

Chitosan scaffolds: Interconnective pore size and cartilage engineering

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Abstract

This study was designed to determine the effect of interconnective pore size on chondrocyte proliferation and function within chitosan sponges, and compare the potential of chitosan and polyglycolic acid (PGA) matrices for chondrogenesis. Six million porcine chondrocytes were seeded on each of 52 prewetted scaffolds consisting of chitosan sponges with (1) pores $\leq 10 \mu\text{m}$ in diameter ($n = 10$, where n is the number of samples); (2) pores measuring $10\text{--}50 \mu\text{m}$ in diameter ($n = 10$); and (3) pores measuring $70\text{--}120 \mu\text{m}$ in diameter ($n = 10$), versus (4) polyglycolic acid mesh ($n = 22$), as a positive control. Constructs were cultured for 28 days in a rotating bioreactor prior to scanning electron microscopy (SEM), histology, and determination of their water, DNA, glycosaminoglycan (GAG) and collagen II contents. Parametric data was compared ($p = 0.05$) with an ANOVA and Tukey's Studentized range test. PGA constructs consisted essentially of a matrix containing more cells than normal cartilage. Whereas very few remnants of PGA remained, chitosan scaffolds appeared intact. DNA and GAG concentrations were greater in PGA scaffolds than in any of the chitosan groups. However, chitosan sponges with the largest pores contained more chondrocytes, collagen II and GAG than the matrix with the smallest pores. Constructs produced with PGA contained less water and more GAG than all chitosan groups. Chondrocyte proliferation and metabolic activity improved with increasing interconnective pore size of chitosan matrices. In vitro chondrogenesis is possible with chitosan but the composition of constructs produced on PGA more closely approaches that of natural cartilage.

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1. Introduction

Tissue engineering is a rapidly developing field, whereby cells are allowed to proliferate and organize their extracellular matrix in a three-dimensional (3-D) lattice to form ex vivo a clinically functional tissue, exhibiting histochemical, biochemical and biomechanical properties identical to native, healthy tissue. The ideal matrix for in vitro chondrogenesis has yet to be determined and the list of biomaterials tested for cartilage repair is extensive [1,2]. Among these, a polyglycolic acid (PGA) mesh has been used extensively as a

resorbable substrate onto which chondrocytes produce implant-free cartilage after 4 weeks of dynamic culture [3,4]. Although this matrix sustains chondrogenesis, its application has been limited by the mechanical properties of engineered constructs and their lack of integration with adjacent tissues [5,6]. Whereas byproducts of polymers used for biomedical applications, such as polylactic and polyglycolic acids may induce a foreign-body reaction, chitosan, a natural aminopolysaccharide, is degraded into neutral or weak-base sugars that are physiologic precursors of natural GAG [7–9]. Chitosan is formed by alkaline deacetylation of chitin, the second most abundant natural polysaccharide, primarily obtained as a subproduct of shellfish, such as crabs and shrimps [10,11]. Its superior biocompatibility has been attributed to its structural similarity with glycosaminoglycans

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(GAG) naturally present in the extracellular matrix of cartilage [12,13]. Another potential benefit of the use of chitosan includes its antimicrobial properties, which result from enhanced migration and activity of neutrophils [14]. The use of chitosan in wound management has been well investigated, and research has more recently focused on its potential applications in orthopedics [15]. Indeed, the similarity of chitosan to GAGs makes it a particularly attractive candidate for cartilage defects repair and much interest is now directed toward its application in cartilage engineering [16–18]. Although chondrogenesis has been described on chitosan sponges [19], its potential for cartilage engineering and the effect of interconnective pore size have not been objectively evaluated.

The objectives of this study were to evaluate the effect of interconnective pore size on the chondrogenic properties of chitosan, and compare the constructs engineered on chitosan or polyglycolic acid scaffolds.

2. Materials and methods

2.1. Scaffolds

Scaffolds of chitosan¹ were prepared by a freeze-drying technique. Chitosan flakes (3%, w/v) were dissolved in aqueous 0.2 M acetic acid. According to the manufacturer, the chitosan had a mean molecular weight of 200,000 and a degree of deacetylation of 85%. The resulting viscous solution was filtered and transferred to cylindrical molds 5 mm in diameter and approximately 3 mm in height. Although the minimum porosity of all scaffolds was maintained at 80%, interconnective pore size was adjusted by varying the freezing temperature. Interconnective pore size was measured with an image analysis software on serial scanning electron microscopic sections throughout the scaffolds. Three types of scaffolds (Fig. 1) were prepared:

- (1) chitosan sponge with pores measuring $\leq 10 \mu\text{m}$ in diameter (CS, $n = 10$);
- (2) chitosan sponge with pores measuring 10–50 μm in diameter (CM, $n = 10$);
- (3) chitosan sponge with pores measuring 70–120 μm in diameter (CL, $n = 10$).

All scaffolds were lyophilized for 4 days to allow complete removal of the solvent.

The second type of scaffolds ($n = 22$) consisted of a 2.5 mm thick, non-woven mesh made of 13- μm -diameter PGA fibers,² with a void volume of 97% (Fig. 2). Cylinders measuring 5 mm in diameter were cut prior to sterilization.

All scaffolds ($n = 52$) were sterilized with ethylene oxide gas and rehydrated through a series of ethanol and phosphate-buffered saline (PBS) solutions (100%, 95%, 75%,

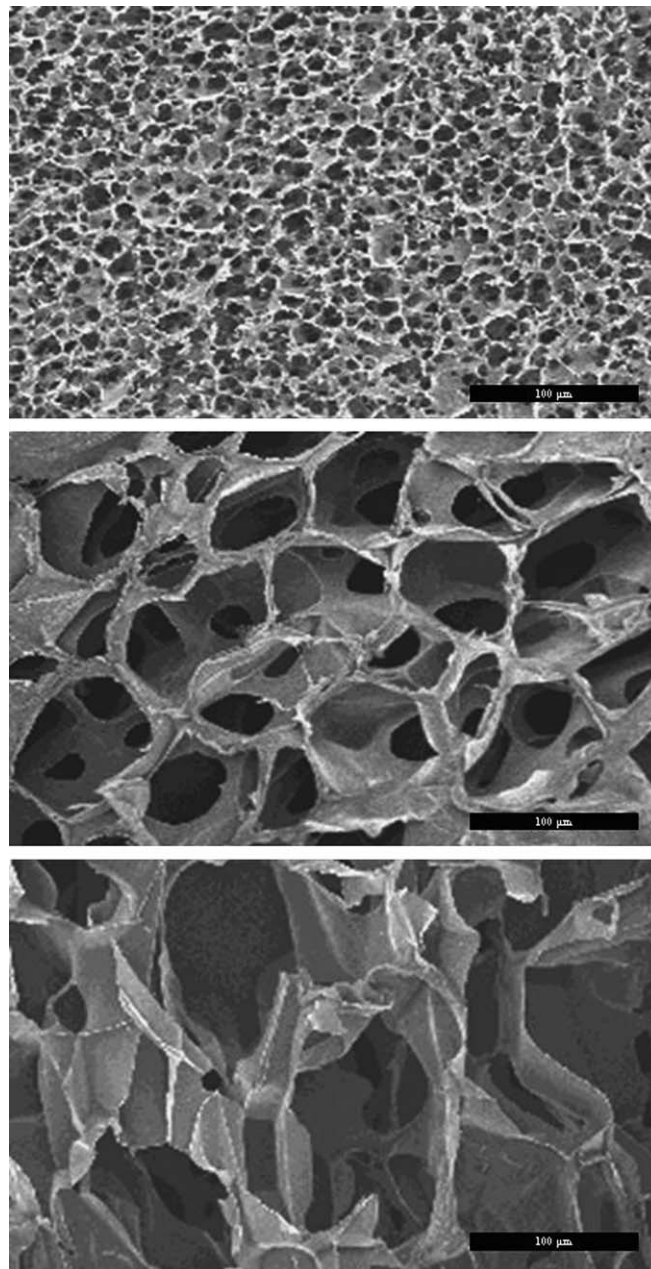


Fig. 1. Scanning electron microscopic examination of chitosan scaffolds with pores measuring $\leq 10 \mu\text{m}$ (top), 10–50 μm (middle) and 70–120 μm (bottom) in diameter.

50%, and 0% ethanol). Polyglycolic acid and chitosan scaffolds were then placed in vacuum tubes that contained sterile deionized water, and maintained on a shaker incubator (100 cycles/min) for 48 h, prior to transfer in medium and overnight incubation in fetal bovine serum [20].

2.2. Chondrocytes

Cartilage was aseptically collected from both stifle joints of four 3–4 week old pigs. Pigs were obtained from the research farm, University of Illinois, after humane euthanasia for reasons unrelated to the study. All procedures in this

¹ Sigma–Aldrich Corp., St. Louis, MO.

² Synthecon Inc., Houston, TX.

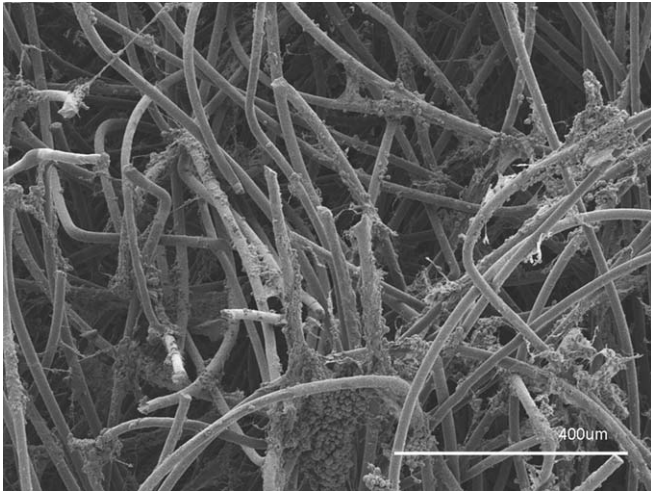


Fig. 2. Scanning electron microscopy of polyglycolic acid mesh (PGA) 48 h after seeding of chondrocytes.

study were approved by the Institutional Animal Care and Use Committee of the University of Illinois. Each sample consisted of cartilage obtained from weight bearing and non-weight bearing surfaces of the femoral condyles and tibial plateau. Cartilage from two pigs was pooled in each experiment, minced in 1-mm³ cubes and rinsed in PBS solution with 2% penicillin–streptomycin prior to overnight digestion in culture medium and 0.15% collagenase type II³ [21]. The culture medium consisted of Dulbecco's modified Eagle's medium that contained 4.5 g of glucose/L, 10% fetal bovine serum, 584 mg/L glutamine, 100 U/ml penicillin, 100 μg/mL streptomycin, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 0.1 mM non-essential amino acids, 0.4 mM proline, and 50 μg of ascorbic acid/mL [21]. Cell number and viability were evaluated via trypan blue exclusion. For each experiment, samples of isolated cells were placed in a 6-well plate to evaluate cell attachment under monolayer static culture. Six samples of pooled fresh cells were also used to determine the DNA content per cell.

2.3. Seeding and culture techniques

Chondrocytes were suspended in culture medium to obtain a cell suspension containing 24×10^6 viable cells/mL; 250 μl of suspension/scaffold (containing 6×10^6 chondrocytes) was used to seed each scaffold using a vacuum and dynamic seeding technique previously developed in our laboratory [22]. Seeded scaffolds were transferred in five rotating bioreactors.⁴ Chitosan scaffolds (CS, CM, and CL) were distributed among three rotating vessels so that each vessel contained 10 scaffolds and at least three scaffolds of each pore size. Seeded PGA scaffolds were divided into two groups of 11 scaffolds each and placed in the remaining two bioreactors. One PGA construct in each vessel was

harvested at 48 h for scanning electron microscopy. All remaining constructs (10 per vessel) remained freely suspended in 60–62 cm³ of medium by adjusting the speed of rotation to match the increasing construct mass (15–28 rpm) [23]. Culture medium was replaced at a rate of 50% every 2–3 days [3]. Constructs were harvested at 4 weeks and divided for analysis.

2.4. Evaluation of constructs

Constructs were weighed before and after dehydration to determine water content. Samples were digested in papain for 16 h at 60 °C and assayed for total sulfated GAG content via spectrophotometry with 1,9-dimethylmethylene blue chloride [24]. Shark chondroitin sulfate (5–50 μg/ml) was used as a standard [25]. The same papain digestion technique was used for a fluorometric assay of DNA with Hoechst 33258 [26]. Collagen II content was determined with an enzyme linked immunosorbent assay.⁵ Previous studies of cartilage engineering have reported hydroxyproline content, which is an indicator of all types of cartilage and therefore lacks specificity. Instead, we measured the collagen II content of our constructs with an ELISA test. Although this kit has been validated in pigs, we also included samples of skin as negative controls, to confirm that collagen I did not interfere with the test. Dedifferentiation of chondrocytes would be expected to affect their ability to produce collagen II.

Four constructs per group were fixed in a 2.5 wt.% gluteraldehyde solution with sodium cacodylate buffer for 2 h. After rinsing with buffer, constructs were submerged in 1 wt.% osmium tetroxide in 0.1 M sodium cacodylate for 90 min. After a buffer rinse, the constructs were dehydrated through an ethanol series. Finally, constructs were placed in hexamethyldisilazane for 45 min and left under a fume hood until completely dry. Each scaffold was bisected, to evaluate the surfaces as well as the central section of the construct. Two samples (superficial and center) were mounted for each construct and sputter coated with gold–palladium prior to examination with a scanning electron microscope at 5 kV.⁶ Criteria evaluated included cell morphology, size, attachment to the support, presence of cytoplasmic extensions, extracellular matrix and remnants of scaffolds.

Slices of cartilage ($n = 3$) and cultured scaffolds of each group ($n = 4$) were fixed in 10% neutral buffered formalin, embedded in plastic and cut via microtome to produce 1-μm-thick sections. A minimum of five sections of each sample were stained with hematoxylin and eosin. Sections of cartilage were examined to confirm the absence of pathology in the cartilage. Sections of engineered tissue were examined to evaluate scaffold morphology, the distribution of cells within the constructs, and the presence of new matrix.

³ Worthington, Freehold, NJ.

⁴ Rotary Cell Culture System, Synthecon Inc., Houston, TX.

⁵ Type II collagen detection kit, Chondrex Inc., Redmond, WA.

⁶ Hitachi S4700, Schaumburg, IL.

2.5. Statistical analyses

DNA, water, GAG, GAG/DNA and collagen II contents were compared between engineered constructs for statistical significance ($p=0.05$). Because data were not normally distributed and could not be transformed, a Kruskal–Wallis test was first used to evaluate overall differences between groups. Data were ranked and statistical differences were evaluated on the ranks with a one-way analysis of variance (ANOVA) and Tukey's highly significant differences (HSD) multiple comparison test [27].

3. Results

3.1. Chondrocyte attachment to the scaffolds

Forty eight hours after seeding, the number of cells counted in the medium of vessels containing chitosan scaffolds was equal to $31.18 \pm 7.48\%$ of the initial number of cells seeded, whereas $7.12 \pm 1.21\%$ of the cells seeded on PGA remained in suspension. Scanning electron microscopy confirmed the attachment of cells to PGA: chondrocytes were grouped around the fibers of the mesh, resulting in a fairly uniform distribution throughout the matrix.

3.2. Histology

All sections of cartilage harvested for this study had a normal histological appearance. The appearance of constructs cultured for 4 weeks varied between types of scaffolds. Whereas the chitosan scaffolds appeared intact, few remnants of PGA fibers were identified on histological sections. PGA constructs consisted essentially of mononuclear cells surrounded by a matrix, with short fragments of polyglycolic acid fibers (Fig. 3). Chondrocytes cultured on chitosan sponges with pores $\leq 10\mu\text{m}$ in diameter formed a film covering the surface of the scaffolds and produced small amounts of matrix. Sponges containing larger pores (CM and CL) appeared to contain more cells surrounded by extracellular matrix. Cells penetrated the pores of chitosan sponges, especially in the group presenting the largest pores. The distribution of cells appeared more uniform throughout scaffolds as pores increased in size.

3.3. Scanning electron microscopy

The appearance of constructs on scanning electron microscopy was consistent with histological findings (Fig. 3). Chitosan scaffolds did not seem to have changed after 4 weeks of culture and contained discrete areas where cells were embedded in matrix. These areas were essentially present at the surface of scaffolds with smaller pores. Cell numbers and penetration within scaffolds appeared to improve in constructs presenting larger pores. PGA constructs appeared to consist essentially of a uniform matrix into which cells and remnants of fibers were embedded. The size and shape of cells were consistent with those of chondrocytes.

3.4. Quantitative analyses

PGA constructs contained an amount of water similar to that of normal cartilage and less than chitosan constructs (Table 1). The DNA content of cells used in our study was equal to $5.13 \pm 0.2\text{ pg}$. This factor was used to calculate the number of cells present in constructs based on their respective DNA content. The cell density and GAG concentration in PGA scaffolds were greater than in any of the chitosan groups. The chitosan sponge with the largest pores (CL) contained more chondrocytes and GAG than the matrix with the smallest pores (CS). Constructs produced with PGA or the macroporous chitosan sponge contained similar concentrations of collagen II, and more than the other two groups. GAG/DNA content was not statistically different between groups.

4. Discussion

4.1. Interconnective pore size and chondrogenesis

Constructs produced on macroporous chitosan sponges (CL) contained more GAG and collagen II than those engineered on chitosan scaffolds with pores measuring less than $50\mu\text{m}$ in diameter (groups CS and CM). However, the GAG/DNA ratio and morphologic features of chondrocytes did not vary between groups, suggesting that the biosynthetic activity and phenotype of individual chondrocytes remained similar between chitosan sponges. Instead, the increase in matrix content most likely reflects the greater cellularity of macroporous chitosan constructs.

Our results differ from a previous study, where the cartilaginous tissue formed on porous titanium alloy discs was thicker and contained more GAG when pores decreased in size [28]. In another study, chondrocytic phenotype and biosynthetic activity were improved in collagen matrices containing smaller pores [5]. These results were attributed to the greater surface area of macroporous matrices, spreading chondrocytes over the relatively flat surfaces of pore walls, thereby affecting cell–cell interactions [5]. Small pores are naturally present in normal cartilage matrix, where their size has been estimated to range from 2.5 to 6.5 nm [29]. Pores smaller than $50\mu\text{m}$ have also been recommended to improve biomechanical strength of engineered constructs [30]. These arguments support chondrogenesis on a scaffold with small pores. However, our study supports the concept that larger interconnective pores improve the cellularity and matrix content within the scaffold, potentially producing a larger construct in less time.

The discrepancy between our results and the two previous studies may be explained by differences in pore sizes tested and culture conditions. The matrices tested in these studies contained pores ranging from 13 to $85\mu\text{m}$ in diameter and were cultured under static conditions [5,28]. In both studies, a film of cartilaginous tissue formed at the surface of matrices, with little penetration into the microporous structures. These results are similar to those obtained with

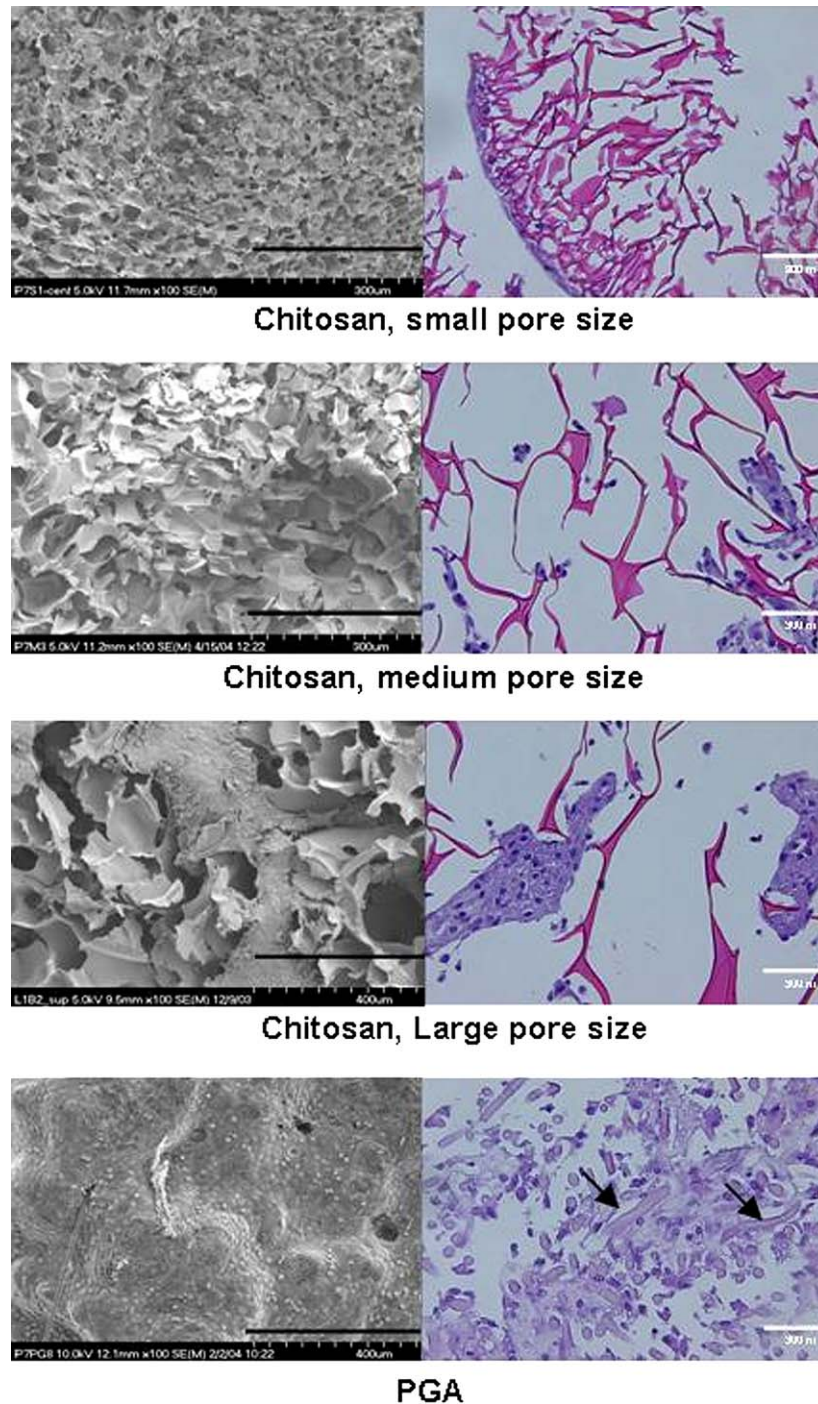


Fig. 3. Histology (H&E, $\times 40$) and scanning electron microscopy of constructs after 4 weeks of dynamic culture. Constructs produced with PGA consisted essentially of a matrix containing more cells than normal cartilage. Whereas very few remnants of PGA remained (arrows), chitosan scaffolds appeared intact. Cells surrounded with extracellular matrix were more obvious in chitosan sponges containing larger pores, especially in the center of scaffolds.

our microporous chitosan sponges (less than $10\ \mu\text{m}$). Lack of cell migration and tissue ingrowth within 3-D scaffolds remains a major constraint in the clinical application of these structures [31]. This is especially relevant under static culture conditions, where high cell density on the surface of the construct may deplete nutrient supply before these nutrients diffuse to cells located deeper within the scaffold [31]. These issues have prompted the search for scaffold

designs and culture techniques improving cell distribution throughout matrices, as a basis for uniform tissue regeneration.

Several “dynamic” culture systems have consequently been proposed to improve the circulation of medium around and within scaffolds cultured for in vitro formation of various tissues [31–33]. Among these, the rotating-wall vessel used in our study has been found to minimize shear

Table 1
Water, cell, glycosaminoglycans and collagen II content of polyglycolic acid and chitosan constructs (mean $\pm$ standard error of the mean)

	CS	CM	CL	PGA
Water (%)	92.02 $\pm$ 0.40 (n = 15) ^b	92.18 $\pm$ 0.21 (n = 17) ^b	91.51 $\pm$ 0.47 (n = 15) ^b	82.59 $\pm$ 0.83 (n = 11) ^a
DNA (μ g/ml)	2.58 $\pm$ 0.31 (n = 8) ^c	2.63 $\pm$ 0.31 (n = 8) ^c	7.18 $\pm$ 0.42 (n = 8) ^b	41.51 $\pm$ 0.14 (n = 5) ^a
Cells (10 ⁶ /ww)	0.36 $\pm$ 0.06 (n = 8)	0.48 $\pm$ 0.11 (n = 8)	1.04 $\pm$ 0.15 (n = 8)	14.06 $\pm$ 0.96 (n = 5)
GAG (%ww)	0.78 $\pm$ 0.31 (n = 8) ^c	0.82 $\pm$ 0.15 (n = 8) ^c	1.3 $\pm$ 0.34 (n = 8) ^b	8.73 $\pm$ 0.46 (n = 5) ^a
GAG/DNA	4.31 $\pm$ 1.44 (n = 8)	4.68 $\pm$ 1.48 (n = 8)	3.42 $\pm$ 1.97 (n = 8)	1.3 $\pm$ 0.19 (n = 5)
Collagen II (%ww)	0.25 $\pm$ 0.06 (n = 7) ^c	0.68 $\pm$ 0.11 (n = 9) ^b	1.76 $\pm$ 0.51 (n = 6) ^a	3.03 $\pm$ 0.40 (n = 4) ^a

CS, chitosan sponge with interconnective pores $\leq 10 \mu\text{m}$ in diameter; CM, chitosan sponge with interconnective pores measuring 10–50 μm in diameter; CL, chitosan sponge with interconnective pores measuring 70–120 μm in diameter; PGA, polyglycolic acid mesh.

^{a,b,c} Within each row, groups with different letters are statistically different ($p < 0.05$).

and turbulence around suspended cells, while providing mechanical stimulation and enhancing cell growth, compared with static cultures [3,34]. This dynamic culture system may explain the presence of cells and matrix within our intermediate chitosan group (CM, pore diameter: 10–50 μm) and the difference with previous results on matrices of similar pore size. Macroporous sponges have been found to improve cell attachment, proliferation and biosynthetic activity, secondary to enhanced diffusion of cells and nutrients into the center of scaffolds [35]. The combined benefits of interconnectivity and dynamic culture explain why cell distribution and function were further improved in chitosan sponges containing the largest interconnective pores in our study.

4.2. Comparison of PGA and chitosan constructs

Our results confirm previous reports of chondrogenesis within chitosan sponges [19]. However, these reports did not include a reference group. Our PGA constructs contained more cells and matrix than any of the chitosan constructs. The decreased water content in the PGA group reflects the fact that (1) PGA was degraded, and (2) PGA was replaced with a matrix that has a water content similar to that of cartilage. The appearance of constructs on SEM and histology was consistent with dissolution of PGA fibers. The degradation of PGA fibers has previously been described as a hydrolytic process influenced by temperature, pH (out of the range of 5.2–7.4), molecular weight and degree of crystallinity [36]. Studies of *in vitro* chondrogenesis on a PGA mesh similar to that used here also documented the degradation of PGA and the presence of PGA remnants in constructs cultured for 28 days under dynamic conditions [37]. The relatively fast degradation of PGA is

believed to enhance cell–cell interactions at high cell densities, thereby stimulating extracellular matrix production [4,38]. However, the GAG/DNA ratio in our study did not support this theory. Instead, the lower cell and matrix contents of chitosan constructs compared to PGA constructs may result from a difference in the number of cells attaching to the scaffolds during the seeding phase. Indeed, the greater number of cells suspended in the medium 48 h after seeding chitosan scaffolds is consistent with a lower attachment of chondrocytes to chitosan compared to PGA scaffolds. This is consistent with our previous evaluation of seeding techniques for PGA and chitosan scaffolds, where a spinner flask and a combined vacuum and dynamic seeding techniques both had a yield rate 10 times lower on chitosan than PGA matrices [22]. In this study, chitosan and PGA scaffolds were placed in the same vessels, creating a competitive environment that may have affected the efficiency of seeding techniques on chitosan, with only 10% of cells attaching to this type of scaffold [22]. Separating chitosan scaffolds from PGA in our study improved the percentage of cells attaching to chitosan to about 70% (based on the number of cells in suspension at 48 h), but did not reach the percentage of attachment obtained with PGA (>90%). The polymers evaluated in our study differed in composition as well as structure. While this affects our ability to distinguish the influence of each factor, it would be impossible to create a sponge with the exact same structural characteristics (porosity, pore size, interconnectivity) as our chitosan scaffolds. Instead, our interest was to identify the scaffold that would best promote chondrogenesis, regardless of structure and composition. This particular PGA mesh was selected for our study because it has extensively been studied under similar culture conditions and can therefore act as a reference [3,4]. Since the composition of our PGA constructs after 4 weeks of culture was identical to that previously reported, we can eliminate issues related to culture conditions as a confounding factor for our results.

The two most commonly used scaffold architectures reported in the literature for cartilage repair consist of porous sponges and non-woven fiber mesh [39]. Although not tested in our study, there is evidence to suggest that chondrocytes may attach preferentially to a mesh rather than foam of similar composition [40]. In a recent study, 75–90% chondrocytes attached to chitosan fibers, suggesting that a difference in structure between the PGA and chitosan constructs in our study may have contributed to our results [18]. In another study, cell adhesivity, proliferation and synthesis of aggrecan were improved when chitosan fibers were covered with 0.04% or 0.07% hyaluronic acid [41]. While none of these studies included PGA mesh as a reference, they provide strategies for improving the potential of chitosan as a matrix for cartilage engineering.

5. Conclusions

Increasing the interconnective pore size of chitosan matrices resulted in the production of constructs containing

more chondrocytes and matrix. This finding most likely results from improved diffusion of nutrients and cells throughout macroporous scaffolds. Although in vitro chondrogenesis is possible with chitosan, the composition of constructs produced on PGA more closely approaches that of natural cartilage. This results from the dissolution and greater cellularity of PGA constructs. Optimizing cartilage engineering, based on cell number/distribution and extracellular matrix production provides a basis for initial selection of scaffolds. However, the biomechanical and biological implications after in vivo implantation will ultimately require further evaluations of final constructs.

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