Characterization of chitosan films for tissue engineering applications

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Abstract: Chitosan (β-(1,4)-2-amino-2-deoxy-D-glucose) is a naturally occurring, abundant biopolymer with desirable biomedical material properties of biodegradability, low toxicity, and good biocompatibility. These properties indicate that chitosan may be suitable as a surface for mammalian cell growth and tissue engineering. The attachment and growth of NIH-3T3 fibroblasts on chitosan films and controls was measured. Chitosan films of 0.5, 1.5, and 3.0% (w/v) support the attachment and proliferation of NIH-3T3 fibroblasts at rates slightly lower than polystyrene controls. The film tensile properties, surface roughness, and surface free energies indicate that the film-formation technique gives films with reproducible physical and chemical properties. Our results indicate that UV–IR treatment of chitosan films can change the water in air (WIA) contact angle and surface free energy (SFE) of the films, and can potentially be used to optimize the attachment and spreading of fibroblasts on these films. The ability of these chitosan films to support cell attachment and growth indicates their potential use as biomedical surfaces. This research may result in the development of biodegradable tissue-engineering matrices.

Key words: Tissue engineering, chitosan, biopolymer.

INTRODUCTION

The goal of this research project was to explore the use of chitosan as a surface for cell attachment and growth. Chitosan has potential as a biomaterial for tissue engineering scaffolds because it is biodegradable, easily formed into structures under mild processing conditions, can be chemically modified, is well tolerated in vivo, and has been reported to have tissue stimulating activity of its own (Klokkevold et al 1996; Muzzarelli et al 1993, 1994). Chitosan has been successfully used in biomedical applications for sutures, wound dressings, and drug delivery (Berscht et al 1994; Bodmeier et al 1989; Hari et al 1996) but has not been investigated within the tissue engineering paradigm.

Chitosan or β-(1,4)-2-amino-2-deoxy-D-glucose is a hydrophobic biopolymer obtained industrially by hydrolyzing the amino acetyl groups of chitin, which is the main component of shells of crab, shrimp, and krill, by an alkaline treatment (Kas 1997). The structure of chitosan is shown in Figure 1.

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Chitosan has been reported to be nontoxic and bioresorbable when used in human and animal models (Lahji et al 2000), and highly deacetylated chitosan has been shown to support the attachment of different cell lines (Prasitsilp et al 2000), indicating good biocompatibility. Chitosan is also easily processed into films and membranes, microparticles and beads, and 3-D scaffolds. Chitosan is similar in structure to glycosaminoglycans (GAGs), which are present in nearly all parts of the mammalian body and are among the essential building blocks of the macromolecular framework of connective and other tissues (Muzzarelli 1992). The healing processes of fetal wounds are mediated in part through the fetal extracelluluar matrix (ECM), which is rich in the GAG hyaluronic acid. Chitosan is structurally similar to hyaluronic acid and could exhibit similar wound-healing effects (Risbud et al 2000). The ability of chitosan to promote wound healing may also be related to its tendency to form polyelectrolyte complexes with the GAG and heparin, which possesses anticoagulant as well as angiogenic properties (promotes tissue vascularization). Chitosan may promote tissue growth and wound healing by forming a complex with heparin and acting to prolong the half-life of growth factors (Lahji et al 2000).

Chitosan-based films were developed and characterized for cell culture and tissue engineering applications. The films were formed by solvent casting, bulk and surface properties were measured, and films were then

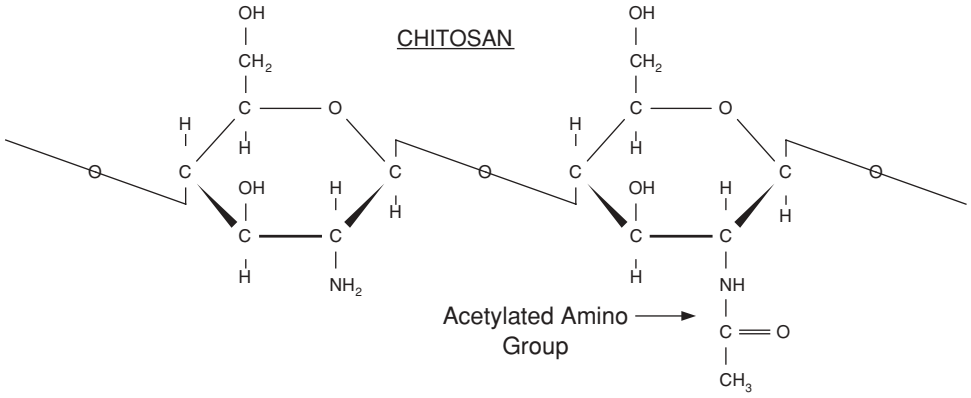

Figure 1 Chemical structure of chitosan.

evaluated for attachment and growth of NIH-3T3 fibroblast cells.

EXPERIMENT

Materials

High molecular weight chitosan (lot no. 04919KU) was obtained from Aldrich Chemical Company (St. Louis, MO). The chitosan prepared from crab shells has an approximate molecular weight of 600,000 g/mol, an 85% minimum degree of deacetylation, and is in the form of white to tan powder and flakes. An acetic acid solution (20% (v/v)) was supplied by VWR Scientific (West Chester, PA). Sodium hydroxide (NaOH) pellets were supplied by Fisher Scientific (Pittsburgh, PA). Ultra-pure distilled water (UP dH_2O) was purified with a Barnstead EASYPure RO compact reverse osmosis water system. A drawdown coater was used for some film-forming applications (RK Print Coat Instruments, Model: Herts SG8 0QZ U.K.).

Fluids utilized for surface free energy (SFE) measurements were glycerol, ethylene glycol, and distilled water. Molecular biology grade glycerol (lot no. 99H0099) was obtained from Sigma Chemical (St. Louis, MO), and analytical grade ethylene glycol (EG; lot no. 5001 T05753) was obtained from VWR Scientific (West Chester, PA). Images were captured onto a standard 120 min VHS tape with a Panasonic CCTV video camera (model no. WV-BD400) with a 2X adaptor fitted with a zoom lens (D.O. Industries model ZOOM 6000 II), connected to a Panasonic video monitor (model no. WV-5740), a video timer (FOR-A Video Timer, model no. VTG-55), and a Panasonic VHS VCR (model no. PV-S4167).

The 490N3T strain of 3T3 fibroblasts were routinely cultured with Dulbecco's Modified Eagle Medium (DMEM, lot no. 1084121) supplemented with 10% calf serum (CS, lot no. 1060198), 100 U/100 μg penicillin/streptomycin (100 U/100 μg Pen-strep lot no. 1079370), all obtained from Life Technologies (Grand Island, NY). Sterile, tissue-culture-grade, Falcon T-25 (25 cm^2) flasks were obtained from VWR Scientific (Bridgeport, NJ). Fifteen-milliliter centrifuge tubes, 50-mL centrifuge tubes, and 1-mL freezer vials were obtained from VWR Scientific (Bridgeport, NJ). Dimethylsulfoxide (DMSO) used for freezing cells was obtained from Sigma (St. Louis, MO). Cells were observed with a Hund-Wetzlar Wilovert AS phase contrast microscope. Manual cell counts were performed with a hemacytometer (Leavy Counting Chamber manufactured by Hausser Scientific) supplied by VWR Scientific (Bridgeport, NJ).

Film formation

Chitosan solutions (0.5, 1.5, and 3% (w/v)) were made by dissolving chitosan in 1.5% (v/v) acetic acid. This solution was strained through cheesecloth to remove any foreign particulates. Films were cast by spreading a control volume of chitosan solution onto a clean, smooth, rectangular glass plate. Once cast onto the glass support, the solution was air dried at room temperature for 48 h to ensure complete solvent evaporation. The dried film was separated from the glass support, and rinsed with 500 mL of 1 M of NaOH. The films were then repeatedly rinsed with distilled water for 30 min to wash away any soluble products. The pH of the film rinse-water was measured after each 30-min rinse, until a constant pH of the rinse water was obtained to ensure that no more soluble products were coming off of the film. Typically, four successive 30-min dH_2O rinses were sufficient to obtain a stable pH near 7. Finally, the wet films were spread out and attached to the clean glass support with clamps and allowed to dry for 24 h at room temperature.

Another method for casting 1.5 and 3.0% chitosan films was also employed using a RK Print Coat Instruments, Herts SG8 0QZ U.K. drawdown coater. The glass support attached to tinfoil was placed on the coater, and the smoothest rod (model no. 1) was placed in the apparatus. The rod was raised to a height of 2 mm above the glass support on the drawdown coater, and 200 mL of chitosan solution was placed on the glass support, just in front of the bar. The 3.0% chitosan solutions were spread at an approximate rate of 3 in./s, and the 1.5% chitosan solutions were spread at an approximate rate of 2 in./s. The film was then placed on a level surface, and subsequently dried and treated as previously outlined for the above film-casting technique.

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All films used in cell culture experiments were sterilized for 1 h in a UV–IR oven. The UV bulbs are GE model RSM operating at 275 W and 110–120 V, and emit light at 296 nm. The IR bulbs were GE model AJ IR reflectors, and were standard tungsten filament bulbs with a thin IR coating, emitting light over a broad spectrum at 250 W and 120 V. Samples were placed in sterile, disposable 15 × 100 mm polystyrene petri dishes, and placed in the oven at 14.5 in. below the lamps. Films were characterized with no UV–IR treatment, as well as with UV–IR treatments of up to 24 h.

Film tensile properties

The chitosan films were evaluated for thickness, Young's modulus of elasticity, yield stress, strain to failure, and surface roughness. A method for testing the tensile properties of the films was adopted according to ASTM standards for testing the tensile properties of paper and paperboard using a constant-rate-of-elongation apparatus (Allan et al 1998). The instrument used to test these properties was an Instron Series 5500 tester. Film thickness measurements were obtained with a Mitutoyo digital micrometer with 1 μm accuracy (No. 293-705 7126095). Similar results for film thickness were also measured with a Starrett 0–1 in. micrometer (model no. 230), with a 12.7 μm accuracy.

Chitosan films were cut into strips of 25.4 mm × 254 mm and placed in the grips of the Instron tester so that distance between the grip clamping zones was 180 mm. An elongation rate of 7 mm/min for 0.5% and 1.5% films and 10 mm/min for 3.0% films resulted in specimen rupture 10–30 s following the initiation of the elongation force. Approximately 20 samples were tested for each concentration to determine the Young's modulus of elasticity, the yield stress, and the strain to failure. All films were tested dry at room temperature.

Substrate average roughness was measured with a Tencor Instruments profilomter (Alpha 200), with a diamond-tipped stylus. Samples were mounted onto a glass slide with double-sided tape. Minimum sample dimensions were 25.4 mm × 25.4 mm. Samples were scanned by dragging the stylus along a 2000 μm line over the substrate at various points. The Tencor Alpha 200 uses internal software to calculate the average vertical variation in the substrate over the range of the scan. An average of at least 10 scans was obtained and used as the value for the average roughness (Ra). Scans were performed on both sides of the films.

Surface free energy estimation by contact angle measurement

A substrate's SFE can be calculated by measuring contact angles of various fluids with different known surface tension characteristics and a generalized version of the Young-Dupre equation for the surface free energy components of a liquid on a solid. Fluids utilized for SFE measurements were glycerol, ethylene glycol, and distilled water.

The contact angles of distilled water, ethylene glycol, and glycerol were measured on chitosan films and polystyrene controls according to the following procedure. Chitosan films were mounted onto a glass slide with double sided tape. One-microliter drops were placed onto films and substrates with a 1 μL Hamilton syringe accurate to 0.02 μL (model 7001). Images were captured onto a VHS tape at 60-125× magnification. Water and ethylene glycol contact angles were captured at 15 s after contact with substrate, which was the time required for the drop to stop spreading. Glycerol contact angles were captured at equilibrium 30 s after contact with the substrate. No significant adsorption of fluids by the chitosan films was observed, as evidenced by no visual change in drop shape or size over the time span of the experiments. The contact angle of all fluids was captured for chitosan films without UV–IR sterilization, as well as for films sterilized for 1, 12, and 24 h, respectively.

The contact angles were measured for both the left and right sides of the drop and averaged. Four tests were conducted on each sample, and four samples were cast for each concentration. The average contact angle of a substrate reported is the average of a minimum of five different drop images on a substrate. Contact angles measured on either side of the film displayed similar results.

Cell culture

The NIH-3T3 fibroblasts were used for experiments between passages 10 and 30. Fibroblasts were maintained at 37 °C in an incubator, equilibrated with 5% carbon dioxide (CO_2), and kept at approximately 95% humidity. Cells were subcultured every 4–6 days. Cell confluency was confirmed visually by observation of the culture flask with a phase contrast microscope.

Cell attachment experiments

To determine the suitability of solvent-cast chitosan films for cell culture, the attachment of NIH-3T3 fibroblasts onto chitosan films of three different concentrations (0.5, 1.5, and 3.0% (w/v)) was measured and compared to polystyrene controls.

Corning six-well tissue culture plates and untreated, sterile polystyrene dishes were obtained from VWR Scientific (Bridgeport, NJ). Silicone O-rings were obtained from McMaster-Carr (ID = 1-1/16 in., OD = 1-5/16 in.). Images were captured with a Minolta 35 mm SLR camera, attached to a Hund-Wetzlar Wilovert AS phase contrast microscope.

Bradford reagent was prepared with Coomassie Brilliant Blue G (lot no. 1838A17), supplied by EM Science (Gibbstown, NJ), certified A.C.S. 85% *o*-phosphoric acid (lot no. 001815) and certified A.C.S. methanol (lot no. 001009), both supplied by Fisher Chemical (Fair Lawn, NJ). Bovine serum albumin (lot no. 78H0696) was obtained from Sigma Chemical (St. Louis, MO) for protein

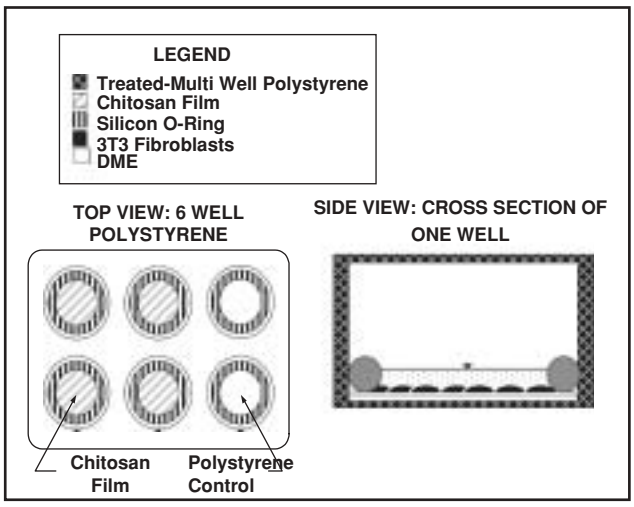

Figure 2 Cell attachment and growth experimental setup.

standards. Sodium hydroxide pellets were obtained from Fisher Chemical (Fair Lawn, NJ).

Chitosan films were prepared and sterilized by UV–IR radiation for 1 h. Films made from chitosan solutions of 0.5, 1.5, and 3.0% (w/v) concentrations were cut into 7.9 cm^2 discs using a stainless steel cutting tool. The cutting tool was autoclaved prior to use.

Sterilized chitosan films were placed into four of the six wells and held stationary with silicone O-rings. Silicone O-rings were also placed in the remaining empty wells, which were used as the tissue-culture grade polystyrene control. Finally, autoclaved silicone O-rings were placed into untreated polystyrene petri dishes used as a second control. The O-rings kept the chitosan films stationary when exposed to cell culture and medium, as well as provided an experimental control for a standard area (5.72 cm^2) to which fibroblasts were exposed during experimentation. Experiments were assembled in six-well tissue culture polystyrene dishes according to Figure 2. The untreated polystyrene controls were separate. Three plates were set up for each film concentration for measurement of cell concentration at 15, 30, and 60 min after inoculation (nine multi-well tissue-culture-grade plates per attachment experiment as well as three untreated polystyrene petri dish controls were used).

Samples and controls were inoculated with 8.8×10^5 NIH-3T3 cells per well (1.1×10^5 cells/cm^2) and supplemented with 2 mL total volume of DMEM with 10% CS and 100 U/100 μg pen-strep, then placed in a 37 °C incubator with 5.0% CO_2 and 95% humidity. This cell density is near the saturation density of the NIH-3T3 fibroblasts on tissue culture grade polystyrene. At sample times of 15, 30, and 60 min after inoculation, samples were removed from the incubator. The DMEM was aspirated, and the number of cells that remained attached to the chitosan films or controls was counted according to the following procedure. The film samples were rinsed twice with 2 mL of sterile D-PBS to remove any unattached cells and media. Cells were loosened from the surface by adding 1 mL of trypsin-EDTA and gently tapping the sides of the sample dishes or plates. Cells were then suspended in 1 mL DMEM with 10% CS and 100 U/100 μg pen-strep. Cells were recovered and quantified by a total protein determination. The total protein concentration of each sample was measured in duplicate using the Bradford assay.

Cell growth experiments

The rate of NIH-3T3 fibroblast growth on chitosan films (0.5, 1.5, and 3%) were measured and compared to untreated polystyrene and tissue-culture-grade polystyrene controls. Chitosan films, polystyrene tissue culture flasks, and petri dishes utilized for the NIH-3T3 growth experiments are the same as those described in the attachment studies. The experimental set-up for the growth experiment is similar to that for the attachment experiment with the following exceptions. Films were inoculated at a lower cell density of 6.0×10^4 cells/dish to avoid contact inhibition. Cell counts were taken at 12 h, 1, 2, 3, and 4 days in culture, using the same methods described in the attachment experiments. Culture medium was changed every other day.

RESULTS AND DISCUSSION

Bulk and surface properties of films

Films were all cast from identical-source chitosan, which was determined to have a DDA of 96% by FTIR analysis (data not shown). The tensile properties of the chitosan films give an understanding to the mechanical properties of the films, as well as a measure of the reproducibility of the film-formation procedure. The results of the tensile properties and standard errors of the varying concentration chitosan films are summarized in Table 1.

The thickness of the chitosan films increases with increasing concentration. This is to be expected, as films were cast with equal solution volumes over equal areas. Yield stress and strain to failure also increase with increasing chitosan concentration. Film roughness (not shown) were all in the nanometer range, which in comparison to 3T3 cell diameter indicates all films can be considered smooth.

The Young's modulus of elasticity of all samples are very similar. These data differ from that of Kienzle-Sterzer (Kienzle-Sterzer et al 1982), who indicated that the Young's modulus of elasticity should increase with increasing chitosan concentration. However, the preparation and testing procedures of Kienzle-Sterzer differed somewhat from those utilized here. First, chitosan films were dried at a higher temperature. Second, tension experiments were performed on chitosan samples that were immersed in deionized water at room temperature. Therefore, the results presented here are not directly comparable to those published by Kienzle-Sterzer. Conversely, Mima and coworkers (Mima et al 1982) characterized both

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Table 1 **Summary of tensile properties of chitosan films**

	0.5% Chitosan film	1.5% Chitosan film	3.0% Chitosan film
Thickness (mm)	0.012 ± 0.0005	0.02 ± 0.0031	0.038 ± 0.0005
Young's modulus of elasticity (MPa)	4240 ± 80	4447.5 ± 160	4290 ± 110
Strain to failure (mm/mm)	0.011 ± 0.001	0.020 ± 0.002	0.027 ± 0.002
Yield stress (MPa)	39.8 ± 0.3	56.5 ± 5.9	62.7 ± 1.0
Elongation (%)	1.74 ± 0.03	1.59 ± 0.02	3.11 ± 0.03

Note. Each result represents the average of two samples, each with a minimum of 10 measurements. Standard deviation is shown.

the wet and dry tensile properties of chitosan films. They found an increase in wet tensile strength with increasing *N*-acetylation and no correlation in dry tensile strength and *N*-acetylation. Although this study did not consider the relation to chitosan concentration, there was still no observable trend in the dry tensile strength that varies with various degrees of *N*-acetylation, partially confirming our observations.

The percent elongation of the chitosan films increases with chitosan concentration. This result might be explained by the greater thickness of the 3.0% chitosan films compared to the 0.5% films. The 1.5% film exhibited high variability in most of the tensile parameters investigated. The wet tensile properties of the films should also be investigated.

Results reported here agree with that of Khan et al (2000) who showed that similarly made chitosan films had a tensile strength of 67.11 MPa. Previous work suggests that the formation of composites made of 20% chitosan, 72% collagen, and 8% chondroitin 4-6-sulphate can form skin scaffolds with elastic modulus of 150 kPa and tensile strength of 400 kPa (Berthod et al 1994). Chitosan scaffolds formed by freezing and lyophilization were shown to have a maximum tensile strength of 5.5 kPa, yield strength of 0.5 kPa (Madihilly and Matthew 1999). Such scaffolds are porous and expected to have less strength than films.

It is recommended that substrates used for skin tissue scaffolds have an ultimate tensile strength of 6.37 MPa, elastic modulus of 35 MPa, and an ultimate tensile strain of 100% (Yamada et al 1970). From this perspective, the chitosan films presented in this report possess adequate mechanical properties for skin tissue engineering. However, it should be noted that the properties will change over time during use as a tissue engineering matrix due to hydration.

Summaries for water in air (WIA) contact angles and surface free energies (SFE) of chitosan films (UV–IR sterilized for 1 h as used in cell culture studies) and polystyrene controls are depicted in Table 2.

The polystyrene controls have moderate wettabilities, as illustrated by the WIA contact angles of 75.6 and 73.2 degrees for the tissue-culture-grade polystyrene and untreated polystyrene, respectively. These values agree with the data of previous studies (Saltzman 1997; Tamada and Ikada 1994) that indicate cell culture substrates with WIA contact angles between 60 and 90 degrees displayed improved cell adhesion compared to substrates with WIA contact angles outside the specified range. All of the chitosan films also have WIA contact angles in this optimum range. This indicates that chitosan films should support cell attachment.

Studies also show that cell culture substrates with SFEs greater than 55 mJ/m^2 (erg/cm^2) support more extensive fibroblast spreading than those with SFEs below 30 (mJ/m^2) (Saltzman 1997; Tamada and Ikada 1994). The SFE of the chitosan films and polystyrene controls described here are within this range, indicating that chitosan substrates will support fibroblast spreading

Table 2 **Summary of WIA contact angle and SFE of substrates**

Sample	Water in air contact angle	Surface energy [γ_s ($mJ\ m^{-2}$)]
Treated polystyrene multiwell tissue-culture dish	75.6 ± 0.4	37.4 ± 1.7
Untreated polystyrene petri dish	73.2 ± 0.3	48.4 ± 0.9
0.5% (w/v) chitosan in 1.5% (v/v) acetic acid	83 ± 4.5	41.5 ± 4.2
1.5% (w/v) chitosan in 1.5% (v/v) acetic acid	79.83 ± 3.1	36.4 ± 11.2
3.0% (w/v) chitosan in 1.5% (v/v) acetic acid	84.1 ± 3.1	36.5 ± 4.7

Note. Each data point is the average of four tests on four samples, with each test representing a minimum of 5 contact angle measurements at various points on the substrate.

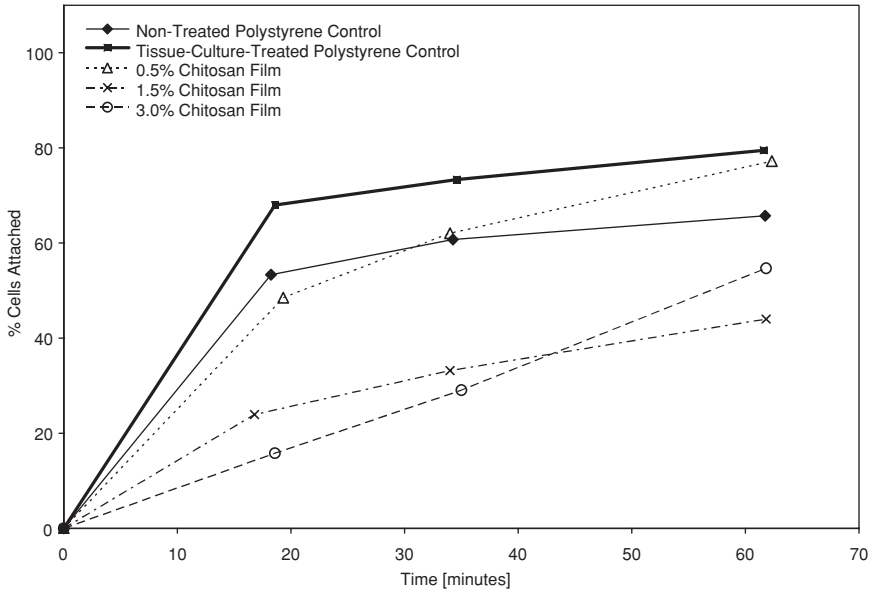

Figure 3 Cell attachment on chitosan films and polystyrene controls. Each point represents at least three different tests, maximum error 5%.

to a lesser extent than substrates with SFEs greater than 55 erg/cm^2. Interestingly, the SFE of the tissue-culture grade polystyrene is lower than that of the untreated polystyrene and the 0.5% chitosan film. The untreated polystyrene dish has the highest SFE at 48.4 mJ/m^2. This suggests that the untreated polystyrene may exhibit better cell spreading than the tissue-culture grade polystyrene. Water in air contact angle and SFE are important properties for cell attachment, but they are not the sole criteria for substrate suitability for cell culture. Additional surface characterization will be performed on the most promising films.

The overall SFE of the chitosan films appears to be affected by the length of time for which the films were subjected to UV–IR sterilization. Films made from 1.5 and 3% chitosan concentration showed a 69 and 48% increase in SFE after 24 h of exposure to UV–IR radiation, respectively. The 0.5% film showed no significant change in SFE when exposed to radiation. This SFE increase in the higher concentration films may be due to crosslinking induced by the UV radiation, or possibly a change in the degree of deacetylation of the chitosan films. It is anticipated that the SFE of chitosan films can be modified using UV–IR treatment to better support cell spreading.

Cell attachment

The attachment experiment results are depicted in Figure 3, which shows the percentage of the total number of cells that attached to the surface compared to the original 8.8×10^5 cells per dish inoculated. Cells attach to all chitosan films and show increasing cell concentration with time over the 1 h duration of the experiment. The polystyrene controls support greater NIH-3T3 cell attachment than the 1.5 or 3.0% chitosan films. However, cell attachment to the 0.5% chitosan films is approximately the same as to the polystyrene controls. The rate of attachment for the 0.5% chitosan film and controls within the first 20 min is greater than that for the next 40 min. The controls and the 0.5% film achieved greater than 50% attachment after 20 min in culture. Attachment to the polystyrene controls and the 0.5% chitosan films approaches a plateau at 60 min in culture, where 65–80% of cells inoculated have attached to the substrate. Cell attachment within the first 20 min shows a direct relationship to chitosan concentration.

Cell attachment shows an inverse relationship to surface free energy, with the higher energy surface (0.5%) supporting greater attachment than the lower energy surfaces. This observation is in agreement with others (Webb et al 1997; Yang et al 2001) which indicate cell attachment increases with surface energy. The potential for increasing SFE of the 1.5 and 3% chitosan films with UV treatment suggests that the higher concentration films may be modified to improve cell attachment.

There are no natural cell-recognition sites on the surface of polymer materials, so the attachment of NIH-3T3 fibroblasts is likely mediated by the adsorption of serum proteins onto the polymer surface. Many polymers will adsorb proteins present in serum to their surface, with maximum adsorption occurring on polymers with intermediate wettabilities. Modification of the surface properties such as wettability, surface energy, and charge may improve cell affinity for a polymer surface via interaction with proteins.

Cell growth

The growth of the NIH-3T3 fibroblasts on the surfaces is depicted in Figure 4. All concentrations of chitosan films

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Table 3 **NIH-3T3 growth parameters on different surfaces**

Sample	Specific growth rate (h^{-1})	Population doubling time (h)
Treated polystyrene tissue-culture	0.0304	22.8
Untreated polystyrene petri dish	0.032	21.7
0.5% (w/v) chitosan	0.0284	24.4
1.5% (w/v) chitosan	0.0224	30.9
3.0% (w/v) chitosan	0.0226	26.1

supported NIH-3T3 cell growth. The polystyrene controls support more rapid cell growth and a higher cell concentration than the chitosan films. The controls also approach the saturation density of 1.2×10^5 cells/cm^2 after 4 days in culture as expected.

The 0.5% chitosan film and 3.0% chitosan films showed similar NIH-3T3 cell growth characteristics over 4 days. The 0.5% film had the highest cell density after 4 days at 7.5×10^4 cells/cm^2, followed by the 3.0% film with 5.2×10^4 cells/cm^2. The 1.5% chitosan film had the lowest cell concentration after 4 days in culture at 3.7×10^4 cells/cm^2, but all chitosan films showed no statistical difference with respect to final cell density.

Specific growth rates of cells on the films are shown in Table 3. All chitosan films exhibited slower growth rates and longer population doubling times (PDT) than the controls. The 0.5% films show the highest cell proliferation rate. This result may indicate that higher concentrations of chitosan inhibit cell growth.

It was considered that the final cell population supported by the controls shown in Figure 3 may be due to the higher cell attachment to these surfaces. On all surfaces, the cell populations decrease initially after inoculation due to less than 100% attachment and growth lag period. Therefore, the growth rate and population doubling times were calculated relative to the number of cells that actually attach (i.e. specific growth rate). Even accounting for differing initial cell atttachment, the cell proliferation is somewhat slower on chitosan films than controls, indicating that although chitosan films have a lower cell attachment rate, the cells on the surface also exhibit a slower growth rate. However, it is very likely that the initial attachment plays a significant role in cell affinity for surfaces.

Results of other researchers indicate that fibroblast growth is inhibited on chitosan substrates. Risbud et al (2000) showed that NIH-3T3 cell growth inhibition resulted from their inability to attach to the chitosan/PVP hydrogel. While the study presented in this paper indicate NIH3T3 cells will proliferate on chitosan, our results that indicate lower attachment to chitosan, and the effect of SFE on attachment, corroborate the observations of Risbud. Chatelet et al (2001) reported increasing fibroblast cell adhesion with decreasing degree of acetylation, and lack of fibroblast proliferation on chitosan surfaces.

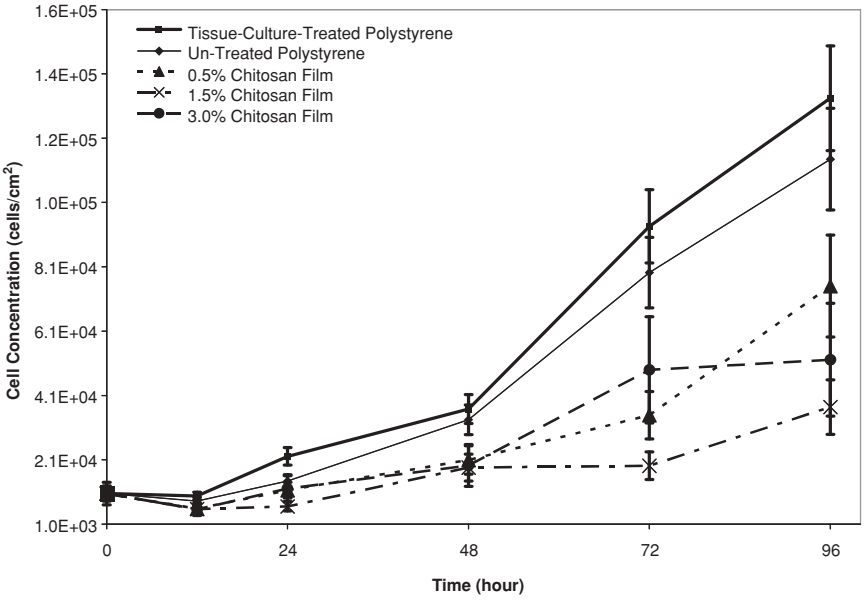

Figure 4 Cell growth on chitosan films and polystyrene controls. Each point represents three different tests, sampled in duplicate. Error bars represent 95% confidence intervals.

CONCLUSIONS

Chitosan films were formed with reproducible tensile properties, and the properties were in the desirable range for tissue engineering. The WIA contact angles of the three concentration chitosan films are in the reported optimum range of 60–90 degrees to support maximum cell adhesion (Tamada and Ikada 1994). All chitosan films as well as polystyrene controls should support cell attachment and adhesion based on the reported WIA contact angles. The estimated SFE of the untreated chitosan films is in a range shown to promote fibroblast spreading (Skakenraad et al 1986).

Chitosan films exposed to UV–IR radiation for 24 h showed an increase in SFE. This change may be due to crosslinking of the chitosan films by the UV radiation or deacetylation of the chitosan molecule. This is a very intriguing result which indicates the potential to increase the SFE of chitosan films with UV–IR radiation. These results suggest the possibility for optimizing cell spreading and growth of NIH-3T3 fibroblasts on these chitosan films by changing the SFE of formed films.

All substrates support the attachment of NIH-3T3 cells. The polystyrene controls support greater cell attachment than the 1.5 or 3.0% chitosan films, however, attachment of cells to the 0.5% chitosan film is similar to the controls. Attachment within the first 20 min is inversely related to chitosan concentration.

The chitosan films also support proliferation of 3T3 cells, with the 0.5% chitosan film showing cell growth rates approaching the controls, and again giving higher growth rates than the other two concentrations tested. The attachment and growth of 3T3 cells on the chitosan films is highest on the lowest concentration of chitosan. These results indicate that chitosan is a suitable substrate for in vitro cell culture.

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