A replica molding technique for producing fibrous chitosan scaffolds for cartilage engineering†

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Cartilage engineering benefits from the fabrication of random fibrous constructs, mimicking the structures found in the extracellular matrix of natural tissue, to support the attachment and proliferation of cells. Here, a novel approach is demonstrated for the production of fibers with controlled dimensions in the micron regime from the naturally derived biopolymer chitosan. The process involves filling an array of microchannels recessed into a mold surface with a solution bearing chitosan, inducing a pH-dependent coagulation, and releasing the structures from the mold into a medium where they assume random orientation. The dimensions and shape of these channels in the master template were defined by a standard photolithographic process followed by anisotropic reactive ion etching of the underlying silicon wafer. Replica castings of this silicon wafer surface in elastomeric polydimethylsiloxane (PDMS) served as the mold for chitosan. The resulting scaffolds were produced with fiber cross-sectional widths of 22 ± 4 μm, 13 ± 3 μm, 4.7 ± 1.6 μm, 1.7 ± 0.6 μm, and 1.1 ± 0.4 μm and were found to consist of 97.8 ± 0.5% medium when hydrated. Study of the nanoscale morphology of the fibers revealed that the effects of liquid surface tension play a significant role in the preservation of this open form and that lyophilization of the product is the preferred long term storage method.

1 Introduction

The response of living cells with high levels of organization in tissue to external stimuli is perhaps the ultimate system to be understood in the biological sciences. Studies have documented that tissue cells react by preferential attachment or even dedifferentiation according to the surface morphology of their environment.[1,2] Such sensitivity to the control of the dimensionality of the surroundings is likely the reason that generating cartilage *ex vivo* has classically been particularly difficult.[3] Evidence indicates that prior attempts to engineer 3-D constructs for chondrocyte cells found in cartilage have met with limited success due to a tendency for dedifferentiation.[4] Study of natural cartilage reveals that the differentiated chondrocytes produce extracellular matrix (ECM) proteins and amino substituted polysaccharides, which take the form of random fibrous networks as shown in Fig. 1.[5] This ECM is essential to the function of cartilage in providing structure to the body. The goal of *in vitro* tissue engineering is to create a construct which is conducive for the cells to develop the ECM as it occurs naturally. A highly desirable approach to the development of cartilage for orthopedic reconstruction is to fabricate a scaffold structure from a suitable material, seed it with donor cells already of the chondrocyte phenotype, allow the cells to populate in culture, and finally implant *in vivo* to encourage integration with the native tissue at the damaged joint.[6] As a result, the requirements for these precursor scaffolds are typically two-fold. The environment must act as an open mesh to permit cells to permeate its entire structure during initial attachment and to ensure that nutrients or waste can diffuse in or out as needed. Also, the material comprising the scaffold should degrade by enzyme activity or some other means at a controllable rate with a time scale that coincides with its replacement with permanent ECM materials, which do not elicit enzyme response.

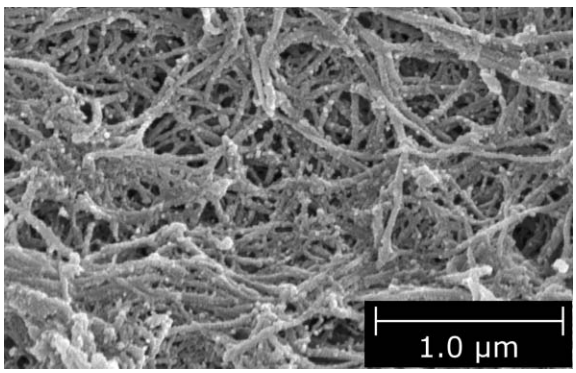

Fig. 1 Scanning electron micrograph exhibiting the nanofibrous morphology of the self generated native environment of chondrocytes, containing a structural matrix of proteins linked to polysaccharides consisting of glycosaminoglycan units.

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Previous approaches to meet these requirements have been based on both synthetic and natural polymeric materials and a variety of techniques with a wide range of sophistication to increase the openness of the scaffold. Spun fibers of polyglycolic acid (PGA) designed as biodegradable sutures have been applied to cartilage engineering, exhibiting favorable chondrocytic attachment.[6] Although the meshes degrade over an acceptable duration by hydrolysis to non-toxic glycolic acid, the erosion occurs at different rates for the amorphous and crystalline regions, resulting in rapid loss of structural stability, and the product's decrease in local pH is potentially adverse to the cells.[7] Further, the extrusion-type process by which the fibers are made is difficult to scale into sub-micron diameter dimensions due to the demands of increasing bulk polymer purity and process pressure. The naturally derived biopolymer chitosan has well-understood biocompatibility as it is chemically similar to complex glycosaminoglycans found in cartilage ECM and degrades predictably by lysozyme activity over a time scale that can be controlled by its chemical properties and surface area. Lyophilizing, or freeze-drying, an aqueous solution of chitosan is known to produce a highly porous sponge. The structure results from micro ice crystal formation, precipitation of the dissolved species surrounding these crystals, and subsequent removal of the water by sublimation.[3] While the pore size can be adjusted to some extent within the micron regime by varying the cooling rate,[8] the method is only capable of producing one general form. These sponges are less conducive to diffusion and mimic the natural ECM less accurately when compared to fibrous structures. A technique known as electrospinning, commonly used to process synthetic polymers into nanofibers, has been adapted to chitosan in an effort to create the preferred fibrous networks. By accelerating the chitosan solution through a spinneret due to a high voltage electric field, fibers in the sub-micron to 40 nm diameter range have been demonstrated.[9,10] Variability in constitution inherent to natural products complicates the matter, often requiring various plasticizers and surfactants to be added to the blend as needed. Biocompatibility of each of these solution modifiers, and combinations thereof, must be considered carefully before application of fibrous products from this process to cell cultures and, further, *in vivo* implantation. Subsequent application of these electrospun scaffolds faces issues of differing water solubilities of the components as in the case of PGA fibers.[10] Other studies have shown that using concentrated acid as the solvent eliminates the need for additives, but reaching pH neutrality by dissolution, necessary for culture preparation, is likely infeasible.[11,12] In all cases high velocities of the product deposition at the target combined with the softness of the chitosan blend also compress the scaffold, detracting from its openness to cell diffusion.

Therefore, the goal of the work presented here is to provide a novel method of fabricating culture scaffolds from chitosan that maintains the openness provided by a random orientation of unbound fibers. This approach utilizes microfabrication methods that are well established in the microelectronic fields to control the geometry of a molded material. This process is accomplished by first defining the fibers as structures protruding from a "master" silicon wafer, which become relief features in a cast polymeric replica mold of the surface. The liquid solution containing chitosan is then allowed to flow over the replica surface and fill all features, which in this case are linear channels with depths and widths in the 1–50 μm size range. Any excess solution not filling the channels in the mold is removed prior to application of another aqueous solution to induce a solidification transition in the molded chitosan. The result is that individual fibers with dimensions controlled according to the master can be released and collected from solution as a random network as illustrated in Fig. 2. Since the defined fiber structures never leave the aqueous phase, they experience no unbalanced forces which cause meshing as in the electrospun scaffolds. By permitting later rearrangement of the network in solution, the overall shape of the scaffold and, thus, the neo-cartilage after culture is more easily manipulated. It is worth noting that once the work of patterning the master is done, multiple replica molds can be produced and replaced as needed. Thus, this process is suitable for production scaleup as the yield is directly proportional to the area of the mold substrate. Also significant to clinical application of this work is the ability to preserve these constructs for extended periods before chondrocyte culture can occur; an experiment to evaluate various storage methods is performed here to address this concern.

Chitosan was selected for this study due to its favorable chemical and biological properties. Chitosan is a polysaccharide chemically similar to cellulose, which suggests that it has good structural properties. This material is obtained by treating chitin, the second most abundant natural biopolymer behind cellulose, with a strong base to induce deacetylation, leaving a terminal amine as the functional group. This reaction

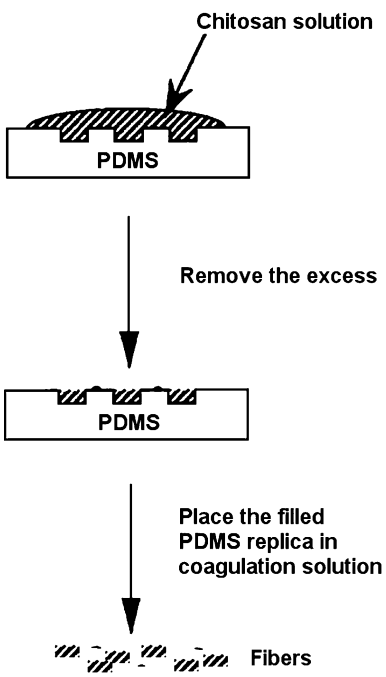

Fig. 2 Schematic illustration of the chitosan solution micromolding process on a polydimethylsiloxane (PDMS) replica surface relief structure, which was previously defined by casting from a silicon master.

almost never reaches completion so chitosan can essentially be thought of as a random copolymer of $\beta(1 \rightarrow 4)$ linked D-glucosamine and *N*-acetyl D-glucosamine units. These amino groups can be protonated by aqueous acids, creating a third subunit illustrated in Fig. 3. Thus, sufficiently low solution pH below the pK_a of the amine, usually cited as being between 6.0 and 6.5, can induce enough cationic charge on the molecules that it becomes energetically favored for water to solvate.[8,13,14] The fraction of possible protonation sites converted to this amino moiety, known as the degree of deacetylation (DDA), becomes a key property in determining the behavior of the material.[3] Further, the pH-dependent solubility provides a basis for coagulation in the molding process. By controlling the aqueous solution pH, this transition from a viscous liquid state, which takes the form of its container, to a solid can be directed. It is also possible to intentionally add chemicals for specific biological activity to the chitosan solution without losing this capability of inducing solidification transitions, which is essential to the molding approach. A wide variety of chitosan composites have previously been studied.[15–17]

2 Experimental

2.1 Solution preparation

"Chito Clear" fg95 (Primex, Siglufjörður, Iceland) was used as the chitosan scaffold material and is specified as having greater than 95 percent DDA and a viscosity of 500 cP at 1% concentration in dilute acetic acid (MW is likely in the order of 500 kDA according to the viscosity). 3% w/v chitosan molding solution was prepared as follows: a mixture of 3.00 g of chitosan flakes to every 100 mL of 0.200 M acetic acid solution was magnetically stirred for 48 h with moderate heat (50 °C plate temperature) and refluxed to restrict concentration changes due to evaporation. The viscous solution was passed through 0.45 μm and 0.2 μm pore nylon membrane syringe filters to remove any insoluble particulates before finally being stored at 4 °C.

Stock glacial acetic acid, hydrochloric acid, pelletized anhydrous sodium hydroxide, and anhydrous sodium sulfate were obtained from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hanover Park, IL). Coagulant solution of 0.150 M NaOH/0.150 M Na_2SO_4 ($[Na^+]$ = 0.450 M; pH = 13.0 accounting for ionic activity corrections) in deionized (DI) water was prepared analytically. 1.0 M HCl was used to clean the chitosan residues.

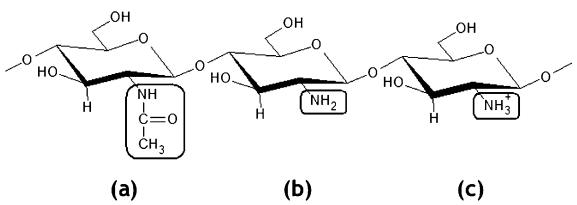

Fig. 3 Molecular structure of chitosan demonstrating the three possible states of the amino functional group: (a) acetylated, (b) terminal, (c) protonated. Deacetylated forms (b) or (c) account for greater than 95 percent of the subunits in the material used in this study.

2.2 Master and replica fabrication

Four inch diameter N-type <100> silicon wafers (Silicon Quest, Santa Clara, CA) were patterned by photolithography with AZ5214E (Clariant USA, Charlotte, NC)/S1805 (Shipley, Marlborough, MA) photoresists and contact printing/GCA Model DSW-6100 stepper projection and etched by the Bosch process in an inductively couples plasma – reactive ion etcher (ICP-RIE) (Surface Technology Systems, Newport, UK). Following solvent and O_2 plasma ashing removal of the photoresist etch mask, silane treatment of the native oxide was accomplished by soaking the etched silicon wafer in a 2% w/v solution of dimethyldichlorosilane in octamethylcyclotetrasiloxane (PlusOne Repel-Silane, Amersham Biosciences, Uppsala, Sweden) for approximately 5 min followed by rinsing with ethanol and DI water. Silane functionalization decreases the surface energy and, thus, reduces adhesion with the replica material. The surface profile consisted of parallel channels equal in width and depth, separated by an approximately equal distance. Silicon wafers produced with 55 ± 1 μm, 30 ± 1 μm, 10.0 ± 0.3 μm, 4.2 ± 0.3 μm, and 2.7 ± 0.3 μm relief channels covering the entire wafer surface, resulting in about 100 to 1000 periods, served as the masters.

Next, multiple elastomeric replicas were fabricated from each silicon master surface by casting and chemically curing polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, Midland, MI) using a 10 : 1 base to curing agent weight ratio. Prior to casting, the PDMS mixture was degassed under vacuum to remove any entrapped air. Approximately 40 g of degassed PDMS was poured onto the surface of the silicon master through the use of a custom aluminum fixture that prevents spreading of the liquid PDMS beyond the wafer edge. Since the surfaces of the silicon masters were patterned entirely to the edges in the lithography process, the fixture confined the pre-cured PDMS to take advantage of as much patterned area as possible. The PDMS curing process was accelerated by heating in air within a 110 °C oven for two h. Channel widths were verified within the margin of error for the masters by using a calibrated light microscope. The finished cured PDMS replicas have a 85 mm diameter and a 7 mm thickness.

2.3 Chitosan molding

O_2 plasma treatment for 120 s at 500 mTorr, 300 W RF power in a Texas Instruments DPR A-24-D etch system was performed on all the PDMS replicas immediately before molding. Chitosan solution was removed from 4 °C storage only to deposit an appropriate amount on the replica surface. An excess of solution was distributed parallel to the channels and carefully removed by hand using the sharp edge of a rubber tool. The replica was inverted into a pool of coagulant at 40 °C for 45 min. Flexing the structure in solution and applying pressure with the same rubber tool facilitated removal of the coagulated fibers. The released fibers were allowed to soak in the coagulant bath for 24 h.

2.4 Scaffold manipulation

Once the fiber forms thoroughly solidified due to the coagulant, this basic solution needed to be removed before

the scaffolds were ready for subsequent characterization and use. The medium, that is, the liquid surrounding and contained within the fiber network, was exchanged with progressively higher concentrations of the desired liquid. Elevated pressures available in a centrifuge provided an efficient method of removing excess liquid and collecting the chitosan fibers over 0.2 μm pore diameter nylon membrane filters, while not being detrimental to the openness of the structure. Exchanges of high purity deionized water (Milli-QUV Plus, Millipore, Bedford, MA) to reach pH neutrality were typical to preserve the product by reducing ionic strength or as an intermediate step in medium changes to solvents in which the salts are insoluble. One such example of the latter case is preparation for scanning electron microscopy (SEM), which requires that the scaffold be moved to a volatile yet chitosan-insoluble liquid such as isopropanol. Rapid evaporation of the isopropanol in a 100 °C constant temperature bath, mounted on an electrically conductive surface, and Au sputter coating for 20 s at 18 mA in 0.2 mbar Ar (SPI-MODULE, Structure Probe Inc., West Chester, PA) complete this procedure. The representative samples undergoing this manipulation and analysis could not be recovered for further use. Also, product yield was quantified according to the wet weight. Since the interwoven fibers could be pulled from the medium as a single coherent piece, the wet weight included both the chitosan and the minimum amount of retained water. Scaffolds regained any lost volume upon return to aqueous medium, making this quantification suitable for all products.

2.5 Storage study

A study to evaluate the viability of various long term storage conditions was carried out. Newly produced scaffolds in water medium were kept for 15 d under one of the following conditions: stored at 4 °C, frozen at −80 °C, frozen at −196 °C (liquid nitrogen), and in a dry environment at 20 °C following lyophilization (frozen at −80 °C). After the samples returned to room temperature or were rehydrated in the case of the lyophilized product, all were prepared as described previously for SEM examination of the nanostructure to be compared against micrographs of newly produced fibers.

3 Results and discussion

3.1 Process optimization

Individual chitosan fibers, which collect into random networks and serve as scaffolds for cells, were produced by this method. Fig. 4 presents a macroscopic view of one such network that results from a single chitosan molding from the surface of one of the PDMS replicas described earlier as well as micrographs of representative samples of networks produced from differing masters. Since the overall arrangement of the fibers follows the same form among the five fiber diameters, it can be assumed that processing chitosan in this way is scalable. Though the fibers intersect and contact at multiple points while in medium, the material is sufficiently coagulated to prevent irreversible adhesion or deformation at these points. Many measures have been taken to ensure consistently high yields and reproducible product quality; these are outlined in the discussion of the process below.

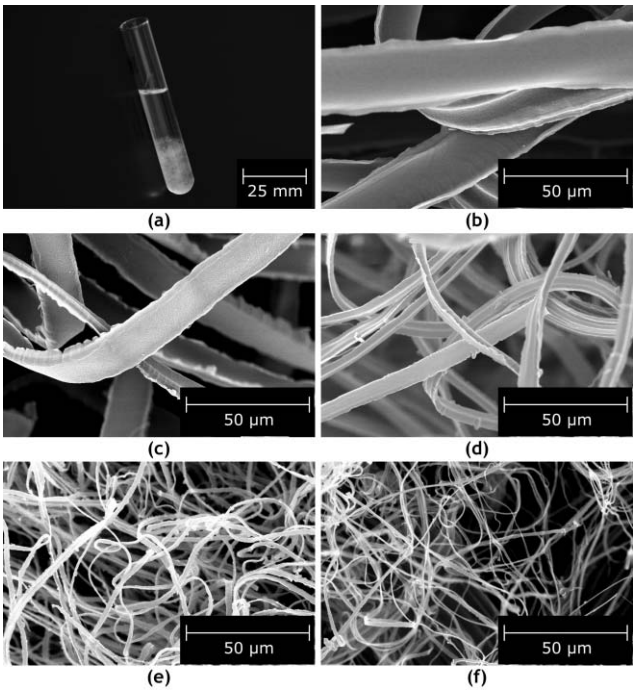

Fig. 4 (a) Appearance of the fibrous chitosan scaffolds in pH neutral water medium prior to preparation for electron microscopy. Representative SEM micrographs of scaffolds molded from (b) 55 μm, (c) 30 μm, (d) 10 μm, (e) 4.2 μm, (f) 2.7 μm channel width masters/replicas. The magnification in (b)–(f) is identical for comparative purposes.

Formulation of the coagulant solution, consisting of the strong base NaOH to deactivate the pH-dependent solubility of chitosan and Na_2SO_4 to increase ionic strength, was found to play a significant role in its effectiveness and, thus, the yield from each procedure. Various concentrations of these two components were tested before those specified in Section 2.1 were standardized, and any increase beyond these levels provided minimal improvement in yield but increased the difficulty in purifying the scaffolds for further use. Concentration of chitosan in the molding solution was similarly optimized. The solubility is effectively limited to approximately 4% w/v despite pH-dependent solubility considerations and the amply low pH of the 0.200 M CH_3COOH solution (2.73); the equilibrium position corresponding to this hydrogen ion concentration is far on the side of amino group protonation according to its acid dissociation constant (pK_a). A minimum solvation length imposed by like-charge electrostatic interactions between the massively multivalent chitosan molecules in solution is one possible explanation.[13,18] This reasoning for solubility limitations would also account for the rapid viscosity increase with chitosan concentration, which is detrimental to wetting properties and ease of molding. Since acetic acid is a weak acid, it may have a buffering effect in resisting pH changes in the system, but a shift in the equilibrium from increased conjugate base concentration leads to a pH increase nonetheless.

Fabrication of the PDMS replicas from silicon masters proceeded with negligible dimensional changes as this method has been established as having extremely high fidelity.[19] In order to fill relief structures in PDMS with a polar solvent-based chitosan solution, it is necessary to modify the surface properties for wettability. This is accomplished by treatment of this material with an oxygen plasma to create a silica-like surface.[20] A dry technique is preferred since it requires only a short amount of time and produces a consistent effect as long as the equipment's process parameters, such as time, power, and gas pressure, are kept constant. Another consideration in choosing these parameters is the possibility for developing surface microcracks.[20] Significant changes in the chitosan solution contact angle and wetting indicate that this surface modification is relatively short-lived. Therefore, performing this procedure on all the replicas immediately prior to molding was necessary for consistency.

3.2 Product characterization

A current limitation of the method for the creation of fibers with dimensions below 1 μm, which is desired to closely mimic the ECM structure, is the incomplete removal of molding solution at the crests or spaces between the channels before drying or coagulation take place, resulting in bundles of fibers attached by a film. A random arrangement of ribbon-like structures does not allow the same access for penetration of medium, not dissimilar from the porous lyophilized sponges, and may also affect the overall density. These undesired effects lower the quality for the purposes of cell environments. The incidence of this sheet formation depends heavily upon the pressure applied with the rubber tool despite the high thickness of the PDMS molds to resist deformation. There is an inherent inconsistency in the manual nature of the procedure as presented, which could be greatly reduced in an industrial production setting with calibrated machinery. This pressure variation coupled with shallow feature relief depths is the largest impediment to producing fibers with diameters below 1 μm. Within the range of channel widths attainable from traditional photolithography (55 μm to 2 μm), it seems that about 5 μm is the practical limit for generating scaffolds with reproducibility suitable for research cell cultures by these manual procedures. Production of fibers with sub-micron dimensions, however, is certainly possible and is demonstrated here [Fig. 4(f)].

The coagulated fiber dimensions are found to decrease by a fairly constant ratio ($\sim$60%) relative to the replica channel sizes according to SEM analysis; that is, fibers from the 55 μm, 30 μm, 10.0 μm, 4.2 μm, and 2.7 μm master patterns averaged 22 $\pm$ 4 μm, 13 $\pm$ 3 μm, 4.7 $\pm$ 1.6 μm, 1.7 $\pm$ 0.6 μm, and 1.1 $\pm$ 0.4 μm, respectively, over 6 random measurements each from image analysis. The effect is confirmed by both SEM analysis and calibrated optical microscopy while still in the medium. Rapid loss of solvent during coagulation of the low chitosan content solution is suspected. Moreover, anisotropy in fiber dimensions exists in some cases. Solution drying in the channels before the transfer into coagulant is a likely cause of these ribbon-like structures, most apparent in Fig. 4(c). While edge rounding is observed at all sizes, at larger fiber diameters it is more apparent which side was on the open end of the mold with artifacts of a liquid meniscus shape.

Since the hydrated scaffolds consist almost entirely of liquid medium, exchanges without disturbing the openness of this form require attention. As long as the scaffold remains wet internally, it is able to recover its original volume once returned to excess liquid and agitated. The application of centrifuge filtration here maintains this minimum hydration level. What occurs when a scaffold in water medium is allowed to evaporate can be seen in Fig. 5. The relatively soft fibers deform and fuse when subjected to internal capillary forces between them during drying.[21] This is not the case when isopropanol, which has a much reduced surface tension, particularly at elevated temperatures, dries from the fibrous matrices. Preparation of the low density scaffolds for UHV SEM analysis then occurs by this less aggressive evaporation for a more accurate representation of the morphology when in medium.

Similarly, quantifying the product in a non-disruptive way presents some challenges. Dehydration for a reliable dry weight is not always an option, and volume and, thus, weight of these highly compressible structures in the wet state lack consistency. For example, given the low density of chitosan relative to the medium, a doubling of water contained within a structure that is currently at 98% results in only about a one percent difference in water content (to 99%). The wet weight determination procedure outlined in the Experimental section was settled upon as the least biased and most reasonable estimation. This density of solid in liquid is also an important quantity that correlates to the openness for cells, for instance, to diffuse in its matrix. Since the structure is truly fibrous in nature, citing porosity in terms of average pore size would be inappropriate. Instead, a measure is determined from comparison of the wet and dry weights. The latter is easily found by dehydrating a scaffold, even by aggressive means, after removal of coagulant salts. Since the geometry of the scaffolds seems to scale directly with fiber dimensions, it is expected that the water content relative to chitosan would remain constant. Among 8 scaffolds measured, all were within 0.5% of the average of 97.8% water content by weight.

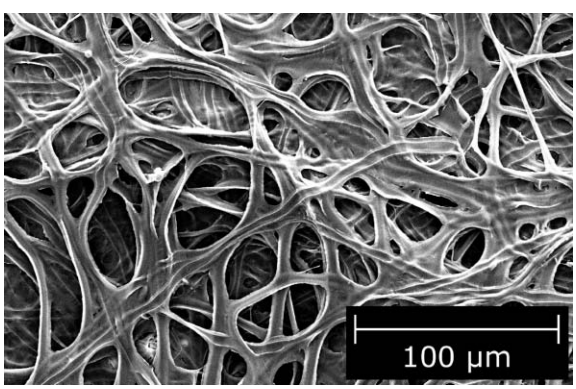

Fig. 5 SEM image of a scaffold (from the 10 μm channel mold) that is directly dehydrated from aqueous medium under vacuum. Forces due to high liquid surface tension result in meshing and compression of the soft fibers.

3.3 Storage study

Long term storage of hydrated chitosan scaffolds proved problematic. Even with reduced kinetics at 4 °C, chitosan fibers increase in nanoporosity and degrade to the point that they lose structural stability after 4 months as depicted in Fig. 6. pH drifts as measured in these samples can lead to protonation and ultimately dissolution of the chitosan fibers. The storage study performed evaluated the feasibility of various techniques involving freezing the aqueous medium against the control. Even after 15 d, the fibers stored under standard 4 °C refrigeration displayed precursors of degradation as surface pores in the order of 10 nm developed as seen in Fig. 7. All of the samples in fact showed signs of surface degradation, but it was least extensive in the lyophilized product, making this the preferred storage method. Macroscopic appearance of product processed by lyophilization is shown in Fig. 8. Since this method involved freezing at −80 °C just as another specimen in the study, it is assumed that this step is responsible for any porosity observed as dry storage does not permit dissolution of the material. Based on

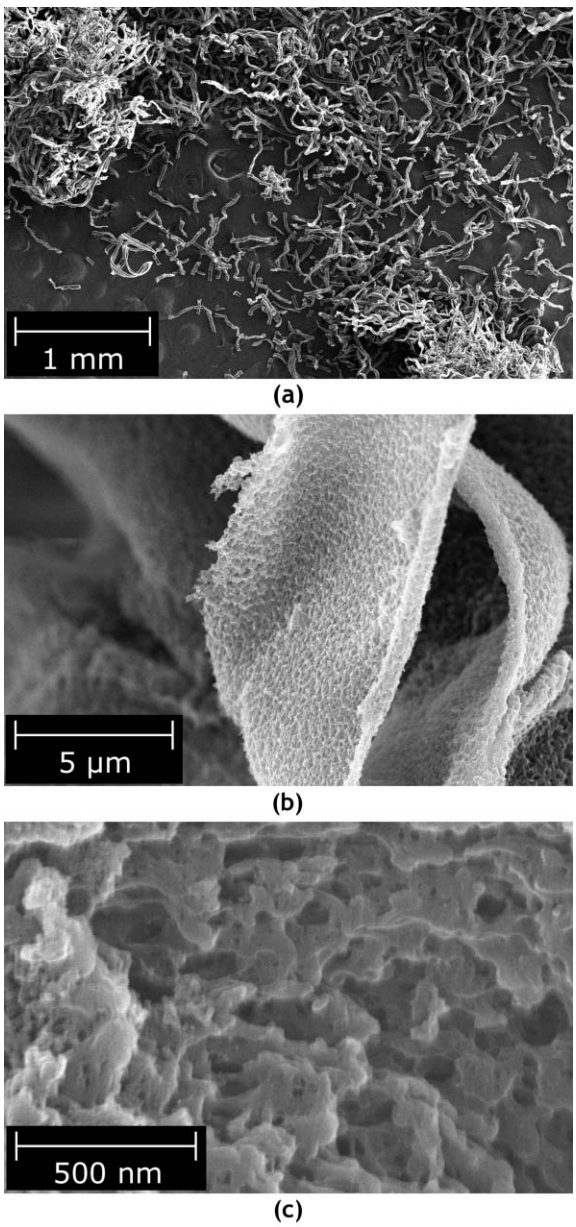

Fig. 6 Progression of SEM micrographs at (a) 20×, (b) 4600×, (c) 52 000× of chitosan fibers (from the 55 μm channel mold) aged for 4 months in water medium. Onset of microporosity leads to the fibers becoming prone to fracture. Dimensional anisotropy in the fiber cross-section is apparent in (b).

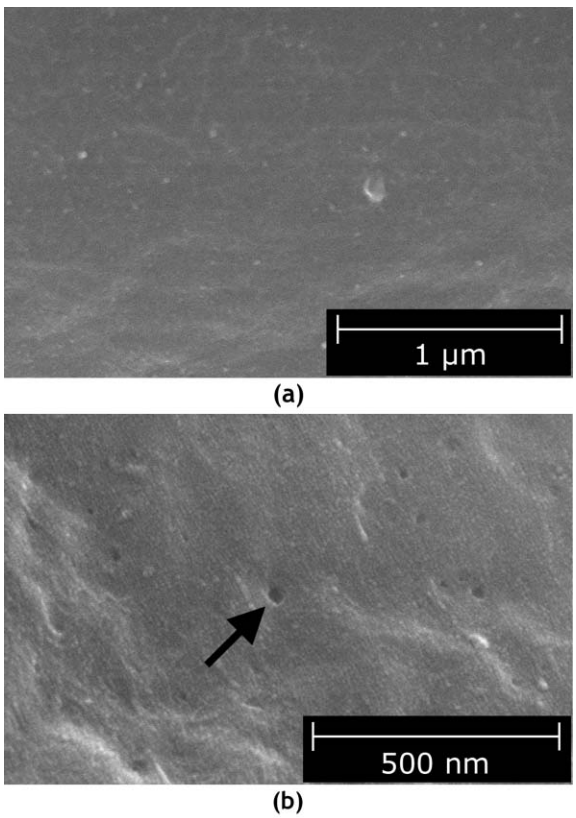

Fig. 7 Comparison of the surface morphology of single fibers in newly molded product as a control (a) and a sample in the storage study aged for 15 d at 4 °C in pH neutral water (b). Formation of pores with diameters of less than 50 nm as highlighted in (b) indicates the onset of dissolution. Any differences in the overall topology are circumstances of the local mold shape and variations in the coagulation process.

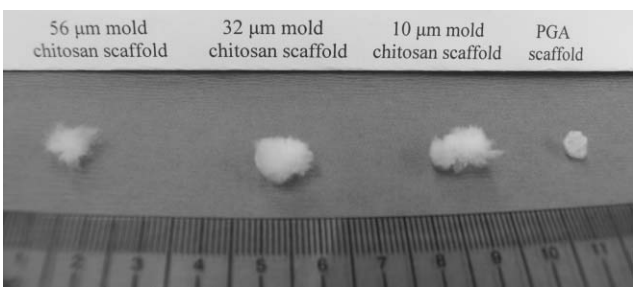

Fig. 8 Size of the lyophilized chitosan scaffolds with non-woven polyglycolic acid (PGA) fibers as a reference, relative to a scale in centimetres. The dry weight of all are standardized to approximately 2 mg.

the mechanism of ice crystal formation inducing as discussed for lyophilization as a scaffold fabrication technique, the fibers themselves must not have much absorbed water. Some penalty still exists, however, for freezing scaffolds, which is more pronounced over extended periods. Therefore, storage of chitosan fibers by lyophilization is worthwhile in the long term (greater than 2 weeks) as dry storage can be extended indefinitely.

4 Conclusions

A protocol for the relief surface molding of chitosan fibers was developed; parameters for the oxygen plasma pretreatment of the PDMS mold surfaces, chitosan solution properties, and coagulant formulation were optimized to yield complete mold cavity filling and release of the structures. The pH-dependence of the solubility mechanism of the chitosan material used is key to allowing the mold cavities to define the shape of the solid, which is essential to the process. The method is in fact relevant to nearly all materials for which a solidification transition can be controlled. Future work will involve seeding the scaffolds with chondrocytes, culturing for extended periods, and performing the pertinent techniques to quantify the cell dedifferentiation and expression of various extracellular matrix proteins. This study would evaluate the merit of fibrous chitosan scaffolds relative to previous constructs as well as any correlation between fiber dimensions and cell activity.

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Authors Queries

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