated that chitosan materials affect the migration of macrophage, leukocytes, PMN, endothelial cells and possibly other cells types. Migration may be influenced by the degree of deacetylation and degradation products of the chitosan material. Yet, migration may not be the only factor behind the positive results seen with chitosan. Chitosan may also play a support role in the production of growth factors, cytokines and collagen by cells.

Cytokines, growth factors and signaling

The synthesis of nitrous oxide (NO) is one of the first responses of cells in the wound healing process (Figure 1.6). Nitrous oxide can stimulate cell proliferation (Witte and Barbul, 2002). Wound healing has been shown to be NO dependent with delayed closure of excision wounds when NO is inhibited (Stallmeyer, *et al.,* 1999). Chitosan has been shown to positively influence the production of NO (Jeong, *et al.*, 2000). Water soluble chitosan with a degree of deacetylation over 90% and a high molecular weight of

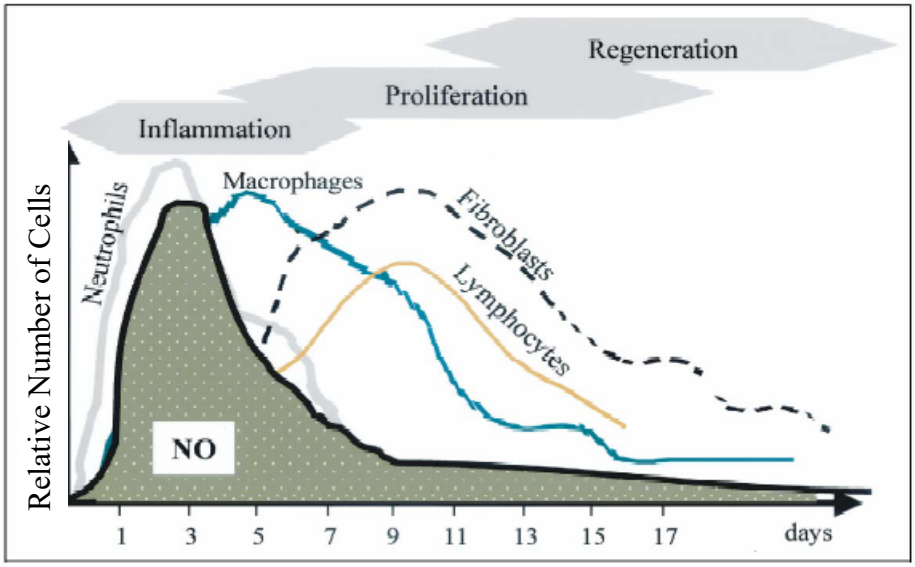

Figure 1.6. Phases of wound healing and the generation of wound Nitrous oxide. Modified from Witte and Barbul, 2002.

30,000 Da was tested for induction of NO production *in vitro*. RAW 264.7 macrophages were introduced to water soluble chitosan with and without priming by recombinant

Interferon-γ (IFN-γ). Water soluble chitosan alone reduced the production of NO by approximately 15 % when compared to control. When primed first by recombinant IFN-γ, the production of IFN- γ was increased by approximately 60 % relative to control (Jeong, *et al.*, 2000).

Following NO expression, macrophages and fibroblasts become involved in wound healing (Witte and Barbul, 2002). Thus, the growth factors and cytokines of interest are those primarily secreted by macrophages and fibroblast such as IL-1, IL-6, IL-8, PDGF, and TNF. Below is a summary of each.

Interleukin-1(IL-1)

Secreted primarily by macrophage/monocyte lineage cells, IL-1 is also produced by keratinocytes in active wounds (Sauder, *et al.*, 1990; Goretsky, *et al.*, 1996). IL-1 activates neutrophils, upregulates adhesion molecules, and promotes chemotaxis (Fong, *et al.*, 1996). Detectable levels of IL-1 occur within the first 24 hours of the wound. Expression tends to peak on the second or third day and then declines rapidly during the remainder of the first week (Goretsky, *et al.,* 1996; Fahey *et al.,* 1990). Interleukin-1 is responsible for the promotion of the secretion of other pro-inflammatory cytokines which further contribute to the local inflammatory response and increased proliferation of fibroblast and keratinocytes as well as increased collagen formation at the wound site (Sauder, *et al.,* 1990). High levels of IL-1 have been shown to have adverse effects on wound-healing in irradiated mice (Vegesna, *et al.,* 1995). Further, elevated levels of IL-1

are associated with chronic non-healing wounds (Trengove, *et al.,* 2000; Barone, *et al.,* 1998).

Interleukin-6 (IL-6)

Interleukin-6 has a multitude of functions. Within wounds it is secreted by polymorphonuclear cells (PMNs) and fibroblasts. Interleukin-6 is evident within 6 hours and expression continues for longer than a week (Goretsky, *et al.,* 1996; Mateo, *et al.*, 1994). Interleukin-6 has proven to dramatically increase the proliferation of fibroblasts. Interestingly, IL-6 is diminished in fetal wounds. Exogenous addition increases scarring. IL-6 levels, both basal and induced, declines with age in fibroblasts. Although IL-6 is not a clear candidate for either up or downregulation, plasma levels indicate the severity of the wound and could be of use as a prognostic marker (Goretsky, *et al.*, 1996; Ueyama, *et al.*, 1992; Frieling, *et al.*, 1995).

Interleukin-8 (IL-8)

Primarily produced by macrophages, IL-8 is also produced by fibroblasts in acute wounds (Clark, *et al.*, 1993; Liechty, *et al.*, 1998). Major effects include increased neutrophil degraulation, increased neutrophil and monocyte chemotaxis, and increased expression of endothelial adhesion cells. The highest levels of IL-8 occur during the first day of injury (Engelhardt, *et al.*, 1998). Interleukin-8 promotes keratinocyte maturation and margination (Engelhardt, *et al.*, 1998; Nanney, *et al.*, 1998). Interleukin-8

production by fibroblasts is increased in patients with psoriasis (Konstantinova, *et al.*, 1996).Low expression of IL-8 mRNA by fibroblasts in fetal wounds supports the theory that pro-inflammatory cytokines are at least partially responsible for the scaring (Liechty, *et al.*, 1998).

Platelet-derived Growth Factor (PDGF)

Platelet-derived Growth Factor is released from platelet alpha granules following injury. This induces the immediate recruitment and activation of immune cells and fibroblasts. After the initial response, PDGF is also secreted by macrophages; and, through macrophages, stimulates collagen and protoglycan synthesis (Beer, *et al.*, 1997; Lepisto, *et al.*, 1996). All naturally occurring isomers have varying degrees of positive influence on wound healing (Lepisto, *et al.*, 1996; Lepisto, *et al.*, 1994). Interestingly, two receptors are also added to the mix. All three PDGF isomers are normally found at very low levels in normal skin and chronic non-healing ulcers (Pierce, *et al.*, 1995). Levels of PDGF receptors have been found to be decreased in impaired wound-healing states (Beer, *et al.*, 1997).

Tumor Necrosis Factor (TNF)

Primarily released by macrophage-monocyte lineage cells, TNF initiates the immune cascade in response to injury or bacterial infection. Its main role is to upregulate cell-surface adhesion molecules which are essential for neutrophil chemotaxis (Omann, *et*

al., 1997; Moser, *et al.*, 1989). Tumor Necrosis Factor is expressed locally with 12 hours of wounding. The expression continues to rise until it peaks at 72 hours. Elevated concentrations of TNF l increase vascular permeability and vascular proliferation (Omann, *et al.*, 1997). Excessive circulation of TNF has been associated with multi-system organ failure and increased morbidity in inflammatory diseases (Girardin, *et al.*, 1988; Marks, *et al.*, 1990; Shalaby, *et al.*, 1991; Waage, *et al.*, 1987). Recombinant TNF applied locally to normal and doxorubicin-impaired animals' wounds increased both wound-disruptive strength and collagen synthesis (Mooney, *et al.*, 1990; Fu, *et al.*, 1996). Yet TNF may also cause decreased collagen synthesis (Rapala, *et al.*, 1997) and be associated with the poor wound healing seen in septic and chronic disease states (Cooney, *et al.*, 1997; Wallace, *et al.*, 1998; Trengove, *et al.*, 2000).

Chitosan's regulation of Cytokines and Chemokines

Saccharide moieties play a crucial role in cell signaling and immune recognition (Suh, *et al.*, 2000). Chitosan, which is a polysaccharide, has been shown to increase the number of PMN, leukocytes, and macrophages and thus enhance the wound healing process (Minami, *et al.*, 1993). The initial increase in immune response is also accompanied by an increase in growth factors such as IL-1, IL-6, IL-8, colony stimulating factor (CSF), TNF, and PDGF. Macrophages exposed to chitosan have been shown to activate IL-1 (Muzzarelli, *et al.*, 1987). By adding powdered chitosan to culture media, growth factors produced by fibroblasts increased. In particular, transforming

growth factor-β-1 (TGF-β-1) and PDGF (Ueno, *et al.*, 2001), and TNF-α and IL-8 (Mori, *et al.*, 1997) increased. Chitosan of 80% deacetylation and dose level of 500 µg/ml was shown to increase IL-6, IL-8, and TNF-α production in HUVECs. It did so at a rate higher than 30% deacetylated chitin (Mori, *et al.*, 1997). Chitosan of approximately 80 % deacetylation with a molecular weight of 50,000 increased TGF-β1 and PDGF expression in L929 fibroblasts exposed to 5 µg/ml, 50 µg/ml, and 500 µg/ml in a dose dependent manner (Ueno, *et al.*, 2001). The production of IL-1 by L929 mouse fibroblasts has been stimulated by exposure to 70% deacetylated chitosan. Activation of CSF was also observed. Interestingly, the production of CSF occurred in a biphasic manner with the highest number of colonies corresponding to the dose of 10 µg per ml. Elevated, but not maximal responses were seen for doses 1, 5, 500, 1000 µg per ml. Neither IL-2 nor TNF was shown to be activated in this study (Nishimura, *et al.*, 1986). In a follow-up study, 80% deacetylated chitosan was shown to activate secretion of IL-1 by macrophages over a 72 hour period when administered to mice at a dose of 2.5 µg per ml. Chitin showed no effect (Nishimura, *et al.*, 1987).

Chitosan may stabilize growth factors and cytokines in the wound environment, increasing their life span and thus impact on the wound. Chitosan is a polyanonic polysaccharide which can bind proteins such as heparin (Denuziere, *et al.*, 1998) or possibly growth factors (Mori, *et al.*, 1997) to form polyelectrolyte complexes. Many growth factors involved in wound healing bind to heparin and are thereby stabilized and activated (Kratz, *et al.*, 1997; Kratz, *et al.*, 1998). In addition, low molecular weight

chitosan was found to have no effect on the growth of vascular smooth muscle unless platelet derived growth factor was present. Inui, *et al.* (1995) suggested that activation of the tyrosine kinase pathway is essential for oligosaccharides such as chitosan to function. While Denuziere *et al.* (1998) observed slightly improved wound healing in Sprague-Dawley rats treated with chitosan when compared with the untreated control in a follow-up *in vitro* study, 100% percent deacetylated chitosan was shown to decrease the proliferation of human foreskin primary keratinocytes cultures by 40% when compared to controls (Denuziere, *et al.*, 1998). The *in vitro* study occurred under serum free conditions. The importance of growth factors can be inferred from the decreased *in vitro* results. Further explanation for the extremely positive results from *in vivo* work that can not be explained *in vitro* is that chitosan has shown the most promise in healing problem wounds (Allan, *et al.*, 1984; Naseema *et al.*, 1995; Prudden, *et al.*, 1970). Chronic wounds have been shown to be deficient in growth factors (Bennett, *et al.*, 1993; Pierce, *et al.*, 1995). A boost in growth factors and cytokines alone in a deficit environment may be responsible for the most spectacular *in vivo* results.

The increase in infiltration of immune cells seen with the administration of chitosan is most likely followed by an upregulation of growth factors. Evidence suggests that chitosan increases the production of growth factors. Further, chitosan may stabilize growth factors and cytokines in the wound bed, increasing their impact on the surrounding cells. One of the effects may be an increase in collagen production.

Extracellular Matrix (ECM)

In response to IL-1, fibroblasts produce collagen which improves the tensile strength to the wound (Muzzarelli, *et al.*, 1998). Polymeric surfaces tend to double cells excretion of ECM and half the amount of cell proliferation when 3T3MC fibroblasts in culture were compared to 3 metals-316L stainless steel, Ti-6Al-4Vand pure tantalum and 3 polymers- high density polyethylene, silicone rubber and polytetrafluoroethylene (Hallab, *et al.*, 2001). The application of 82% deacetylated chitosan increased the production of type III collagen *in vivo* (Ueno, *et al.*, 1999). A follow-up study *in vitro* examining the production of types I and III collagen by L29 fibroblasts showed no significant increase with exposure to chitosan (Ueno, *et al.*, 2001).

The degradation process and its possible positive effects

For use as a biomaterial, acute systematic toxicity tests performed in mice did not show any significant toxic effects (Rao and Sharma, 1997). The degradation products of chitosan are amino-sugars which can be incorporated into glycosaminoglycan and glycoprotein metabolic pathways or excreted (Pangburn, *et al.*, 1982). In the body, chitin and its derivatives are degraded by the chitinase and lysozyme contained by macrophages (Boot, *et al.*, 1995). Several hydrolases can also break down chitin to varying degrees depending on the percentage of deacetylation. Most of the studies on chitin and its derivative's degradation focus on the activity of lysozyme. Conflicting findings on the effect of the degree of deacetylation have been reported. Neutrophils, a

major producer of the lysozymes involved in inflammation, are said to more easily phagocytize cationic agents such as chitosan than neutral molecules such as chitin (Usami, *et al.*, 1994). Yet, nearly 100% deacetylated chitosan can not be degraded by lysozyme (Pangbourn, *et al.*, 1982). These studies show that the lower the degree of deacetylation degrades faster than a higher level of deacetylation, if all other factors are equal. Another study found that 70% deacetylated chitosan, out of a range from 45% to 95% deacetylated chitosan, degraded the fastest (Shigemasa, *et al.*, 1993). If molecular weight and degree of deacetylation are held constant, homogenously prepared samples were more susceptible to degradation than where heterogeneous samples (Shigemasa, *et al.*, 1993; Lee, *et al.*, 1995) suggesting that lysozymes need at least three consecutive N-acetyl residues to be most effective. Block segments of N-acetyl sugars are more common in homogenously produced chitosan samples. *In vivo* degradation products are simple oligosaccharides which undergo further degradation by β-glucosamines before being removed from the body (Onishi and Machida, 1999). Interestingly, water soluble chitosan also degrades faster than normal chitosan losing approximately 70% of its mass when subjected to enzymatic degradation for 60 minutes. During the same time, unaltered chitosan degraded less than 20% (Cho, *et al.*, 1999). The degradation produced monomers and oligomers of both chitin and chitosan showed positive effects on migration (Okamoto, *et al.*, 2002) and growth factor production (Mori, *et al.*, 1998).

Surface Properties

While most polysaccharides are negatively charged, chitin and its derivatives have a positive charge. The positive charge of chitin and its derivatives attracts negatively charged objects, such as cell walls. Cells are reported to have a high affinity for chitosan (Muzzarelli *et al.*, 1994; Muzzarelli *et al.*, 1988). The ability to attract and bind cells was theorized to support proliferation. However some chitosan films are theorized to hold some cells lines too tightly to allow for cell proliferation. As a general rule, adhesion for fibroblasts was approximately twice that seen with keratinocytes for chitin films of 52.5 to 97.5% deacetylated (Chatelet, *et al.*, 2001). For the keratinocytes, better adhesion produced higher proliferation rates; the inverse was true for fibroblast (Chatelet, *et al.*, 2001). Differing proliferation results occurred when chitosans over similar ranges were either added to the media as powder or films used as substrates (Chatelet, *et al.*, 2001; Chung, *et al.*, 1994; Howling, *et al.*, 2001). The higher levels of deacetylation showed increased fibroblast proliferation when a chitosan powder was added to the media (Chung, *et al.*, 1994; Howling, *et al.*, 2001); but, when chitosan of similar levels of deacetylation was employed in a film form, a decrease in fibroblast proliferation was observed (Chatelet, *et al.*, 2001). This suggests that surface interaction may influence the response seen.

Surface energy and surface roughness play an important role in biomaterial properties. Cellular adhesion strength and proliferation were influenced more by surface energy than surface texture when comparing metal and polymer materials. However,

polymers generally have a lower surface energy. At low surface energy, an increase in cellular adhesion strength was associated with increased surface roughness (Hallab, *et al.*, 2001). Very little attention has been given to surface roughness with chitosan samples. Chatelet, *et al.*, 2001 noted that the film that supported the greatest amount of cellular proliferation was also the smoothest one. On rough surfaces of chitosan sponges, the morphology of fibroblasts remains spherical, instead of the more extended spindle shape seen on tissue culture plastic (Ma, *et al.*, 2001). The spherical shape has been credited by some authors as being indicative of a more stem/progenitor type cell in function. Others argue that an elongated cell, as seen in the body, is more desirable. When exposed to 91% deacetylated chitosan derived from *P. blakesleeanus,* elongated, interwoven F100 fibroblasts were seen. This natural morphology was not seen in the parts of the dish lacking chitosan (Chung, *et al.*, 1994).

Crystallinity is a material property which is also a function of the degree of deacetylation. Both 0% deacetylated chitin and 100% deacetylated chitosan are said to have maximum crystallinity (Suh, *et al.*, 2000). Crystallinity changes as a polymer erodes in two ways. First, degradation generates crystallized monomers and oligomers. Secondly, the overall crystallinity of the polymer changes (Göpferich, 1996).
As chitosan degrades the crystallinity index increases while the molecular weight decreases (Struzezyk, *et al.*, 1994).

Hypothesis

The hypothesis for this study is that macrophage, osteoblast and fibroblast attachment, growth and release of extracellular molecules will be increased on 95% deacetylated chitosan materials as compared to lower percent deacetylated chitosans. As of yet there is no study known to the author which compares proliferation, adhesion, cell spreading and the production of extracellular molecules for well characterized chitosans. This is surprising since much speculation as to the role the degree of deacetylation, molecular weight, etc, of chitosan materials plays in the biological response. Therefore, for this study, it was hypothesized that macrophage, osteoblast and fibroblast attachment, growth and release of extracellular molecules will be increased on 95% deacetylated chitosan materials as compared to lower percent deacetylated chitosans.. To test this hypothesis, films of 76, 78, 80, 87, 91, 92 and 95% deacetylation chitosan will be evaluated in cell culture models.

CHAPTER II

FIBROBLAST RESPONSE

Abstract

Chitosan has a long history as a wound treatment; however, it is not yet widely accepted in the United States. Recent studies have reported varied results with fibroblasts exposure to chitosan. Chitosan's material characteristics are thought to play a role in cellular response. The aim of this study was to qualify cellular responses with known material characteristics. To achieve this aim, chitosan was characterized and made into films. The films were seeded with fibroblast cells and evaluated for adhesion and proliferation over the course of 5 days. This study found no clear linear relationship between the degree of deacetylation (DDA), adhesion, and proliferation. The material characteristics of DDA, molecular weight (MW), contact angle, and swelling index, could not predict proliferation rates of fibroblasts. Molecular weight corresponded to adhesion of the fibroblasts to the films. Ash content may influence proliferation. The chitosan film with the highest ash content supported the greatest amount of growth on day 3. A general trend of increasing proliferation with increasing ash content was observed.

Introduction

Chitin and chitosan have been used to enhance wound healing throughout history. Ancient Japanese fishermen placed powdered crab shells on lacerations (Li, *et al.,* 1997). The United States Army newest haemostatic agent to treat battlefield injuries contains chitosan (Becker, 2003; Pusateris, *et al.,* 2003). As a wound dressing, chitosan has shown improved and scarless healing potential due to: higher numbers of mitotic cells in the wound bed, greater macrophage infiltration into the site, faster re-epithelialization of the wound, increased angiogensesis, and greater collagen deposition resulting in enhanced healing rates and wound strengths (Biagini, Bertani, *et al*, 1991, Biagini, Pugnaloni, *et a.l*, 1991; Braye, *et al.*, 2000; Cho, *et al.*, 1999; Mi *et al*., 2000; Ueno, *et al.*, 1999). Possible mechanisms for enhanced wound healing include increased cell attachment, binding and increased retention of cytokines and growth factors, and structural similarity to hyaluronic acid (Bumgarnder, *et al.*, 2003; Usami, *et al.*, 1994; Mori, *et al.*, 1997; Kratz, *et al.*, 1998; Muzzarelli, *et al.*, 1999). Chitosan may also provide analgesic effects for serious burns (Kifune, 1992).

Chitosan is a co-polymer of N-acetyl-glucosamine and N-glucosamine units. Either an acetamido group (-NH-$COCH_3$) or an amino group (-NH_2) is attached to the C-2 carbon of the glucopyran ring. When more than 50% of the C-2 attachment is an amino group, the material is termed chitosan. The degree of deacetylation (DDA) represents the percentage of amino groups. Figure 2.1 represents a subunit of 100% DDA chitosan. Ideally, chitin is a linear polysaccharide of β-(1-4)-2 acetamido-2-deoxy-

D-glucopyranose where all residues are comprised entirely of the acetamido group -NH-COCH3. This is termed fully acetylated or 0% DDA. (Figure 2.1).

While preliminary data from *in vivo* models is promising, there has not been a definitive study on the influence of the DDA and molecular weight of chitosan materials on the observed biological/clinical outcomes for soft tissue applications. Chitosans of approximately 82-100% DDA implanted in connective tissues have shown increased angiogenesis, promotion of stromal cell migration and differentiation, and reorganization of collagen extracellular matrix (Ueno, *et al.*, 1999; Muzzarelli *et al.*, 1987; Mi, *et al.*, 2000; Cho, *et al.*, 1999). However when chitosans of varying DDAs were examined in bone applications, chitosan implants of the highest DDA, 94 and 100 %, were associated with fibrosis (Hidaka, *et al*, 1999).

In order to correlate DDA with *in vivo* performance, researchers have attempted to characterize chitosan material properties and cellular response. *In vitro* cell culture studies also show promising yet conflicting data. For example, fibroblasts proliferation was increased on 89 and 91% DDA (Chung, *et al.*, 1994; Howling, *et al.*, 2001) while chitosan films ranging from 52.5 to 97.5% DDA were found to inhibit fibroblasts proliferation (Chatelet, *et al*, 2001). Differences in these studies may be due in part to the source of chitosan (fungal vs arthropod) and types of cells (primary vs transformed). A thorough investigation of how chitosan's material properties affect cultured cells may help determine the appropriate type of chitosan to use in wound healing applications. Therefore the aim of this study was to evaluate material properties of chitosans such as

DDAs, molecular weights, and surface properties in relation to fibroblasts attachment and proliferation.

Materials and Methods

Materials

Chitosans

Chitosan powders of crab origin were obtained from Vanson HaloSource (Redman, WA) through generous donations and purchase. Films were made by solution casting 1% chitosan dissolved in 0.2 M acetic acid (Sigma-Aldrich St. Louis, MO) in 96 well culture plates. The plates were dried in a laminar flow culture hood for the first and last 24 hours of a one week period. Between drying periods, they were stored at a constant temperature of 21°C. A longer drying period was employed in an attempt to increase the films' crystallinity. The films were then rinsed in copious amounts of phosphate buffered solution (PBS) and sterilized in ethylene oxide gas.

Cells

Normal Adult Human Dermal Fibroblasts (NAHDF) (Clonetics/ Cambrex, Baltimore, MD) were maintained at 37°C in a 5% CO_2 atmosphere under sterile conditions. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and 1% antibiotic–antimycotic (Gibco/

Invitrogen Carlsbad, CA). Cells were subcultured with 1% trypsin-EDTA (Gibco/ Invitrogen Carlsbad, CA). Only cells between the third and sixth passage were used.

Material Characterization

Degree of Deacetylation Determination

The powders were tested for degree of deacetylation by titration (Broussignac, 1970). Chitosan (0.5 g) was dissolved in 20 ml of 0.3N hydrochloric acid (Sigma-Aldrich St. Louis, MO). After adding 400 ml of distilled water, this solution was titrated with 1N NaOH solution (Sigma-Aldrich St. Louis, MO). A titration curve of pH vs. NaOH titration volume was generated. The curve's inflection points were found for each indicated transition. The volume of NaOH at the each inflection point was applied to the equation:

$$\%NH_2 = 16.1*(y - x)/M \qquad (1)$$

where M is the weight of chitosan used (0.5 grams, in this study), x is the first inflection point on the graph of measured pH vs. titration volume, y is the second inflection point (Broussignac, 1970). Figure 2.2 represents a sample titration curve. The DDA was determined from 3 samples of each chitosan lot.

Molecular Weight Measurement

Molecular weight was determined by dilute solution viscometry as described by Knaul, *et al*, 1998. Briefly, solutions of various chitosan were dissolved in 0.25M acetic acid and 0.25M sodium acetate (Sigma-Aldrich St. Louis, MO) solvent to concentrations

of 0.0025 to 0.03 g/dl, depending on the sample. The samples were analyzed at 25 °C using an Ubbelohde viscometer (Cannon, State College, PA). The intrinsic viscosity was calculated. The molecular weight was found by using the Mark-Houwink equation:

$$[\eta] = K'M^{\alpha} \qquad (2)$$

where [η] is viscosity and M is molecular weight. The constants, K' and α, are $1.14*10^{-4}$ and α=0.83, respectively (Knaul, *et al.*, 1998). Figure 2.3 is an example of a viscometry graph used to determine the molecular weight. The molecular weight was determined from five samples for each DDA of chitosan.

Contact Angle Measurement

Sessile drop air /water contact angle measurements were performed on non-hydrated films using a contact angle goniometer (Rame'-Hart model 100; Mountain Lake, NJ). The contact angle was determined from five samples for each DDA of chitosan.

Ash Content Determination

Chitosan ash content was determined using a constant weight crucible. The crucible weight, W_0, was stabilized to a tolerance of ±0.5mg by repeatedly placing it into an oven at 550°C ± 20°C for 30 min and allowing it to cool for 30 minutes until the weight was constant. Chitosan (2-5g) was combusted in the constant weight crucible and placed in an oven at 550°C ± 20°C for 3 hours. The sample was removed, cooled in a desiccator for 30 minutes, and re-weighed (W_1). This heating and cooling process was

repeated every hour until a constant weight was established (W_2). The ash percentage was calculated by the equation:

$$Ash\% = \frac{W_2 - W_0}{W_1 - W_0} \times 100 \qquad (3)$$

where W_0 is the constant weight of crucible, W_1 is the weight of sample and crucible, W_2 is the weight of assay and crucible (Tingda, 2001). The ash content was determined from five samples for each DDA of chitosan.

Protein

Film protein concentrations were determined by BCA assay (Pierce, Rockford, IL). The films were tested with the BCA reagents according to the manufacture's instructions and read at 560 nm (μQuant Universal Microplate Spectrophotometer; Bio-Tek Instruments, Inc., Winooski, VT. The protein was determined from five samples for each DDA of chitosan.

Cell Adhesion Assay

Normal adult human dermal fibroblasts cells were exposed to the chitosan films for 30 minutes in a serum-free media. Then the media and non-adherent cells were removed. The films were rinsed twice with PBS. Media was added to the chitosan films. After one hour at 37 °C, Promega CellTiter (Promega, Madison, WI) was added. The cell number was then assessed by reading the plate at 490 nm on a μQuant Universal

Microplate Spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). Tissue culture plastic was used as the control substrate.

Cell Proliferation Assay

Growth on the chitosan films was determined by cell counting at 3 and 5 days after seeding 5000 or 1000 cells/cm^2, respectively. Cell proliferation was determined by the addition of Promega's CellTiter MTS Cell Proliferation Assay Kit (Promega, Madison, WI) which was read after one hour by the microplate spectrophotometer at 490 nm. Absorbance values were converted to cell number using a standard curve of known cell number vs. absorbance**.**

Statistical Analysis

Triplicate samples of each film were used in cell culture studies. Cell culture studies were repeated three times. Post-hoc multi-comparison tests using a F-protected Least Significant Differences were used to determine where statistical differences exist. Statistical differences were declared at $p<0.05$.

Results and Discussion

Material Characteristics

The DDA, molecular weight, ash content, contact angle, and protein for the test chitosan materials are shown in Table 2.1. The DDAs obtained by titration were similar to those reported by Vanson HaloSource (Redman, WA). The molecular weight ranged

form 8.22 *10^6 D for the 80% DDA chitosan to 1.99 *10^6 D for the 91% DDA chitosan. The molecular weight found by dilute solution viscometery is an estimated molecular weight that is between the weight average molecular weight and the number average molecular weight. The same K and a values for the Mark-Houwink equation were used for all DDA. These values have been previously reported to be valid for the range between 71 and 95% DDA (Knaul, *et al.,* 1998). At this time there is no universal standard set for chitosan quality grades known to the authors. Instead, each manufacture sets their own standards, which vary from company to company. The ash content for all chitosan samples was above allowable levels for medical/pharmaceutical grade chitosan (<0.2 %) according to the standards set by the chitosan manufacturer Dalwoo (Dalwoo, Seoul, Korea). However, all samples are within acceptable range of ash content of 2% or less with the exception of 78 and 87% set by LipoSan Ultra (Primex, Siglufjordur, Iceland). The sample with the highest ash content, 78% DDA, had 3.57% ash. The lowest ash content sample, 80% DDA, had only 0.24% ash. The contact angles measured ranged from 87.7° for 80% DDA to 62.1° for 95% DDA. These contact angle measurements fall within the range reported by others (Bumgardner, *et al.,* 2003). There was a general trend of contact angle decreasing as the DDA of the chitosan increased (Figure 2.4). It is theorized that the changing electrophysical properties influence the material characteristics causing the surface energy to increase with an increase in the DDA. Residual protein content varied from a negligible concentration on the 92% DDA films to 287.1 micrograms/cm^2 for 78% DDA. With a few exceptions, molecular weight

generally decreased with increasing DDA. This was expected due to the increased processing required to deacetylate the chitosan (Khor, 2001).

Adhesion

Fibroblasts cell adhesion for all chitosan films was statistically greater than cell adhesion to the control substrate (Figure 2.5). The chitosan films' promotion of cell adherence consisted of three statistical groups. Chitosan films of 76, 80, 87, and 92% DDA had the lowest number of adhering cells. Yet, these films did attract 2.5 times more cells than the control. The 76 and 78% DDA films showed the next highest amount of cell adhesion which was 3 times more than the control. The 91 and 95% DDA had the highest number of cells adhering to the surface, averaging more than 4 times that of the control.

The degree of deacetylation did not predict the level of adhesion as earlier reported (Figure 2.5) (Chatelet, *et al.*, 2001). Cell adhesion to the 91% DDA chitosan film was statistically greater than cell adhesion to the tested chitosan films of lesser and greater deacetylation levels. This may indicate that the DDA is not the only factor affecting cellular adhesion to chitosan films examined in this study. However, a general trend may be present. The highest cell adhesion did occur for 91 and 95% DDA, two of the three highest DDA tested. While these films also had the highest water contact angle, which generally suggests low wettability, the high DDA content does suggest a high net positive charge due to the amine groups. The high positive charge of the chitosan films would have a high electrostatic attraction for negatively charged cells, resulting in the

high number of cells attaching to the chitosan materials and this attraction may be increased with increasing DDA. Overall, there was not a strong relationship between DDA and cell adhesion for the chitosan materials tested (Figure 2.6). Molecular weight correlated well with adhesion (Figure 2.7). As molecular weight increased, adhesion decreased. A relationship between molecular weight and adhesion can be seen in other studies (Chatelet, *et al.*, 2001).

Proliferation of NAHDF

Proliferation of NAHDF cells varied on the films of different DDA (Figure 2.8). For the 3 day point, there were 4 statistical groups, performing above, at and below control proliferation levels. The performance of the first group, comprised of 76, 91, and 95% DDA, was below control levels. The second group, 80 and 95% DDA films, supported slightly less growth compared to the control. Films of 87 and 92% DDA supported the same amount of growth as the control. Growth on 78% DDA was 3.5 times greater than control. Interestingly, 78% DDA had the highest ash content.

Not all samples supported increased growth during the longer 5 day proliferation period (Figure 2.9). It should be noted that the initial seeding was not the same for the two studies. However, the relative difference between the films in question and the control, show a decrease in relative proliferation especially for 78%. This could be due to overgrowth and subsequent cell death. Chitosan films of 92 and 95% DDA showed the greatest growth on the 5th day. Their proliferation level was comparable to control. Chitosans films of 78, 80, and 87% DDA exhibited reduced proliferation compared to

control. Chitosans of 76 and 91% DDA continued exhibit the lowest cell numbers, considerably below control.

Comparing Adhesion, Proliferation Data, and Material Characteristics for NAHDF

For NADHF cells, adhesion to the chitosan film does not appear to be directly related to proliferation. The most adhesive film, 91% DDA chitosan, was the second least supportive of proliferation at 3 days. The least adhesive film, 87% DDA chitosan was the second most supportive of proliferation at the 3 day point. Both 76 and 78% DDA chitosan supported cell adhesion to the same level. Yet, at the 3 day point, 76% DDA chitosan supported the least amount of proliferation while 78% DDA chitosan supported the greatest amount of proliferation. There was no clear relationship between proliferation and DDA with the samples examined in this study as previously reported [20]. Hence this data does not clarify conflicting reports in the literature on fibroblasts' response to chitosan. For fibroblasts cell lines, inhibition of growth as seen in 76% DDA has been reported by others (Mori, *et al.*, 1997; Chatelet, *et al.*, 2001). Yet other groups have shown an increase in fibroblasts proliferation at certain degrees of deacetylation, as seen with the 78% DDA (Howling, *et al.*, 2001; Chung, *et al.*, 1994).

Contact angle did not relate to proliferation or adhesion data. Contact angle does correlate to DDA (Figure 2.2). With the exception of 87% DDA, the contact angle decreases as the DDA increases. Protein of the films with the exception of 78% DDA was an indicator for cell proliferation. As the amount of protein of the film decreased, proliferation increased (Figure 2.10).

Conclusion

This study found no clear linear relationship between the DDA, adhesion, and proliferation. The material characteristics of DDA, MW, contact angle, protein and ash content could not predict proliferation rates of fibroblasts. Molecular weight did correspond to adhesion of the fibroblasts to the films. Ash content and protein may influence proliferation. A general trend of increasing proliferation with increasing ash content was observed. Protein related inversely to the amount of proliferation observed for all but one sample. Additional studies examining other factors that may contribute to fibroblasts' response to chitosan exposure need to be performed to truly understand the relationships between chitosan's physical and chemical properties to cellular response.

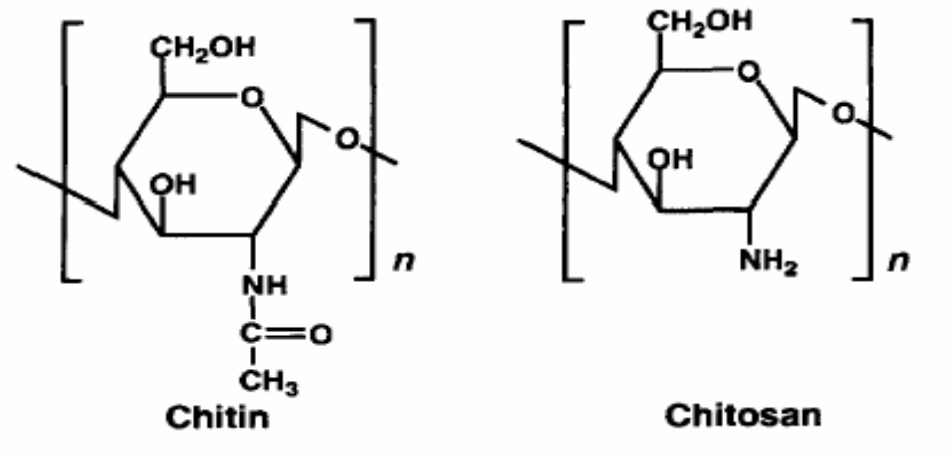

Figure 2.1. The chemical structure of Chitin and Chitosan. When more than 50% of the acetyl group, -CO-CH3, are removed from the chitin polysaccharide molecule, the molecule is referred to chitosan.

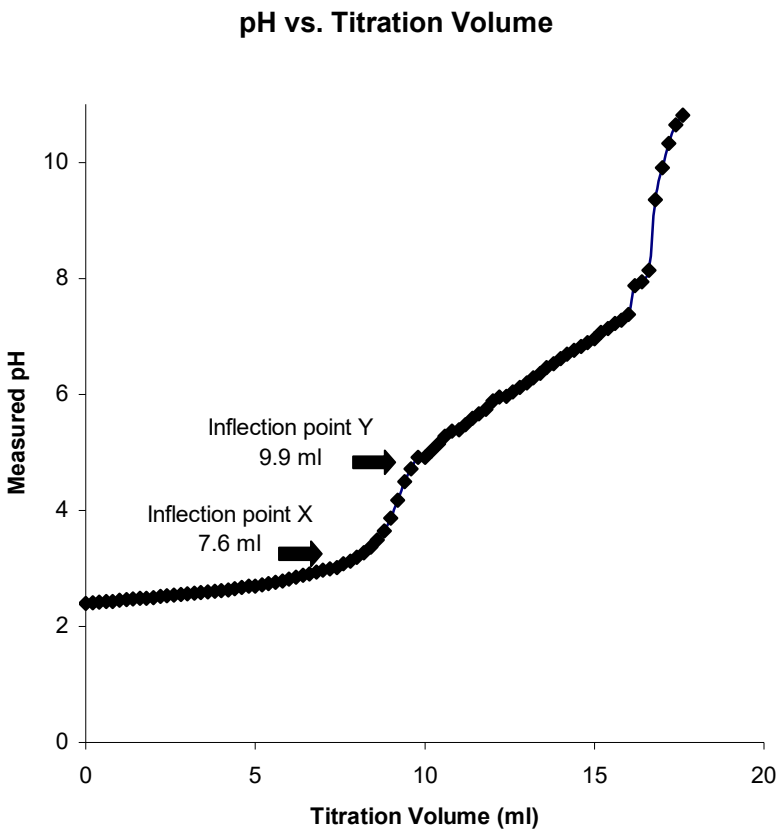

Figure 2.2. Example Titration Curve for determining the degree of deacetylation of chitosan materials. The inflection points were determined from the second derivative. For $\%NH_2 = 16.1*(y - x)/M$, x=7.6 ml, y=9.9 ml, M=0.5 grams. This example is one of the runs for 76% DDA. This method found the DDA to be 74.06%.

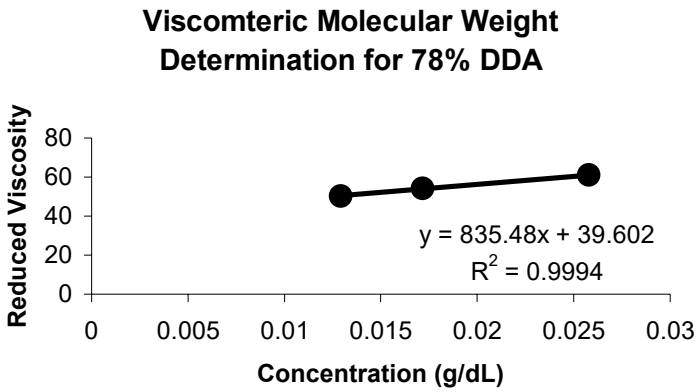

Figure 2.3. Example graph for Molecular Weight Determination. For 87% DDA chitosan sample. The intrinsic viscosity was found by calculating the y intercept of the trend line. This value is 39, [η]. [η] = $K'M^a$ where K=1.4x10-4 g/dl and a=0.83 T (Knaul, *et al*,1998) M=3.7 *10^6 Daltons.

Table 2.1. Material Characteristics

Vanson Lot Number	Vanson DDA (%)	Titration DDA (%)	Molecular Weight ($*10^6$ Daltons)	Ash content (%)	Contact Angle (degree)	Protein (Micrograms/cm^2)
VNS-389	76.1	76.21 ± 1.86	3.20	0.932 ± 0.026	85.6 ± 0.7	159.4 ± 10.4
03-ASDQ-122	78.7	78.85 ± 4.32	3.75	3.574 ± 0.006	72.5 ± 1.9	287.1 ± 44.8
02-CISC-0920	80.6	82.66 ± 1.87	8.22	0.239 ± 0.002	73.7 ± 2.3	101.0 ± 14.0
03-ASSQ-0212	87.7	85.85 ± 3.68	7.47	2.456 ± 0.019	89.7 ± 1.6	67.6 ± 17.5
00-CESC-0915	91.9	91.92 ± 2.67	1.99	0.766 ± 0.044	73.9 ± 0.7	142.1 ± 23.4
01-CESQ-1415	92.9	92.31 ± 3.72	7.52	0.524 ± 0.009	63.9 ± 0.5	2.4 ± 1.4
98-AECQ-0136	95.6	96.50 ± 3.23	2.43	0.408 ± 0.001	62.1 ± 1.1	110.5 ± 56.2

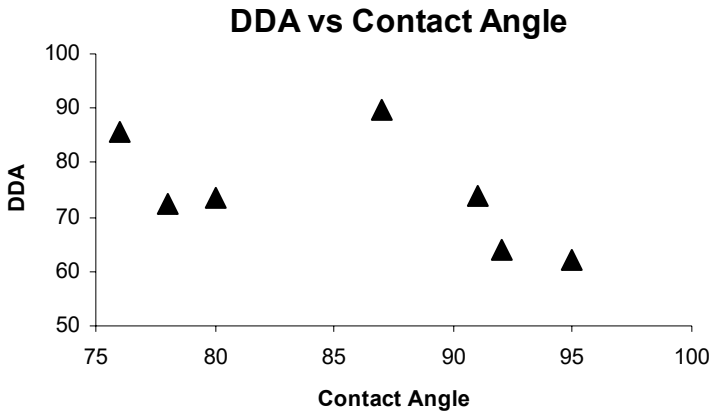

Figure 2.4. DDA vs. Contact Angle. Contact Angle decreases as the DDA of the chitosan films increases.

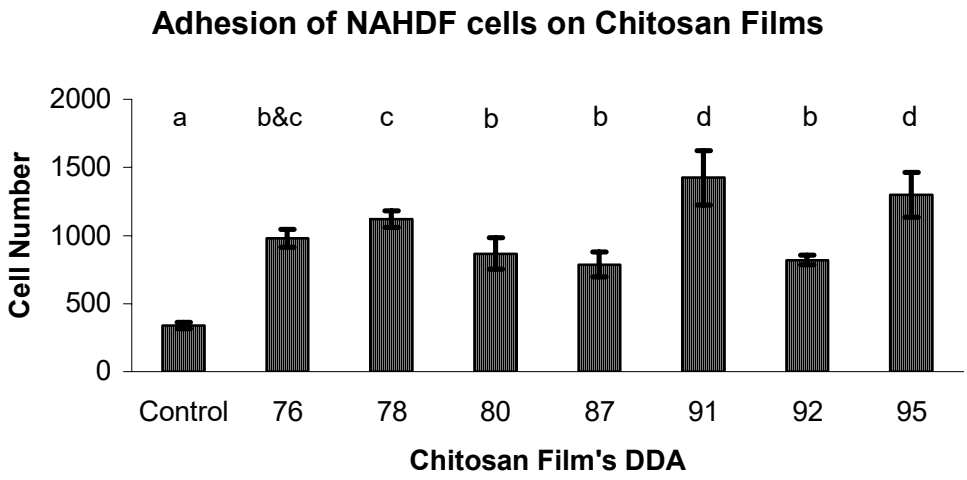

Figure 2.5. Adhesion of NAHDF cells on Chitosan Films. Examined after 30 minutes in serum-free medium exposed to the chitosan film. Columns with different superscripts are significantly different. $P<0.05$.

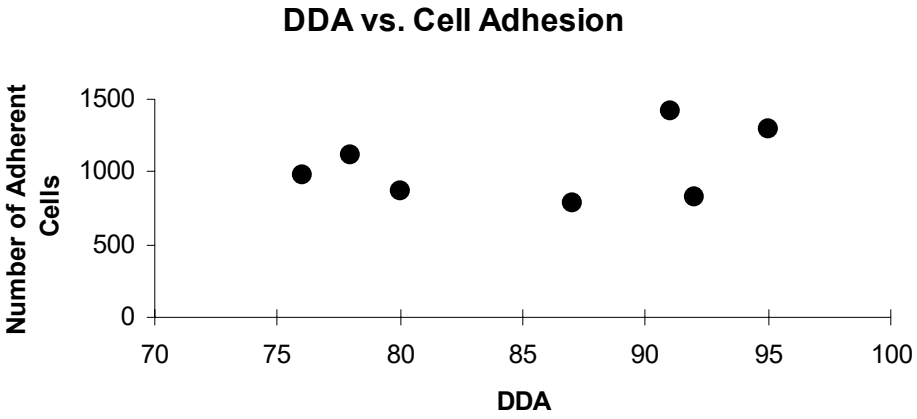

Figure 2.6. Scatter plot of the DDA of the test chitosan materials vs. the Number of Adherent Cells. Data does not indicate any relationship between adhesion and DDA of these test chitosan materials.

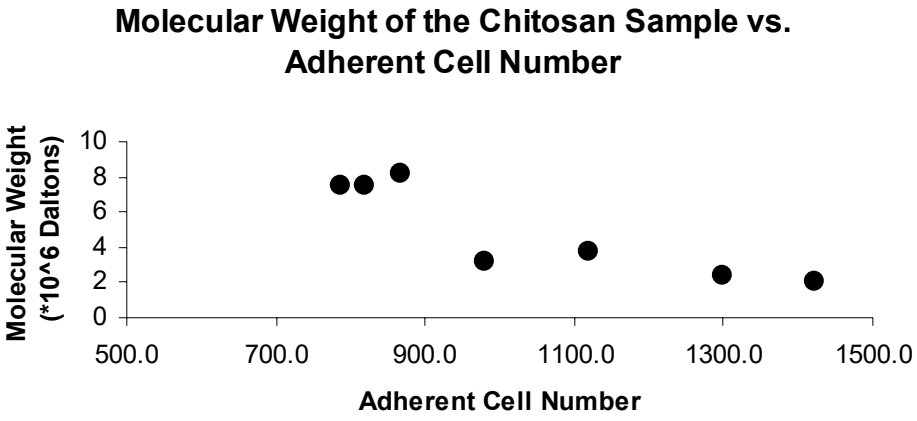

Figure 2.7. Molecular Weight of the Chitosan vs. Cell Adhesion. An inverse relationship between molecular weight and adherent cell number is observed. As molecular weight increases, adherent cell number decreases. A linear trend line including all points is provided by the equation: $y = -0.0097x + 15.029$ which has a corresponding $R^2 = 0.793$.

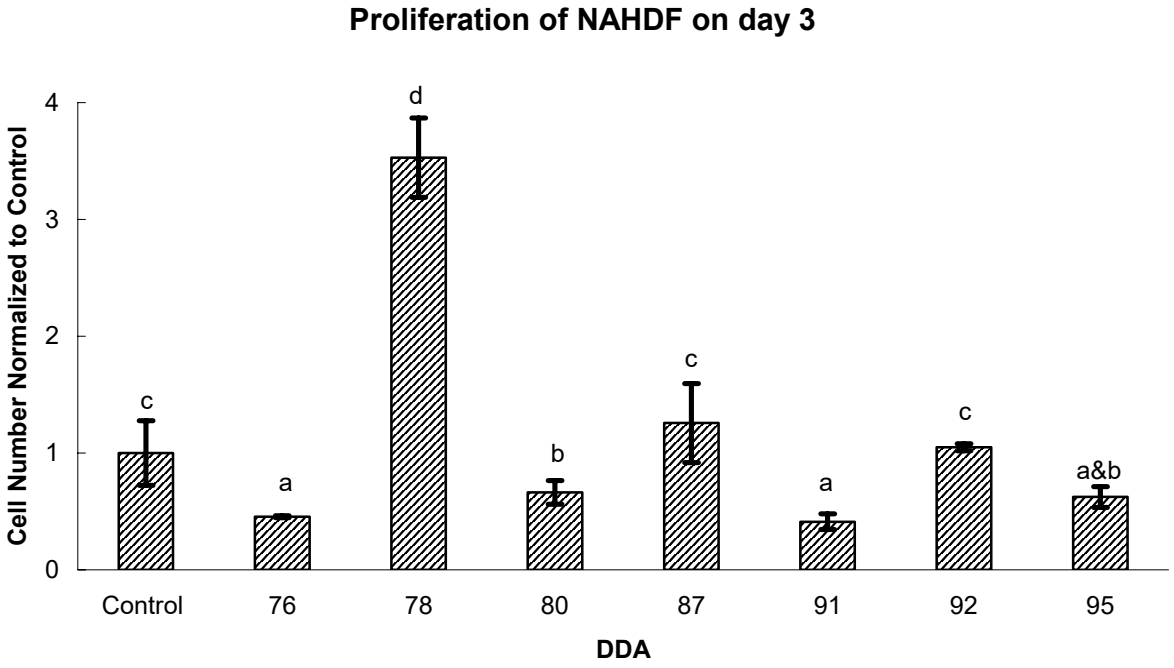

Figure 2.8. Proliferation of NAHDF on Chitosan Films. The cell number was assessed on the 3rd day of the study. Columns with different superscripts are significantly different. $P<0.05$.

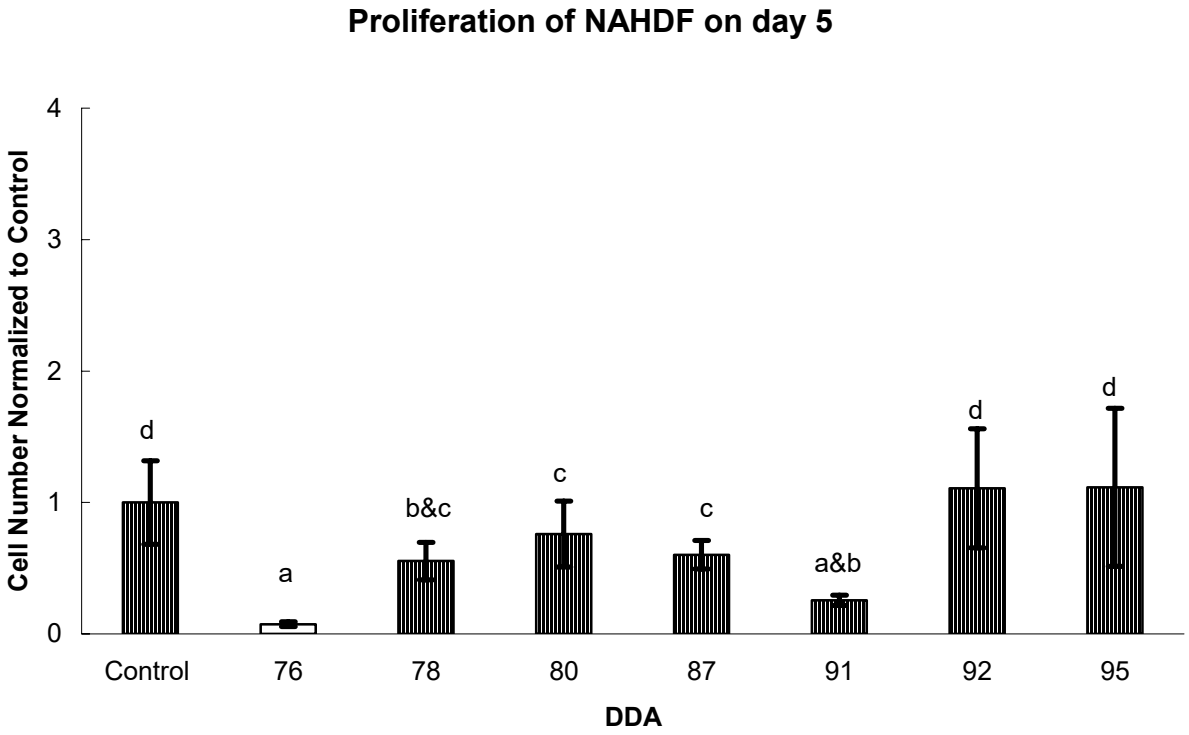

Figure 2.9. Proliferation of NAHDF on Chitosan Films. The cell number was assessed on the 5th day of the study. Columns with different superscripts are significantly different. $P<0.05$.

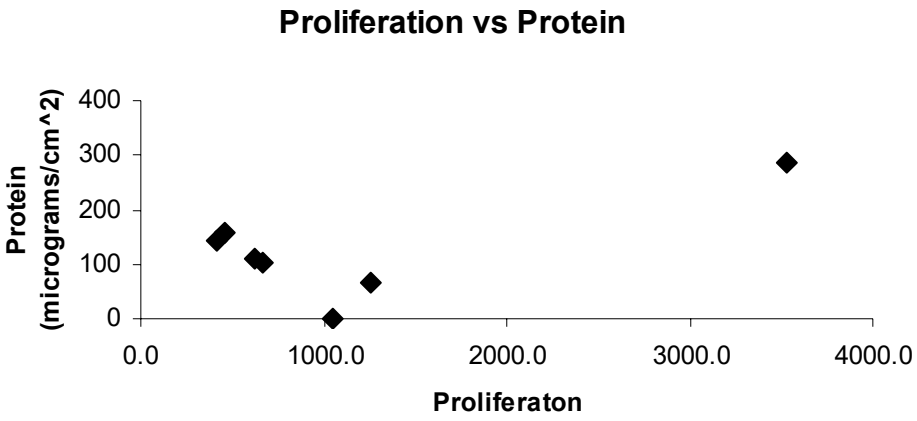

Figure 2.10. Proliferation vs Protein. With the exception of the 78% DDA chitosan sample, as the protein decreases, the proliferation of NADHF increases.

CHAPTER III

BONE RESPONSE

Abstract

Chitosan has shown great promise as a biomaterial in particular as a wound dressing material. Interest in chitosan for bone applications is growing. This study examines the *in vitro* responses of two bone cell lines, NHOst and UMR-106, to chitosan of known material characteristics. The aim of this study was to qualify cellular responses of bone cells with well characterized chitosan materials. To achieve this aim, chitosan films were seeded with bone cells and evaluated for adhesion and proliferation over the course of 5 days. Proliferation and adhesion varied greatly between the cell lines and also the different chitosans. For NHOst, 95 and 78% DDA films increased proliferation by more than 7 times that of the control material. But for UMR-106, films of 95 and 76 % DDA were comparable to control.

Introduction

Chitosan is emerging as a biomaterial. It has been shown to enhance wound healing rates (Biagini, *et al.,* 1991; Braye, *et al.,* 2001; Pruden, *et al.,* 1970; Ueno, *et al.,* 1999) and wound strength (Cho, *et al.,* 1999). Chitosan has similarities to extracellular matrix molecules such as hyaluronic acid (Muzzarelli, *et al.,* 1999) and heparin which

stabilize and activate chemokines and cytokines (Biagini and Muzzarelli, 1992, Denuziere, *et al.,* 1998; Kratz, *et al.,* 1997; Kratz, *et al.,* 1998). Its high cationic nature is reported to promote cell adhesion (Muzzarelli *et al.,* 1994; Muzzarelli *et al.,* 1988). Degradation products are simple oligosaccharides which may be processed by normal metabolic pathways down to CO_2 and water (Onishi and Machida, 1999). For these reasons, chitosan is a promising material for biomaterial applications.

The *in vitro* data indicates chitosan is a suitable material for osteogenic applications. Normal human osteoblast proliferation was increased when exposed to chitosan films of greater than 90% deacetylation (Lahiji, *et al.,* 2000). UMR-106 osteoblast cells exhibited greater growth and attachment on titanium coupons bonded with chitosan films composed of 91.2% deacetylated and 200,000 molecular weigh chitosan than the control titanium coupons (Bumgardner, *et al.,* 2003). Chitosan alone or as a coating has shown potential in osteogenic applications.

Chitosan has shown increased osteogenic activity in animal models. When chitosan was tested in an adult rabbit critical-size-defect model, a marked increase in calcification and bone union was noted over a 12-week period when compared to the untreated control. Osteogenic activity was noted, not only in the periosteal, cortical, and marrow elements as expected, but also from the surrounding soft tissues such as tendon and muscle in chitosan treated models (Borah, *et al.,* 1992). An 8 mm defect in a sheep's femur healed within 40 days when treated with chitosan, having a level of deacetylation greater than 99%, an average molecular weight of 200,000 and a modification to increase

cationicity. The untreated control showed no osteoblastic activity (Muzzarelli, *et al.,* 1994). Hidaka, *et al.* (1999) studied osteogenesis in rat calvaria treated with chitosans membranes of 65, 70, 80, 94, and 100% deacetylation. All samples showed signs of osteogenesis to varying degrees. One-week post implantation, samples of 65, 70, and 80% deacetylation showed an induction of neutrophil accumulation, slight inflammation with immature granulation tissue, and the most prominent signs of osteogensis. The 94% and 100% deacetylation samples showed mild inflammation surrounded by granulation tissue. Fibrous connective tissue and mild to moderate immune reactions were observed in samples of 60 and 70% deacetylated chitosan implants on weeks 4 and 8. At the same time, components from the 80% deacetylated chitosan implant remained. Macrophages containing cell debris were still present for the 80% deacetylated chitosan implant. For 90 and 94% deacetylated chitosan membranes, the membranes remained in tact and were encapsulated.

These studies suggest that the degree of deacetylation may play a role in the suitability of chitosan for osteo-applications. This is not surprising since the degree of deacetylation influences antimicrobial activity, degradation rate, immune reaction and mechanical properties such as strength and elongation (Zivanovic, *et al.,* 2004; Suh, *et al.,* 2000; Hidaka, *et al.,* 1999; Tomihata, *et al.,* 1997). This study aims to compare the known material characteristics of chitosan films to the cellular response elicited.

Materials and Methods

Materials

Chitosan

Chitosan powders of crab and shrimp origin were obtained from Vanson HaloSource (Redman, WA) through generous donations and purchase. Films were made by solution casting 1% chitosan dissolved in 0.2 M acetic acid (Sigma-Aldrich St. Louis, MO) in 96 well culture plates as described previously (Chapter 2). The films were then rinsed in copious amounts of phosphate buffered solution (PBS) and ethylene oxide gas sterilized.

Cells

Normal Human Osteoblast (NHOst; CC2528, Cambrex, Baltimore, MD) and UMR-106 (ATCC Manassas, VA) were maintained at 37°C in a 5% CO_2 atmosphere under sterile conditions. NHOst, human derived cell line of fully differentiated osteoblasts capable of making and depositing calcium phosphate mineral, were cultured in Osteoblast Medium (OGM; Cambrex, Baltimore, MD). The UMR-106 cells, a rat osteosarcoma cell line capable of making and depositing calcium phosphate mineral, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and

1.0 mM sodium pyruvate 10% fetal bovine serum and 1% antibiotic –antimycotic (Gibco/ Invitrogen Carlsbad, CA). NHOst cells were used between the second and fifth passage.

Material Characterization

Degree of deacetylation, viscometric molecular weight, ash content, water contact angle, protein and moisture content of films were determined and reported previously (Chapter 2).

Cell Culture Experiments

Adhesion and proliferation studies were performed using Promega CellTiter (Promega, Madison, WI) as previously reported (Chapter 2).

Statistical Analysis

Triplicate samples of each film were used in cell culture studies. Cell culture studies were repeated three times. Post-hoc multi-comparison tests using a F-protected Least Significant Differences were used to determine where statistical differences exist. Statistical differences were declared at $p<0.05$.

Results and Discussion

Material Characteristics

The DDA, molecular weight, ash content, protein, and contact angle for the tested chitosan are shown in Table 1. The DDAs obtained by titration were similar to those reported by Vanson HaloSource (Redman, WA). The molecular weight ranged form 8.22 $*10^6$ D for the 80% DDA chitosan to 1.99 $*10^6$ D for the 91% DDA chitosan. The ash content for all chitosan samples was above allowable levels for medical grade chitosan (<0.2%) according to the standards set by the manufacturer Dalwoo (Dalwoo, Seoul, Korea). The sample with the highest ash content, 78% DDA, had 3.57% ash. The lowest ash content sample, 80% DDA, had only 0.239% ash. The contact angles measured ranged from 87.7° for 80% DDA to 62.1° for 95% DDA. There was a general trend of contact angle decreasing as the DDA of the chitosan increased. Protein varied from a negligible concentration on the 92% DDA films to 287.1 micrograms/cm^2. With a few exceptions, molecular weight decreases with increasing DDA. This is expected due to the increased processing required to deacetylate the chitosan. Contact angle decreases as the DDA of the chitosan samples increases.

Adhesion

Adhesion of NHOst cells to chitosan films varied greatly among the different percent DDA chitosan materials studied (Figure 3.1). The majority of chitosan films, 78,

80, 87, 92, and 95% DDA, had a lower number of adhering cells than the control. Control and 91% DDA were statistically indistinguishable. 76% DDA increased the adhering cell number by 3 times that of the control. There is no general trend between the DDA and the adhesion of NHOst cells to chitosan films in this study. Other studies have reported a relationship between chitosan films DDA and adhesion for fibroblasts and keratinocytes (Chatelet *et al.,* 2001). Our study of fibroblasts (Chapter 2) did not find a correlation between adhesion and DDA. Instead, a relationship between molecular weight and adhesion was observed. Re-examining the data presented in Chatelet *et al.* (2001) a relationship between the molecular weight and adhesion was also present in this study. However, for the NHOst cells, a relationship between molecular weight or DDA and adhesion was not observed.

Adhesion for UMR-106 cells was increased above the control for most of the chitosan DDAs with the exception of 76 and 95% DDA (Figure 3.2). 78, 80, 87, 91, and 92% DDA increased the number of adhering cells above control, averaging 17.5% more attached cells than the control. There may be a trend between molecular weight and adhesion for the UMR-106 cells (Figure 3.3).

Proliferation

Proliferation of NHOst on day 3 for all chitosan films was greater than control, as others have shown (Borah, *et al.,* 1992; Lahiji, *et al.,* 2000; Muzzarelli, *et al.,* 1994) (Figure 3.4). Films composed of 76, 80, 87, 91, and 92% DDA increased proliferation

an average of 3.5 times control. An increase of more than 7 times control was seen for NHOst cells on films of 95 and 78% DDA.

At the 5 day point, all chitosan films supported more proliferation of NHOst cells than the control (Figure 3.4). The first statistical group comprised of 76, 80, 87, 91, and 95% DDA films increased proliferation levels 9 times control. An amazing increase in cell number 16 times control was seen in the last statistical group comprised of 78 and 92% DDA.

Proliferation for UMR-106 cells on chitosan films was very different from the NHOst cells (Figure 3.5). On day 3, only three chitosan films supported more growth than the control. Films of 87 and 91% DDA supported an average of 23% more growth than the control. Films composed of 78% DDA supported approximately 30% of the growth seen on the control. Films of 95, 92, and 76% DDA were comparable to the control.

By day 5, the control's proliferation levels were higher than the proliferation levels observed for every chitosan film. The relative decrease between the control and chitosan films may be due to overgrowth in these wells. Since the cells were not performing well above the control level, this is not thought to be the case. The best performing film was 76% DDA which supported slightly less than 40% of the growth seen on the control. Both 78 and 92% supported less than 80% of the growth observed on the control.

The difference in the cell lines, normal and cancerous, may be the cause of the differences in proliferation seen between the two lines. Chitosan is used to target cancer cells due to its increased incorporation into the cell over normal cells (Nsereko and Amiji, 2002; Chen, *et al.,* 2002). From the earlier findings, the prediction would be that chitosan should support cancer cell line growth as much as a normal cell line growth. However, this result was not observed in this study. No *in vitro* studies of cancer bone line proliferation with exposure to chitosan are known to the author. This study highlights the proper choice of *in vitro* model cells.

Comparison of Material Characteristics and *In Vitro* Responses

NHOst

With the exception of 78% DDA, the proliferation levels of the NHOst increases with the DDA (Figure 6). There is no strong general trend between cell adhesion and proliferation. If the proliferation levels and adhesion were ranked, there is a suggestion of an inverse relationship for some of the samples. While 92 and 95% DDA promoted little adhesion, they supported elevated proliferation levels. Films of 76 and 91% DDA supported more adhesion than the control and ranked low in cell proliferation when compared to other chitosan films.

UMR-106

In general, the higher DDA films tended to support more cell proliferation (Figure 3.7). There is no relationship between cell adhesion and proliferation. But, cell adhesion was increased statistically above control only for the middle DDA, 87% DDA. Adhesion statistically peaks at the central DDA level, 87% DDA (Figure 3.2).

Other material characteristics do not seem related to proliferation and adhesion, however there are some interesting correlations. Films composed of 78% DDA chitosan increases proliferation the most for NHOst, just as with the fibroblasts (Chapter 2), is also the highest in ash content (Table 3.1). However, there is no trend throughout the data. Neither protein nor contact angle display a general trend with proliferation or adhesion behavior for either cell line.

Conclusion

Many material characteristics contribute to a biomaterial's suitability in particular applications. The degree of deacetylation influences proliferation in both types of bone cells studied. Adhesion should play a role for *in vivo* application suitability. But, a relationship between adhesion and proliferation was not observed in this study. Therefore it may be concluded that other characteristics not addressed in this work must play a role in predicting the proliferation and adhesion of cell lines to chitosan films.

Table 3.1. Material Characteristics

Vanson Lot Number	Vanson DDA (%)	Titration DDA (%)	Molecular Weight (*10^6 Daltons)	Ash content (%)	Contact Angle (degree)	Protein (Micrograms/cm^2)
VNS-389	76.1	76.21 ± 1.86	3.20	0.932 ± 0.026	85.6 ± 0.7	159.4 ± 10.4
03-ASDQ-122	78.7	78.85 ± 4.32	3.75	3.574 ± 0.006	72.5 ± 1.9	287.1 ± 44.8
02-CISC-0920	80.6	82.66 ± 1.87	8.22	0.239 ± 0.002	73.7 ± 2.3	101.0 ± 14.0
03-ASSQ-0212	87.7	85.85 ± 3.68	7.47	2.456 ± 0.019	89.7 ± 1.6	67.6 ± 17.5
00-CESC-0915	91.9	91.92 ± 2.67	1.99	0.766 ± 0.044	73.9 ± 0.7	142.1 ± 23.4
01-CESQ-1415	92.9	92.31 ± 3.72	7.52	0.524 ± 0.009	63.9 ± 0.5	2.4 ± 1.4
98-AECQ-0136	95.6	96.50 ± 3.23	2.43	0.408 ± 0.001	62.1 ± 1.1	110.5 ± 56.2

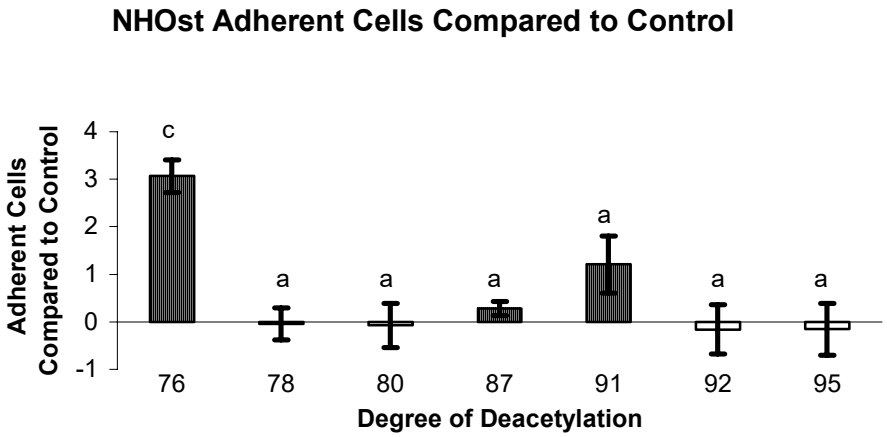

Figure 3.1. Adherent NHOst Cells on Chitosan Films. Films examined after 30 minutes of exposure to the films in serum free media. Statistical Groups are: Control- b, 76- c, 78- a, 80- a, 87- a, 91- b, 92- a, 95- a.

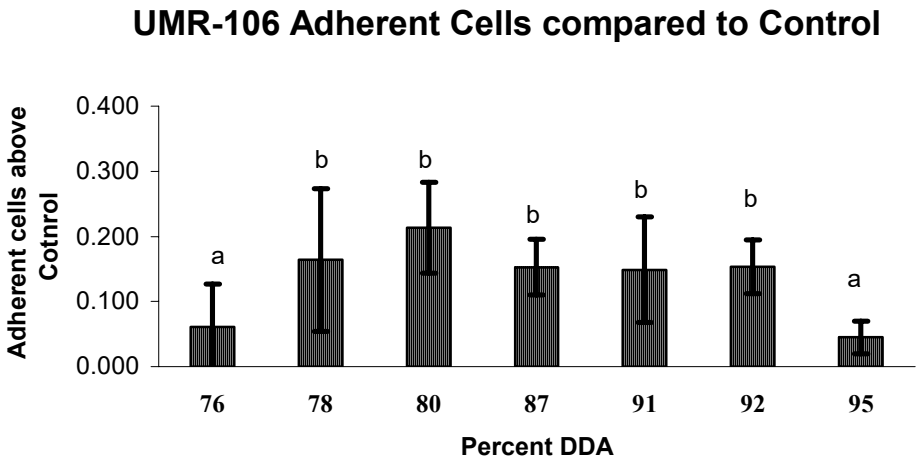

Figure 3.2. UMR-106 Adherent Cells. Films examined after 30 minutes of exposure to chitosan films in serum free media. Statistical groups are: Control- a, 76- a, 78- b, 80- b, 87- b, 91- b, 92- b, 95- a.

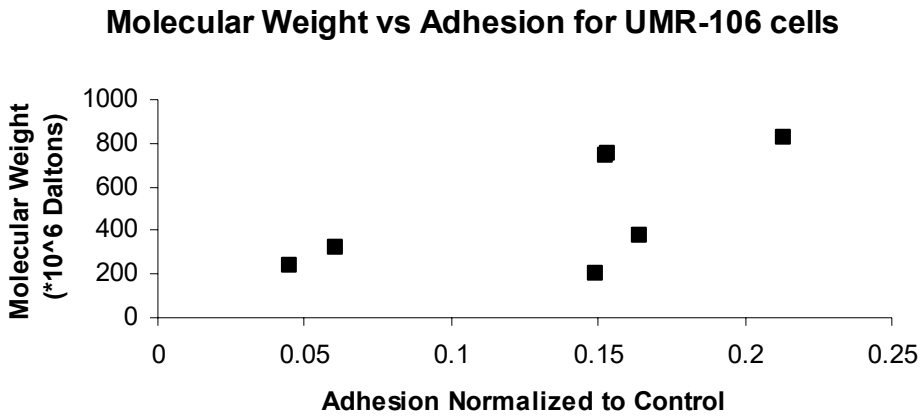

Figure 3.3. Molecular Weight vs. Adhesion for UMR-106 cells. As molecular weight increases, the adhesion of UMR-106 Cells to the chitosan films increases.

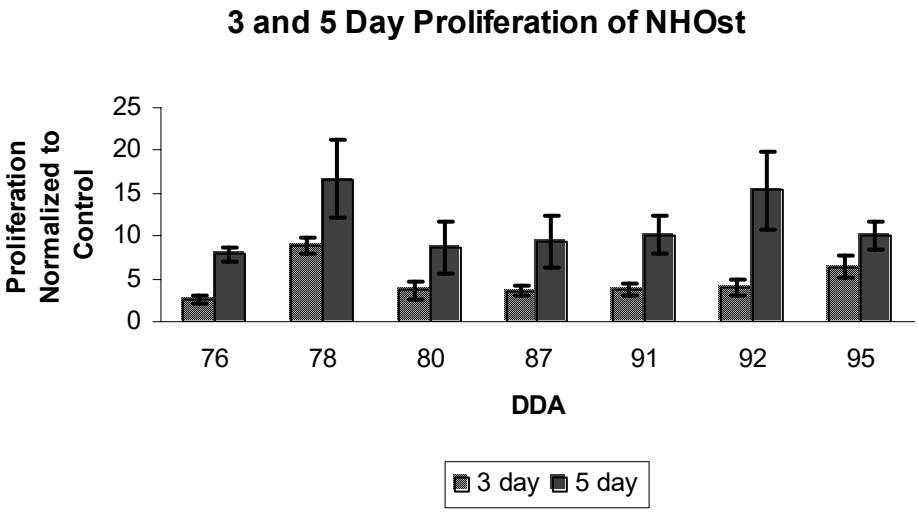

Figure 3.4. Proliferation of NHOst cells on Chitosan Films of varying DDA. The proliferation of NHOst cells on chitosan films were assessed on day three and five by a MTS assay. The statistical groups for 3 day: Control- c&d, 76- a&c, 78- a, 80- b, 87- e, 91- e, 92- c&d, and 95- d&e. The statistical groups for 5 day: Control- b, 76- c, 78- a, 80- a, 87- a, 91- b, 92- a, and 95-a.

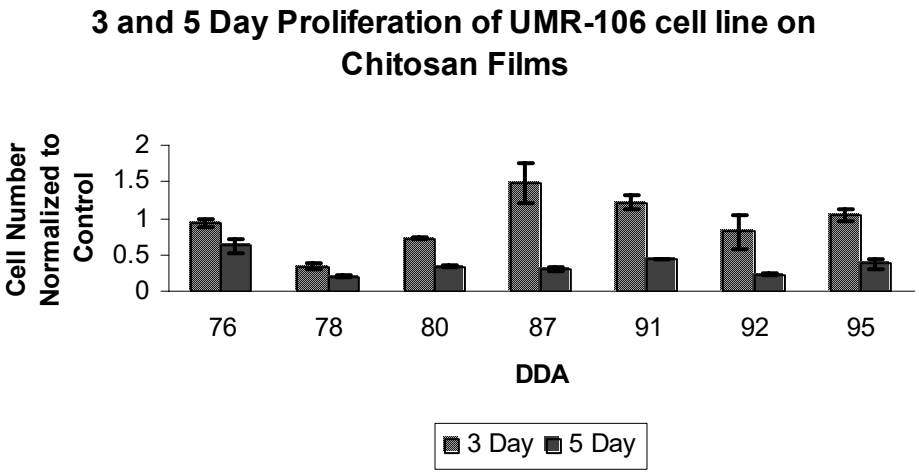

Figure 3.5. Proliferation of UMR-106 cell line on Chitosan Films. Proliferation was assessed on day three and day five by an MTS assay. Statistical groups for 3 day: Control- c&d, 76- b&c, 78- a, 80- b, 87- e, 91- e, 92- c&d, 95- d&e. Statistical groups for 5 day: Control- e, 76- d, 78- a, 80- b, 87- b, 91- c, 92- a, and 95- b&c.

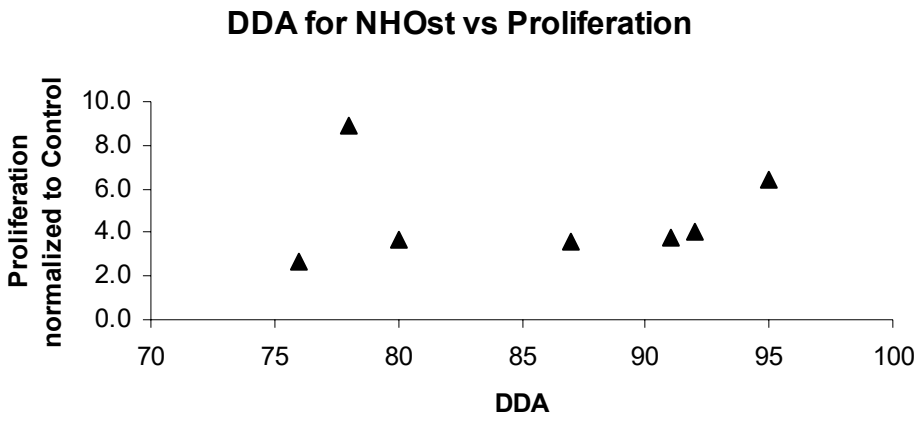

Figure 3.6. Proliferation vs. DDA for NHOst cells. As the DDA of the chitosan films increases, the proliferation levels of NHOst increases. The one exception is 78% DDA which has an extremely high ash content compared to the other chitosan samples. When 78% DDA is excluded from the data, the linear trend line would be $y = 68.387x - 3680.7$ with an $R^2 = 0.6832$

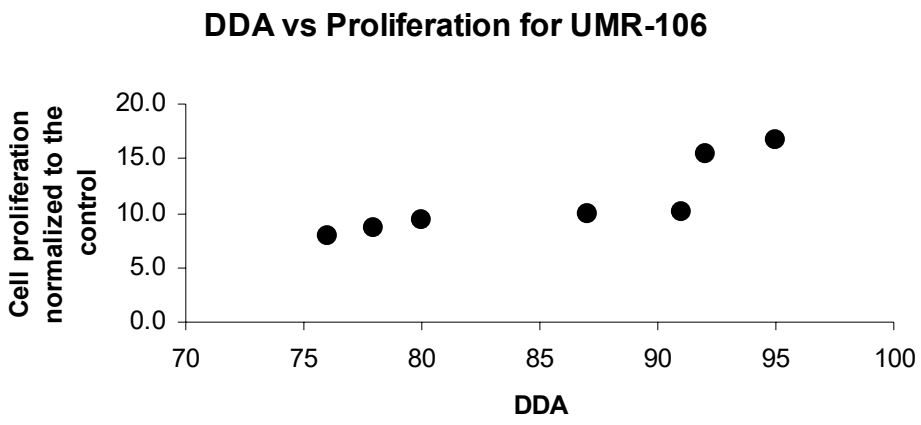

Figure 3.7. DDA vs Proliferation on day 3 for UMR-106. As the chitosan films DDA increases, the proliferation of the UMR-106 increases in a linear manner. The linear trend line is y = 0.3885x - 22.117 with an $R^2 = 0.7203$

CHAPTER IV

MACROPHAGE RESPONSE

Abstract

Chitosan has been shown to increase immune cell number and cytokine and chemokine production in a wound environment. The aim of this study was to qualify the reaction of a TIB-71 cells reaction to chitosan exposure. Proliferation, interleukin- 1β (IL-1 β) production, and nitric oxide (NO) levels were examined. Proliferation over 3 days was increased from 30 to 200% above control. IL-1β production was varied. An increase of 40% greater than control was observed as well as a decrease of 70% less than control. NO production was comparable to the control. Additional studies are warranted.

Introduction

Powdered cartilage from sharks and exoskeletons of crustaceans has been employed to heal wounds in ancient Japan (Khor, 2001). This material was largely forgotten until the later half of the twentieth-century (Prudden, *et al.,* 1970), when a renewed interest in the medicinal use of natural materials began. The active biological component from powdered cartilage and exoskeleton is chitin. While the exact mechanism by which wound healing is promoted has not yet been determined, chitin and its derivatives have shown promise in multiple biomedical applications such as wound

repair, tissue engineering, and drug delivery (Khor, 2001; Kumar, 2000; Suh and Matthew, 2000).

The structure of chitin and chitosan is shown in Figure 4.1. Either an acetamido group (-NH-$COCH_3$) or an amino group (-NH_2) is attached to the C-2 carbon of the glucopyran ring. When more than 50% of the C-2 attachment is an amino group, the material is termed chitosan. Ideally, chitin is a linear polysaccharide of β-(1-4)-2 acetamido-2-deoxy-D-glucopyranose where all residues are comprised entirely of the acetamido group -NH-COCH3. This is termed fully acetylated. Chitosan is a linear polymer of β-(1-4)-2 acetamido-2-deoxy-D-glucopyranose where all the residues are comprised entirely of the amino group -NH_2. This is termed fully deacetylated.

Chitosan has yet to gain full acceptance as a biomaterial. Scientific studies have reported conflicting and/or inconsistent results. For example, chitosan has been cited as inhibiting fibrosis (Bartone, *et al.,* 1988), while others have noted fibrous tissue formation with the use of chitosan materials (Hidaka, *et al.,* 1999; Lu, *et al.,* 1999). The differences in the biological responses observed could be attributed to differences in the degree of deacetylation, molecular weight, source, and preparation of the samples in the different studies (Chatelet, *et al.,* 2001; Chung, *et al.,* 1994; Nunthanid, *et al.,* 2001). Very little attention has been given to the interaction of the immune system and chitosan in the evaluation of chitosan as a biomaterial.

Chitosan has been shown to increase immune cell number and cytokine and chemokine production in a wound environment. Chitosan, which is a polysaccharide, has been shown to increase the number of leukocytes and macrophages and thus enhance the wound healing process (Minami, *et al.,* 1993). The initial increase in immune response is also accompanied by an increase in growth factors such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), colony stimulating factor (CSF), tumor necrotic factor (TNF), and platelet derived growth factor (PDGF) (Muzzarelli, *et al.,* 1999; Mori, *et al.,* 1997; Nishimura, *et al.,* 1986; Ueno, *et al.,* 2001). There have been varying responses of immune cells to chitosan materials. For instance, Nishimura, *et al,* (1986) found that TNF was not activated with exposure to 70% deacetylated chitosan for L929 mouse fibroblasts. Yet, Mori, *et al.* (1997) observed an increase in production of TNF with the exposure of HUVECS to of 80% deacetylation chitosan. Chitosan has also been reported to increase NO production *in vitro* (Jeong, *et al.,* 2000). Therefore the aim of this study was to qualify the reactions of macrophage cells to chitosan exposure. The levels of proliferation, IL1-β, and NO production were studied.

Materials and Methods

Materials

Chitosans

Chitosan powders of crab and shrimp origin were obtained from Vanson HaloSource (Redman, WA) through generous donations and purchase. Degree of deacetylation, viscometric molecular weight, ash content, water contact angle, and moisture content of films were determined and reported previously (Chapter 2). Films were solution cast in 96 well plates from 1% chitosan in 0.2 M acetic acid solutions as previously described (Chapter 2). The films were rinsed in copious amounts of phosphate buffered solution (PBS) and ethylene oxide gas sterilized for use in cell culture tests.

Cells

TIB-71, murine alveolar macrophages (RAW 264.7; ATCC, Manassas, VA) were maintained at 37°C in a 5% CO_2 atmosphere under sterile conditions. The TIB-71 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine, 1.5 g/L sodium bicarbonate and 1% antibiotic –antimycotic (Gibco/ Invitrogen Carlsbad, CA).

Cell Culture Experiments

Proliferation studies were preformed using Promega CellTiter (Promega, Madison, WI) as previously reported (Chapter 2).

IL-1 β Production

Culture supernatant from the 3 day proliferation study was added to the Endogen Mouse IL-1β Colorimetric ELISA kit (Endogen, Rockford, IL). The manufacture's instructions were followed. A standard curve was established at the same time the samples were run. The plate was then assessed by reading the plate at 450 nm and 540 nm on a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). The microplate data was then converted to IL-1β (pg/mL) concentrations. Triplicate repetitive readings from each of three experiments were used to establish the IL-1β concentrations. IL-1β concentrations released normalized to cell number from 3 day growth tests

NO Production

Culture supernatant from the 3 day proliferation study was added to a Griess reagent kit (Molecular Probes, Eugene, OR) per manufacture's instructions. The plate was read on the microplate reader at 548 nm. Using a standard curve, the microplate readings were converted to NO concentrations (μM). The NO level was determined using data from three experiments with triplicate samples and normalized to cell number.

Statistical Analysis

Triplicate samples of each film were used in cell culture studies. Cell culture studies were repeated three times. Post-hoc multi-comparison tests using a F-protected Least Significant Difference were used to determine where statistical differences exist. Statistical differences were declared at $p<0.05$.

Results and Discussion

Material Characteristics

The DDA, molecular weight, ash content, contact angle, and protein for the tested chitosan are shown in Table 4.1. The DDAs obtained by titration were similar to those reported by Vanson HaloSource (Redman, WA). The molecular weight ranged form 8.22 $*10^6$ D for the 80% DDA chitosan to 1.99 $*10^6$ D for the 91% DDA chitosan. The ash content for all chitosan samples was above allowable levels for medical grade chitosan (<0.2%). The sample with the highest ash content, 78% DDA, had 3.57% ash. The lowest ash content sample, 80% DDA, had only 0.239% ash. The contact angles measured ranged from 87.7° for 80% DDA to 62.1° for 95% DDA. There was a general trend of contact angle decreasing as the DDA of the chitosan increased. Protein varied from a negligible concentration on the 92% DDA films to 287.1 micrograms/cm^2. With a few exceptions, molecular weight decreases with increasing DDA. This is expected due

to the increased processing required to deacetylate the chitosan. Contact angle decreases as the DDA of the chitosan samples increases.

Proliferation

For the 3-day proliferation, two films were comparable to the control (Figure 4.2). These films were 76 and 91% DDA. Exposure to films of 78% DDA increased the proliferation levels to 2 fold greater than control. The other films increased proliferation between 30 and 40% above control.

For 5-day proliferation, most films were comparable to control (Figure 4.2). Films of 87, 95, and 92% DDA increased proliferation levels approximately 35% above the control. The greatest increase of 75% above control was seen on the 78% DDA film.

IL-1β Production

IL-1β production varied among the films (Figure 4.3). While most films did not alter IL-1β concentrations from control levels, exposure to 78, 91, and 95% DDA films resulted in concentrations of IL-1β that were statistically different from control. Interleukin-1β production by TIB-71 cells was greatly decreased on the 78% DDA films. The production levels were 70% less than those observed in the control. For 91 and 95%DDA, the production of IL-1β increased more than 40% above the control production level.

NO Levels

There was little variation in NO levels between the control and chitosan films (Figure 4.4). Both of the statistical groups include the control. Chitosan did not modulate NO levels in this experimental design.

Material Properties

Many material properties did correlate with cellular responses. The DDA correlated to the levels of IL-1β (Figure 4.5). As the DDA of the films increased, the amount of IL-1β produced by each cell increased. No trend was apparent between molecular weight and proliferation, IL-1β or NO production. Ash content correlated to IL-1β levels (Figure 4.6). And, again, the chitosan sample with the highest amount of ash content produced the greatest cell proliferation (Chapter 2 and 3). With the exception of 78% DDA, there was a trend that as protein decreased, TIB-71 cell proliferation increased (Figure 4.7). No relationship was observed with either of the material characteristics of swelling index or contact angle to the cellular responses of proliferation, IL-1β, or NO levels. The cellular response of IL-1β predicted proliferation (Figure 4.8). As the levels of IL-1β produced by each cell increased, proliferation decreased.

Conclusion

Films comprised of 78% DDA chitosan supported the greatest increase in TIB-71 proliferation of the chitosan films studied. This is the chitosan sample with the highest ash content. There is no direct correlation between proliferation and DDA. As DDA increased, the production of IL-1β by each cell increased. An increase in the IL-1β production corresponded to a decrease in proliferation. Surface protein also influenced proliferation. As surface protein decreased, proliferation increased.

Additional investigations with chitosan activated by lipopolysaccharide (LPS) (Feng, *et al.,* 2004) or primed first by r IFN-γ (Jeong, *et al.,* 2000) may reveal much different data than reported in this study. This is hinted at by an earlier study which found chitosan did increase NO production *in vivo.* Yet, an increase was not observed in unactivated macrophage cells studied *in vitro* (Peluso, *et al.,* 1994). Chitosan has been shown to enhance cytokine production by inflamed or activated macrophages (Porporatto, *et al.,* 2003); but, chitosan has also been shown to reduce production of the cytokine IL1-β in LPS activated cells (Chu, *et al.,* 2003). Repeating this experiment with activated cells may aid in answering the questions regarding macrophages' role in chitosan enhanced wound healing.

Interestingly, chitosan has done extremely well in chronic wounds (Allan, *et al.,* 1984; Naseema *et al.,* 1995; Prudden, *et al.,* 1970). Recreating a chronic wound environment *in vitro* may aid in evaluating the mechanisms by which chitosan produces the reported increased wound healing.

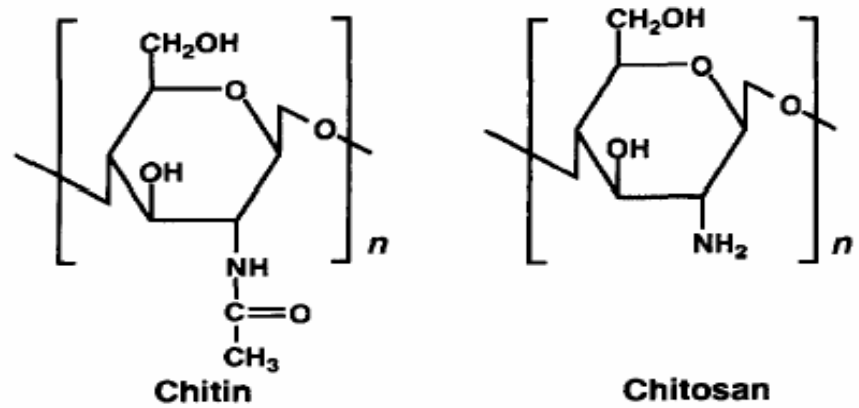

Figure 4.1. The chemical structure of Chitin and Chitosan. When more than 50% of the acetyl group, -CO-CH3, are removed from the chitin polysaccharide molecule, the molecule is referred to chitosan.

Table 4.1. Material Characteristics

Vanson Lot Number	Vanson DDA (%)	Titration DDA (%)	Molecular Weight (*10^6 Daltons)	Ash content (%)	Contact Angle (degree)	Protein (Micrograms/cm^2)
VNS-389	76.1	76.21 ± 1.86	3.20	0.932 ± 0.026	85.6 ± 0.7	159.4 ± 10.4
03-ASDQ-122	78.7	78.85 ± 4.32	3.75	3.574 ± 0.006	72.5 ± 1.9	287.1 ± 44.8
02-CISC-0920	80.6	82.66 ± 1.87	8.22	0.239 ± 0.002	73.7 ± 2.3	101.0 ± 14.0
03-ASSQ-0212	87.7	85.85 ± 3.68	7.47	2.456 ± 0.019	89.7 ± 1.6	67.6 ± 17.5
00-CESC-0915	91.9	91.92 ± 2.67	1.99	0.766 ± 0.044	73.9 ± 0.7	142.1 ± 23.4
01-CESQ-1415	92.9	92.31 ± 3.72	7.52	0.524 ± 0.009	63.9 ± 0.5	2.4 ± 1.4
98-AECQ-0136	95.6	96.50 ± 3.23	2.43	0.408 ± 0.001	62.1 ± 1.1	110.5 ± 56.2

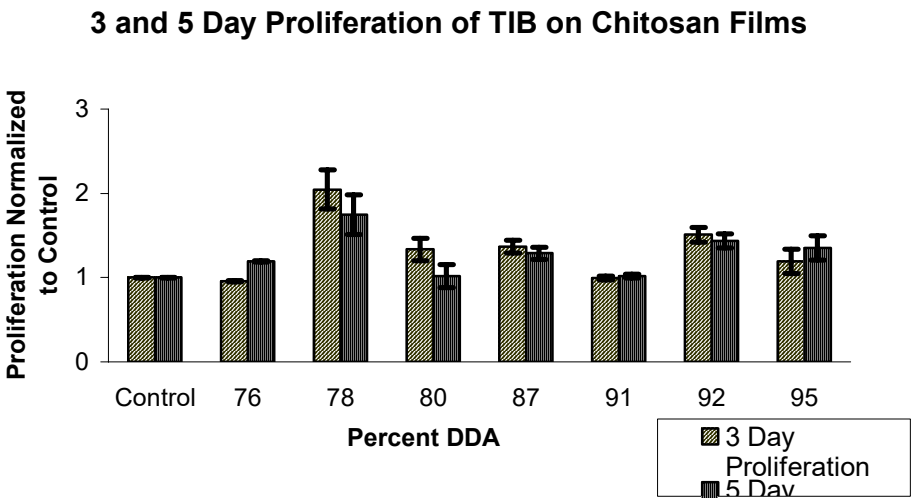

Figure 4.2. Proliferation of TIB-71 on Chitosan Films. Proliferation was assessed on days three and five by a MTS assy. Statistical groups: for 3 day: control- a, 76- a, 78- d, 80- c, 87- c, 91- a, 92- b&c, 95- d. For 5 day: Control- a, 76- a, 78- c, 80- a, 87- a&b, 91- a, 92- b, 95- b.

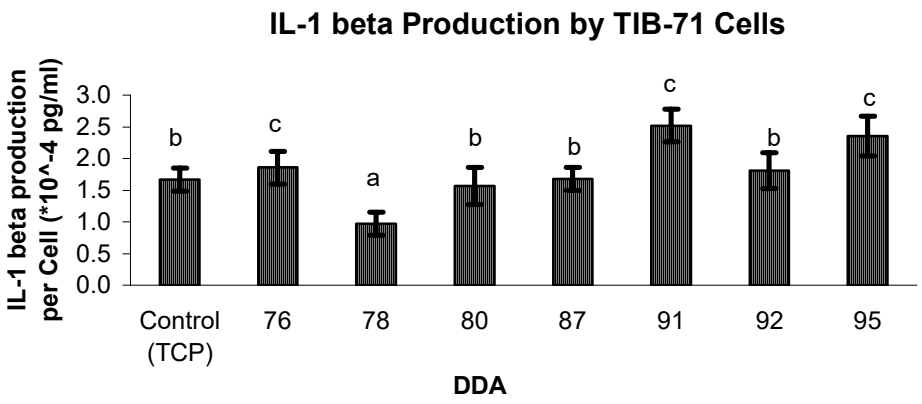

Figure 4.3. IL-1β levels produced by TIB-71 Cells. The cell supernatant was tested by an ELISA. The IL-1β concentration was normalized to the amount produced per cell. Statistical groups: Control- b, 76- c, 78- a, 80- b, 87- b, 91- c, 92-b, 95- c.

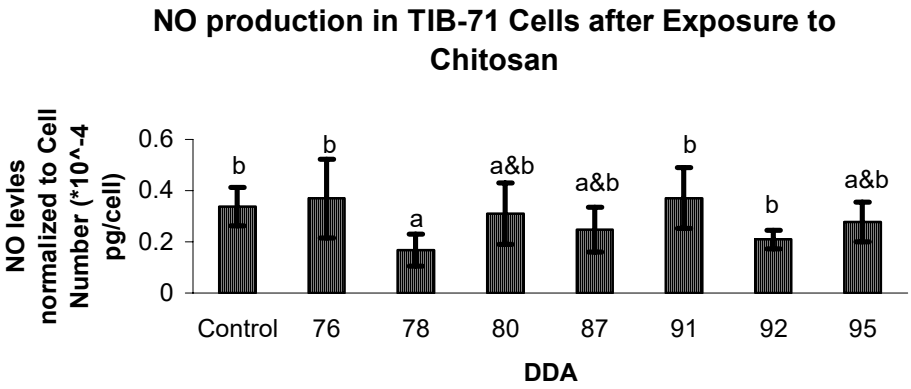

Figure 4.4. NO production in TIB-71 cells after Exposure to Chitosan. The cell supernatant was tested by the Griess method. The NO concentration was normalized to the amount produced per cell. Statistical groups: Control- b, 76- b, 78- a, 80- a&b, 87- a&b, 91- b, 92- b, 95- a&b.

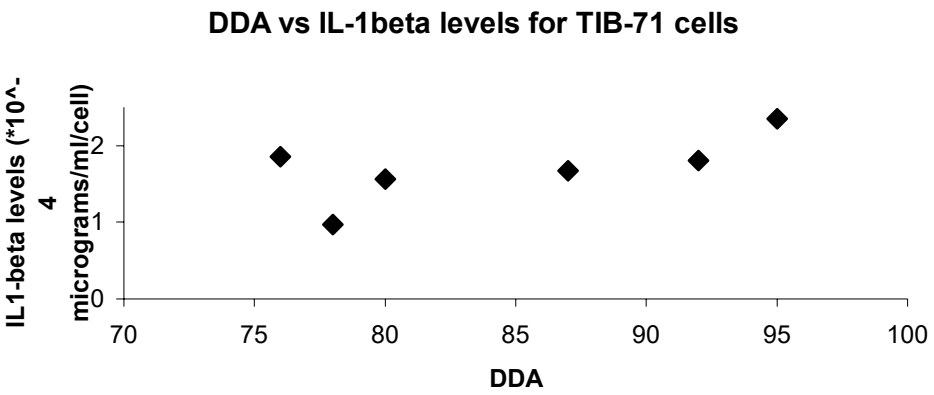

Figure 4.5. DDA vs IL-1beta levels for TIB-71 cells. As the DDA of the chitosan films increases, the TIB-71 cells increase their IL-1β production. A linear trend line would be represented by y = 5E-06x - 0.0002 with $R^2 = 0.4739$.

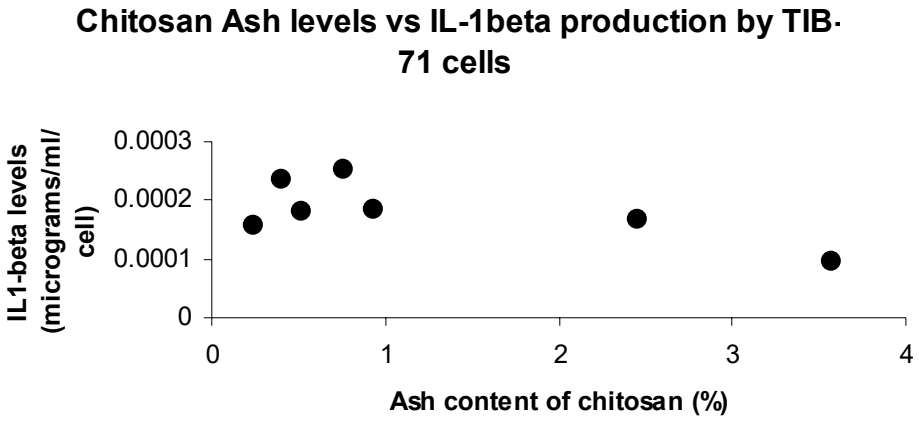

Figure 4.6. Chitosan Ash levels vs IL-1beta production by TIB-71 cells. All the chitosan of 1% ash content or less cluster around the 0.002 level of IL-1β. As the ash content of the chitosan increases, the IL-1β production by the TIB-71 cells decreases.

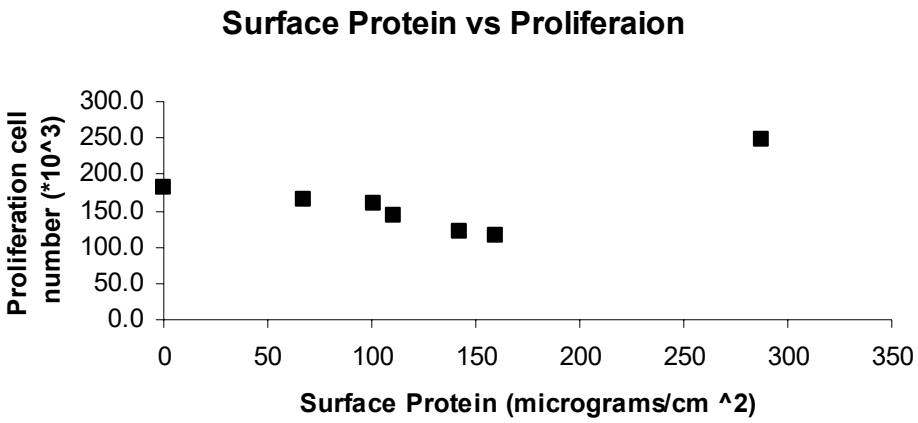

Figure 4.7. Surface Protein vs Proliferation. With the exception of the 78% DDA chitosan film, the proliferation level of the TIB-71 cells increases as the surface protein of the films decreases. Excluding 78% DDA, a linear trend line would be represented by y = -0.432x + 189.6 with R^2 = 0.8836.

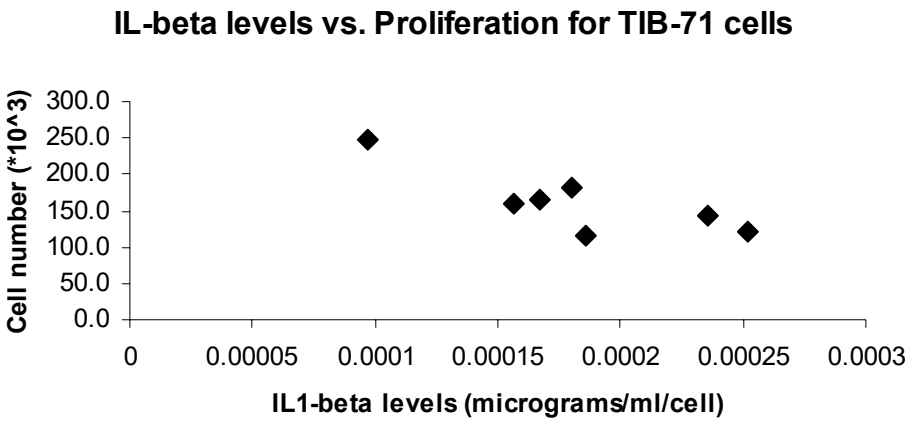

Figure 4.8. IL-beta levels vs. Proliferation for TIB-71 cells. The IL-1β production by the TIB-71 cells predicted the cell number at the three day point. The lower the IL-1β levels, the higher the cell number. A linear trend line would be y = -712676x + 291.66 with R^2=0.6793.

CHAPTER V

SUMMARY

Summary

Chitosan must be examined as an implant material and for its possible role in tissue healing. Implant materials should try to avoid or minimize an inflammatory response. The biological disease of arthritis demonstrates how activated immune cells can destroy the body's tissue resulting in a deleterious condition. Chronic inflammation has been associated with non-healing wounds. Neither of these situations is desired in an implant environment. High levels of most of the known cytokines and chemokines is not desired since they have been found to contribute to chronic/severe inflammatory responses. Yet, low levels can also result in poor healing. Ideally, chitosan would not provoke the inflammation responses seen with many current implants (Uo, *et al.,* 2001). This inflammation response can cause implant failure (Bauer and Schils, 1999). But, it would stabilize the good cytokines and chemokines that aid structurally organized, functional tissue wound healing as it is credited to do in bone and skin applications (Mi, *et al.*, 2000; Muzzarelli, *et al.*, 1998).

Chitosan's promise of scarless healing means more than a beautiful outcome. Fibrous tissue impairs the function of the tissue whether the tissue in question is skin,

bone, cartilage, or muscle. Examining fibroblasts' role in tissue repair for the two areas studies, soft tissue and bone will illuminate this point. Fibroblast initial activity in the wound bed is a support role providing structure for the initial matrix and chemokines and cytokines to produce a mature tissue. A prolonged proliferation of fibroblast leads to scarring. Fibroblasts are needed only in a support role and should not be the dominate cell type in the resulting tissue. An initial establishment of the matrix for the tissue and the production of chemokines and cytokines which call in the appropriate cells and signal appropriate proliferation and angiogenesis are desired for proper wound formation. Yet, if the fibroblast cells continue to grow, scars form. This scarring often results in non-functional tissue composed of cells often incorrectly oriented to provide maximum strength. Even in only a protective skin function, scar tissue can fail. With time, scars revise. The revised scar has decreased strength that can lead to re-injury if the demands on the tissue become too great. Scars are not only aesthetically unpleasing, but ultimately non-functional. Implants need to address the body's tendency to form these non-functioning scared areas. Ideally, the chitosan chosen would not support over proliferation of fibroblast. Instead, it would increase the much needed support role of chemokine and cytokine production and matrix formation. It is theorized that the correctly chosen chitosan does do this. The early reports of chitosans glowing success feature chronic wounds which have been documented to be lacking in chemokines and cytokines. A handful of *in vitro* studies have shown chitosan to increase chemokine and cytokine production. Care would also need to be taken that the levels of these

chemokines and cytokines do not increase to the point triggering unwanted responses such as apoptosis.

For a bone application, the chitosan selected needs to support the of functional bone cells. Fibroblasts play a role in laying down collagen matrix for future bone formation; but bone cells need to dominate the mature tissue. Ultimately, a bone application implant should favor bone type cells over other cell types especially what has been termed "weed-like" fibroblast. Special attention would need to be paid to chitosan's ability to attract and proliferation bone cells as well as chitosan's influence on cell signaling in regards to bone formation.

Chitosan has shown great promise as a biomaterial. It is commercially available in India as sutures and in China as a wound dressing. Additional applications being investigated include coatings for titanium implants (Bumgardner, *et al*, 2003), non-viral gene delivery (Guang, *et al*, 2002 and Romoren, *et al*, 2002), drug delivery (Kast, *et al*, 2002 and Risbud, *et al*, 2000), and tissue constructs (Cast, *et al*, 2001, Risbud, *et al*, 2001, Risbud, *et al*, 2002, Nettles, *et al*, 2001, Elder, *et al*, 2004). Yet, chitosan has not gained acceptance in the United States market. Problems such as conflicting reports in the literature may be the problem. Chitosan data varies. For instance, *in vivo* data ranges from claims of scarless healing (Braye, *et al*, 2000) to no difference in the wound healing process and results (Okamoto, *et al*, 1995) to fibrous tissue formation with the use of chitosan materials (Hidaka, *et al*, 1999 and Lu, *et al*, 1999). *In vitro* cell culture studies report fibroblast proliferation was increased with exposure to 89 and 91% DDA (Chung, *et al*, 1994 and Howling, *et al*, 2001) while chitosan films ranging from 52.5 to 97.5%

DDA were found to inhibit fibroblast proliferation (Chatelet, *et al*, 2001). Many of the early reports failed to examine the material characteristics of chitosan used. While there is growing recognition of material variation, some current studies have also failed to report simple chitosan characteristics such as molecular weight and the degree of deacetylation.

The hypothesis for this study is that macrophage, osteoblast and fibroblast attachment, growth and release of extracellular molecules will be increased on 95% deacetylated chitosan materials as compared to lower percent deacetylated chitosans. This study examined the material characteristics of DDA, molecular weight, ash content, contact angle, and swelling index and the cellular responses of adhesion (for adherent cell lines), proliferation for four cell lines, NAHDF, UMR-106, NHOst, and TIB-71. NO and IL-1β production was also examined for TIB-71 cells. The data revealed that the responses of the cells was not always dependent on DDA of the chitosan material.

NAHDF showed a variety of responses to varied chitosans tested. For adhesion, there was no correlation between DDA and adhesion as previously reported (Chatelet, *et al*, 2001). Yet, molecular weight did correlate to the adhesion of NAHDF to the chitosan films. DDA and adhesion did not correlate to proliferation as previously reported (Chatelet, *et al*, 2001). But, adhesion did vary among the different films. All DDA attracted a higher number of cells than the control. The increase in attached cells varied from 2.5 to 4 times the levels observed on the control. The highest adhesion was seen for 91 and 95% DDA. Films attracting only 2.5 times control level were 76, 80, 87, and 92% DDA. Results for proliferation also differed among the various chitosans. These

variations correlated to the molecular weight of the chitosan. Interestingly, when the data presented by Chatelet, *et al*, 2001 was re-examined, molecular weight also correlated with adhesion. Proliferation varied from more then 50% below the control level for 76% DDA to greater then 400% above the control level for 78% DDA when examined on the third day. However, there was no general trend between DDA and proliferation levels. Surface protein levels greatly influenced proliferation. As the surface protein levels decreased, proliferation increased. One sample, 78% DDA, broke this trend. 78% DDA had the highest ash content. The highest ash content tended to influence proliferation positively. No relationship was noted between the swelling index or contact angle to the adhesion or proliferation. This data suggest that other factors such as MW, protein and ash content of chitosan materials, may also play a part in the attachment and growth of fibroblast cells. This information is important in the selection of the chitosan for specific applications. For an osteogenic applications, fibroblast should be limited to a support role. Altering a chitosan of a chosen DDA to continue less ash or more protein to discourage overgrowth of fibroblast in the implant area may lead to a more successful outcome.

Of the two bone lines examined, UMR-106 and NHOst, results varied. The difference in the lines with UMR-106 being an osteosarcoma line and NHOst being a normal osteoblast line are credited for the variations in the cell response. .

UMR-106 cell lines adhesion was increase for all samples except 76 and 95% DDA which were statistically indistinguishable from control. This increase ranged from only 15% 92% DDA for to 20% for 80% DDA. Again, molecular weight predicted cell

adhesion. As the chitosan molecular weight increased, UMR-106 attached cell numbers increased. Proliferation was not increased on all chitosan samples. Only films of 87% DDA and greater supported more proliferation than the control on the third day. The increase in proliferation was 20 to 30%, a great deal less than the 400% increase reported in the NAHDF. The DDA did predict cell proliferation. As the DDA increased the UMR-106 cell proliferation increases. Proliferation levels on the fifth day were less than the control for all DDAs. The decrease was 40 to 80% below control levels. The lack of UMR-106 proliferation on the chitosan films may seem alarming. It should be remembered that this line is a cancerous cell type and not the best *in vitro* model. Swelling index, surface protein, contact angle, and ash content could not predict adhesion or proliferation levels for UMR-106. For UMR-106, the material characteristic of DDA seems to strongly influence cell proliferation. This response was not seen with the fibroblast. Response to material characteristics is cell type dependent. In addition, the cell line model also influences the responses.

NHOst showed a better response to the chitosan films than the UMR-106 cells. Adhesion was near control levels for most the DDA levels. Only 76 and 91% DDA attracted a greater amount of cells than the control. But, it should be noted that the 76% DDA film attracted three times the amount of cells that the control did. Molecular weight did not predict adherent cell numbers to chitosan films. Proliferation of NHOst cells increased above control levels for all films. Most films showed an increase in proliferation to levels 3.5 times the control. 78 and 95% DDA showed an increase of 7 times control on the third day. These increased continued on day 5 where the increase in

proliferation 16 times the control level. There was a general trend of NHOst proliferation increasing as the DDA of the chitosan film increased, just as in the UMR-106 cells. No trends between proliferation and adhesion were noted between the swelling index, ash content, surface protein, or contact angle. High ash content did correlate to the 78% DDA films breaking general trends in proliferation data.

TIB-71 macrophage cell line showed a range of reactions with chitosan exposure. Proliferation at the 3 day point showed increased from 30 to 200% above control. The highest proliferation was observed on the film composed of 78% DDA chitosan. Discarding the high ash content 78% DDA films proliferation levels, a linear trend can be observed between surface protein and proliferation levels of TIB-71 cells. As surface protein concentrations increase, proliferation levels of the TIB-71 cells increases. This is exactly the opposite linear relationship observed with the NAHDF fibroblast cells. IL-1β production was varied. An increase of 40% greater than control was observed as well as a decrease of 70% of control. A 70% decrease was observed in the cells exposed to 78% DDA chitosan. IL-1β related to two material characteristics: ash content and DDA. IL-1β also correlated to proliferation. As the ash content of the chitosan films increased, the IL-1β production levels per cell decreased. As the DDA increased, the IL-1β levels increased in a linear fashion. Chitosan films which invoked lower levels of IL-1β production per cell supported more proliferation. There was an inverse correlation between IL-1β levels and proliferation. Swelling index, molecular weight, and contact angle did not relate to proliferation and adhesion. Additional studies with TIB-71 conditioned media after exposure to chitosan being added to the cell culture of cell types

in question, for example fibroblast and NHOst cell lines for bone healing, would answer the question of what the signaling levels may mean *in vivo*.

Due to the fact that no solid trend was observed in any of the cell lines which would predict cell proliferation, it may be that there are other unstudied factors influencing proliferation levels. Yet, there are some general trends observed in this data. For one, 78% DDA chitosan from this study increased proliferation the most in the NAHDF, the NHOst, and the TIB-71 cell lines. Molecular weight could predict the adhesion of NAHDF to chitosan films. The molecular weight of the chitosan may have influenced the structure of the chitosan films. This influence on the films structure in turn influences the ability of NAHDF to bind to the films. Ash content greatly influenced NAHDF proliferation. The ash is thought to be a carbonate. Carbonates are common additions to cell culture media used for buffering or for incorporation into extracellular matrix e.g. mineralization in bone lines. At this point the mechanism behind the ash contents influence can not be determined; but a positive influence on the cell culture conditions is suspected for the increase in proliferation. It is interesting to note that the highest amount of ash content corresponded to the greatest amount of growth for the NAHDF, NHOst, and TIB-71. With the exceptions to general trends observed in the other cell lines studied always matching the high ash content samples, it is thought that ash content also played a role in the other cell lines responses, although it was more subtle than seen in the NAHDF. Additional studies of chitosan material characteristics and chitosans where only one characteristic is varied are needed to make more conclusive conclusions.

The above issues in selecting a suitable chitosan for the desired application addressed the influence of basic material characteristics. Ash content and protein levels in the chitosan may contribute to the cell behaviors of adhesion and proliferation. These levels are determined in the manufacturing process. DDA and molecular weight are also determined by the processing of the materials. All these process determined factors influence cellular response. The source of chitosan from nature may also be a factor. All chitosans examined in this study came from a crab source. However, those in the literature examined range from shrimp, fungal, crab, and theorized squid bone (while not reported, the manufacture cited specializes in chitosan from squid bone). This study indicates DDA, molecular weight, ash content, and surface protein are important in selection chitosan for a specific application. Indications are present that multiple factors are determining the outcome. In the present experimental design, it is difficult to separate the multiple factors and the antagonistic or synergistic interactions that may be occurring. Additional studies are needed examine other factors which may influence biological outcome before an appropriate chitosan for each application can be recommended. Presently, more refined and defined chitosan has become commercially available at extremely high prices. This could be of use in future studies.

In conclusion, the hypothesis that macrophage, osteoblast and fibroblast attachment, growth and release of extracellular molecules will be increased on 95% deacetylated chitosan materials as compared to lower percent deacetylated chitosans was not supported. Adhesion and proliferation of fibroblast cells did not correlate to DDA of test chitosans. Factors such as molecular weight and ash content better predicted cellular

responses for NAHDF. For the bone cell lines, there was a trend for proliferation to increase with increasing DDA, suggesting that these materials may be appropriate of hard tissue applications. For the TIB cells, while no correlation between cell growth and DDA was found, the low release levels of pro-inflammatory compounds suggest that chitosan materials may not elicit strong inflammatory responses. While the initial data is promising, further studies are needed to characterized chitosan material characteristics and cellular response.

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