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Accelerated chondrocyte functions on NaOH-treated PLGA scaffolds

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Abstract

Compared to conventional poly(lactic-*co*-glycolic acid) (PLGA), previous studies have shown that NaOH-treated PLGA two-dimensional substrates enhanced functions of osteoblasts (bone-forming cells), vascular and bladder smooth muscle cells, and chondrocytes (cartilage-synthesizing cells). In this same spirit, the purpose of this in vitro study was to fabricate three-dimensional NaOH-treated PLGA scaffolds and determine their efficacy toward articular cartilage applications. To improve functions of chondrocytes including their adhesion, growth, differentiation, and extracellular matrix synthesis, PLGA scaffolds were modified via chemical etching techniques using 1 N NaOH for 10 min. Results demonstrated that NaOH-treated PLGA three-dimensional scaffolds enhanced chondrocyte functions compared to non-treated scaffolds. Specifically, chondrocyte numbers, total intracellular protein content, and the amount of extracellular matrix components (such as glycosaminoglycans and collagens) were significantly greater on NaOH-treated than on non-treated PLGA scaffolds. Underlying material properties that may have enhanced chondrocyte functions include a more hydrophilic surface (due to hydrolytic degradation of PLGA by NaOH), increased surface area, altered porosity (both percent and diameter of individual pores), and a greater degree of nanometer roughness. For these reasons, this study adds a novel tissue-engineering scaffold to the cartilage biomaterial community: NaOH-treated PLGA. Clearly, such modifications to PLGA may ultimately enhance the efficacy of tissue-engineering scaffolds for articular cartilage repair.
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1. Introduction

Perhaps due to its complex structure and composition, articular cartilage has been known to be one of the most common sources of pain and suffering in the human body. For example, over 1 million surgeries to heal damaged soft tissue components of the knee occurred in the year 2000 alone [1]. Moreover, over 150,000 total knee replacement procedures were performed per year in the US [1]. Many types of mechanical trauma as well as diseases, including osteoarthritis, can lead to degradation of cartilage. Further complicating matters, the ability of cartilage to regenerate or "heal"

itself decreases with age. This is because with age, chondrocyte (cartilage-synthesizing cells) numbers decrease, structural organization of cartilage becomes altered, and a well-defined calcified zone arises in cartilage [2]. In addition, collagen fibers in cartilage generally have thin diameters (0.5 nm) but become thicker (however, still in the nanometer regime) with age and disease [2]. Therefore, finding a suitable material to repair damaged articular cartilage is crucial.

Various materials and techniques have been developed in the past to repair articular cartilage defects. One of the methods investigated includes injection of naturally derived hydrogels (e.g., fibrin glue and alginate glue) [3] or synthetic polymer hydrogels (such as poly(ethylene oxide)) into damaged cartilage areas [4]. Numerous problems exist with the use of natural materials as cartilage replacements such as limited

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availability, antigenicity, and possible transfer of disease [5]. Synthetic materials do not pose some of the same risks as the natural materials; however, sterilization methods can be an issue, in addition to detrimental effects of unpolymerized monomer or polymer debris in vivo [6].

Since some synthetic biodegradable materials such as poly(lactic-co-glycolic acid) (PLGA) copolymers [7] are used and studied for various medical applications demonstrating biocompatibility [8–10], they have been widely investigated in conventional form to serve as cartilage prostheses. According to Ma et al. [8], on poly(glycolic acid) (PGA) scaffolds, chondrocytes have been shown to proliferate and produce an extracellular matrix that has a similar aggregate modulus as normal bovine cartilage after 12 weeks of in vitro culture. Clearly, such biodegradable polymers are crucial to completely reconstructing articular cartilage from synthetic scaffolds. PLGA is also one of a few synthetic materials that have been used in products approved by the FDA for clinical applications.

However, PLGA in the form as used today, does not mimic the topographical features that collagen and other extracellular matrix proteins create in cartilage tissue. Several studies have shown that such biologically inspired nanometer surface roughness promotes the adhesion, proliferation, and subsequent functions of various cell lines like osteoblasts (bone-forming cells) as well as vascular and bladder smooth muscle cells [11–13].

For these reasons, this study focused on treating conventional PLGA scaffolds with NaOH in an attempt to create an optimal surface to improve chondrocyte functions pertinent for more effective cartilage tissue-engineering applications.

2. Materials and methods

2.1. Polymer preparation

PLGA (50:50 wt% PLA:PGA; 12,000–16,500 kDa; Polysciences Inc.) copolymer scaffolds were fabricated using chloroform (Mallinkroft, Paris, KY) and heat treatments. Polymer pellets (0.5 g) were dissolved in 6 ml of chloroform in heat (below 60 °C) for 30 min and a salt-leaching method [14,15] was applied to introduce pores into the substrate. For this process, 4.5 g of salt (NaCl, up to 250 μm diameter; Sigma) was added to the polymer solution, mixed with a stirring rod and collectively poured onto a Teflon-coated Petri dish (VWR). The polymer solution was dried for 24 h in air and then 48 h in a vacuum oven (15 in Hg). Then the polymer was soaked in water for 3 days to remove the salt. Some of the resulting scaffolds were soaked in 1 N NaOH for 10 min at room temperature. Substrates were then rinsed with distilled water several times until the

supernatant pH stabilized at 7.4. For sterilization purposes, both untreated and NaOH-treated PLGA scaffolds were soaked in 70% ethanol for 15 min.

Glass coverslips were used as reference substrates. Borosilicate glass coverslips (18 mm diameter; Fisher Scientific) were soaked in acetone (Sigma) for 10 min, sonicated in acetone for 10 min, soaked in 70% ethanol (Pharmco Products) for 10 min, and sonicated in ethanol for 10 min. Distilled water was used to rinse the coverslips in between each step. They were then etched in 1 N NaOH (diluted in dH₂O) for 1 h and rinsed with distilled water. For sterilization, coverslips were autoclaved at 255 °F for 35 min. All samples were used within 1 week after sterilization.

2.2. Scanning electron microscopy

Topography and roughness of the substrates were evaluated using scanning electron microscopy (JOEL JSM-840). Average pore diameter and percent porosity were calculated using these images. Samples were coated with gold via a sputter-coater at ambient temperature. Micrographs were taken at 500× and 2500× with 5–7 kV.

2.3. Brunauer, Emmett, and Teller surface area measurement

Total surface area was determined via a Coulter SA 3100 Series Surface Area and Pore Size Analyzer (Beckman Coulter) with Brunauer, Emmett, and Teller (BET) calculations according to the manufacturer's instructions. Specifically, nitrogen gas was allowed to adsorb onto open pores of the substrate from a flowing mixture of nitrogen adsorbate in an inert, non-adsorbable, helium carrier gas. A glass cell containing the substrate was placed into liquid nitrogen for gas adsorption onto each PLGA substrate. After adsorption of nitrogen gas by each substrate was completed, the glass cell containing substrate was placed in a water bath at ambient temperature and desorption of gas was calculated. Surface area was measured on at least three samples for each type of scaffold.

2.4. Articular chondrocyte cell culture

Human articular chondrocytes (cartilage-synthesizing cells; Cell Applications Inc.) were cultured in Chondrocyte Growth Medium (Cell Applications Inc.) on 100 mm Petri dishes (VWR). Cells were incubated under standard cell culture conditions: (specifically) a sterile, humidified, 5% CO₂, 95% air, 37 °C environment. Chondrocytes used for the following experiments were at passage numbers below 10. The phenotype of these chondrocytes has previously been characterized by synthesis of Chondrocyte Expressed Protein-68

(CEP-68) for up to 21 days in culture under the same conditions [16].

2.5. Chondrocyte numbers

Chondrocytes were seeded at 50,000 cells/scaffold or glass substrate and were allowed to attach and grow for time points of 1, 3, and 7 days. At the end of each time point, non-adherent cells were removed by rinsing with phosphate buffered saline (PBS) solution. Cell counts were determined via the Cytotox 96 assay (Promega) according to the manufacturer's instructions. Specifically, substrates were placed into clean wells, frozen down at $-70\,^{\circ}C$ for 30 min, and then incubated for 15 min at $37\,^{\circ}C$ to lyse cells. The resulting solution was centrifuged at $250 \times g$ for 4 min and 50 μl of it was placed into a well of a 96-well plate. In all, 50 μl of Substrate Mix (Cytotox 96, Promega) was added and the plate was incubated for 30 min at room temperature, protected from light. After the incubation, a Stop Solution (Cytotox 96, Promega) was added to each well and light absorbance was determined using a microplate reader and a spectrophotometer (SpectraMax 190, Molecular Devices Corp.) at 490 nm using computer software (SoftMax Pro 3.12, Molecular Devices Corp.). The resulting light absorbance was compared to a standard curve to calculate the number of cells. A standard curve was constructed by linear regression analysis, using light absorbance of cell lysates at known concentrations. All experiments were run in triplicate and repeated at least three times.

2.6. Confocal images of chondrocytes

Following the same procedures described in Section 2.5, confocal microscopy (MRC 1024, BioRad) along with Confocal Assistant software (BioRad) was utilized to view and capture three-dimensional images of the scaffold and cells at magnifications of $200 \times$ and $600 \times$. For this purpose, Hoescht 33258 dye (Sigma) was used for cell nuclei staining, and Nile Red (Molecular Probes) was used for lipophilic cell body and scaffold matrix staining. For nuclei staining, cells on the substrates were submerged in 0.1% Hoescht stain for approximately 10 s and rinsed with PBS twice. For lipophilic body staining, substrates with cells were immersed in 0.5% (5 μg/ml) Nile Red stain for 5 min and rinsed with PBS twice. Z-series images were taken using the confocal microscope, in which images of horizontal planes were taken for approximately 100 consecutive vertical steps of 1 μm. Duplicate images of each substrate were obtained.

2.7. Extracellular matrix detection assays

Some of the proteins synthesized by chondrocytes in situ (specifically, total intracellular collagen and glyco-saminoglycans (GAGs)) were investigated as measures of cell differentiation when cultured on the PLGA scaffolds of interest to this study.

2.7.1. Total intracellular protein

Total amount of intracellular proteins from the chondrocyte lysates was determined using a commercially available kit (BCA Protein Assay Reagent Kit; Pierce Biotechnology) with a microplate reader and a Molecular Devices SpectraMax 190 spectrophotometer with SoftMax Pro software (Molecular Devices). Following the manufacturer's instructions, the protein-eluted solution was mixed with copper sulfate and bicinchoninic acid and incubated at $37\,^{\circ}C$ for 30 min. This process allowed for reduction of Cu^{+2} to Cu^{+1} by a protein (if present) in an alkaline environment, and the cuprous cation could be detected colorimetrically via an agent containing bicinchoninic acid [17]. Absorbance was measured at 562 nm on the plate reader. The amount of protein adsorbed onto each surface was determined by comparing measurements to a standard curve obtained from the light absorbance of predetermined albumin (Pierce Biotechnology) concentrations. The total amount of protein was normalized to cell number and volume. The experiments were performed in triplicate and repeated at least three times.

2.7.2. Total intracellular collagen

For intracellular collagen concentration measurements, Sirius Red dye (Direct Red; Sigma) was used to stain collagens and a spectrophotometer was utilized to determine the concentration [18]. For this purpose, the cells from the experiments outlined in Section 2.5 were lysed using freeze–thaw methods. Specifically, the cell extracts (50 μl/well) were placed in 96-well plates in triplicate per substrate type. The plates were placed in a humidified incubator (at $37\,^{\circ}C$) for 16 h and then in a dry incubator (at $37\,^{\circ}C$) with desiccant. Each well was washed with 200 μl dH_2O three times for 1 min/wash. In each well, 100 μl of 0.1% Sirius Red stain (0.05 g Sirius Red powder per 50 ml picric acid) was allowed to sit for 1 h at room temperature. Using 200 μl of 0.1 M HCl the plates were washed five times with 10 s/wash. The stain was then washed with 200 μl of 0.1 M NaOH for 5 min and mixed well. The stain was placed into a second plate to read the absorbance in a microplate reader at 540 nm. A standard curve was constructed as microgram of collagen versus absorbance at 540 nm. For the standard curve, 0.1% collagen type I solution (Sigma) was diluted at small increments and the light absorbance of the Sirius Red stain in these dilutions was recorded. The total amount of collagen was normalized to cell number and volume. The experiments were performed in triplicate and repeated at least three times.

2.7.3. Sulfated intracellular glycosaminoglycan

GAGs were determined also using a commercially available spectrophotometric assay (Blyscan assay kit, Biocolor) according to the instructions from the cell manufacturer. For this process, 50 μl aliquots of cell extracts obtained from cell experiments described in Section 2.5 were placed into a set of 1.5 ml microcentrifuge tubes. The tubes were filled with PBS to fill up to 100 μl. Blyscan dye (1 ml) was added to all tubes and the tubes were placed on a shaker for 30 min. At this point, tubes were centrifuged at $10,000 \times g$ for 10 min and the dye solution was carefully removed without disturbing the precipitated GAG on the bottom of the tube. Then, 1 ml dissociation reagent was added to the tubes and mixed well using a vortex mixer for 10 min. Each sample (200 μl) was transferred to a well in a 96-well plate and absorbance was read at 656 nm using a spectrophotometer. GAG content was normalized to cell number and volume. The experiments were performed in triplicate and repeated at least three times.

2.8. Statistical analysis

Data were analyzed using one-tailed, standard Student's *t*-tests with $p < 0.05$ indicating statistical significance. Values with $p < 0.1$ were reported as well.

3. Results

3.1. Substrate characterization

Distinct topographical differences were observed on the NaOH-treated compared to non-treated PLGA scaffolds. Specifically, NaOH-treated PLGA scaffolds exhibited considerably more surface roughness (see Fig. 1). Total surface area, average pore diameter, and percent porosity of each scaffold are shown in Table 1. The differences due to NaOH treatment suggest that NaOH-treated PLGA had more and larger pores compared to non-NaOH-treated PLGA. Specifically, the average pore diameter of the NaOH-treated scaffold was twice that of the non-treated scaffold. In addition, a

Table 1
Scaffold porosity characteristics of NaOH-treated and non-treated PLGA

PLGA chemical treatment	Average pore diameter (μm)	Porosity (%)	Surface area (m^2/g)
Non-treated	98.75 ± 37.05	30	96.91 ± 22.07
NaOH treated	$215.0 \pm 55.08^*$	50	$441.25 \pm 76.75^*$

Note: Surface area, pore diameter, and percent porosity of the NaOH-treated scaffolds were significantly greater than those of the non-treated PLGA scaffolds.

$^*p < 0.05$ (compared to the respective value for non-treated scaffolds).
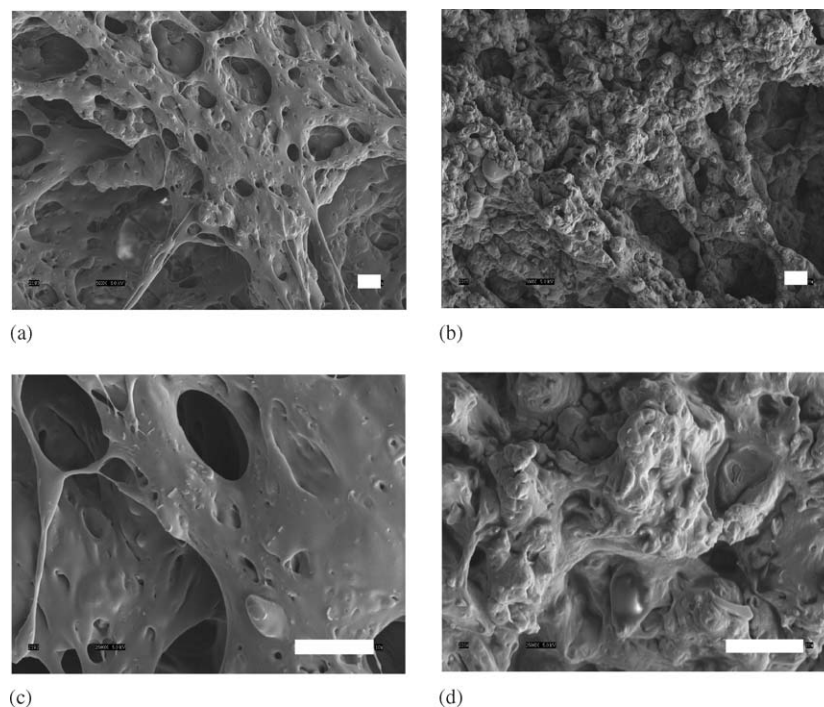

(a)

(b)

(c)

(d)

Fig. 1. SEM images of NaOH-treated (b, d) and non-treated (a, c) PLGA scaffolds. Increased surface roughness was evident on NaOH-treated compared to non-treated PLGA. Top pictures are at a lower magnification, and the bottom pictures are taken at a higher magnification. Bar = 10 μm.

20% difference in percent porosity was observed between the two scaffold types. It is speculated that NaOH treatments created pores and allowed smaller pores to merge into larger pores. Due to more and larger pores, the NaOH-treated PLGA had significantly ($p < 0.05$) more surface area when compared to non-treated PLGA.

3.2. Chondrocyte numbers

At 1 day, significantly ($p < 0.05$) greater numbers of chondrocytes were observed on NaOH-treated PLGA compared to non-treated PLGA (Fig. 2). This trend continued as chondrocyte numbers were greater ($p < 0.1$) on NaOH-treated compared to non-treated PLGA after 3 and 7 days. However, cell numbers did not significantly increase in either PLGA scaffold from 1 to 3 to 7 days. Cell numbers were statistically ($p < 0.01$) greater on glass after 7 days compared to 1 day of culture.

3.3. Cell and scaffold morphology

Confocal images of chondrocytes cultured on the scaffolds after 7 days confirmed the previously reported rough surface features, as well as altered porosity, of the NaOH-treated compared to non-treated PLGA scaffolds (Fig. 3). Greater pore diameter and porosity shown in the NaOH-treated compared to the non-treated PLGA scaffold facilitated penetration of the cells into the scaffold, as indicated by the arrow in Fig. 3.
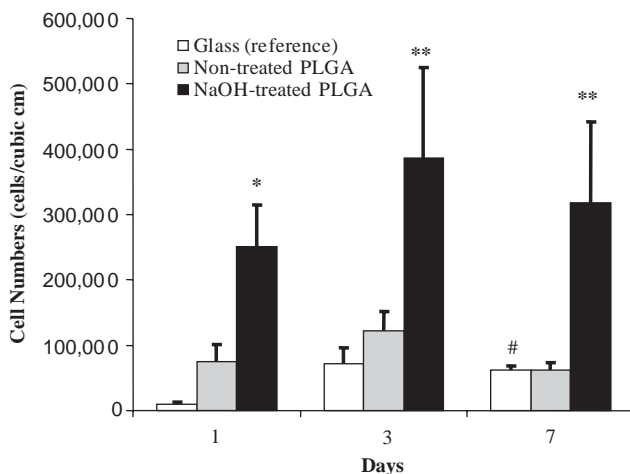

Fig. 2. Cell numbers on NaOH-treated were greater than on non-treated PLGA scaffolds at each time point. Values were normalized to the substrate volume, except for the reference glass, which was normalized to substrate area. Values are mean ± SEM, $n = 3$, $*p < 0.05$ (compared to cell density on non-treated PLGA on the respective day), $**p < 0.1$ (compared to cell density on non-treated PLGA on the respective day), $^{\#}p < 0.01$ (compared to cell density on day 1 on the respective substrate).

3.4. Extracellular matrix component synthesis

To observe subsequent chondrocyte functions, total intracellular protein, intracellular GAGs, and total intracellular collagen content studies were conducted as previously stated. The results demonstrated that compared to non-treated PLGA, chondrocytes cultured on NaOH-treated PLGA synthesized greater amounts of total intracellular proteins, as shown in Fig. 4. Specifically, significantly ($p < 0.05$) greater amounts of total intracellular proteins were measured on NaOH-treated compared to non-treated PLGA on day 3. No statistical differences were observed among the various PLGA scaffolds on day 1. In addition, the amount of synthesized intracellular protein increased ($p < 0.05$) from day 3 to 7 on non-treated PLGA scaffolds.

As demonstrated in Fig. 5, intracellular collagen was synthesized at significantly ($p < 0.01$) larger amounts when chondrocytes were cultured on NaOH-treated compared to non-treated PLGA scaffolds on days 3 and 7. Specifically, collagen amounts from NaOH-treated PLGA were 16 times greater on day 3 and 11 times greater on day 7 compared to the non-treated PLGA. The amount of collagen produced on non-treated PLGA was almost twice as much on day 7 compared to day 3 ($p < 0.01$).

Similar to collagen amount, intracellular GAG content in chondrocytes cultured on NaOH-treated PLGA scaffolds was significantly ($p < 0.01$) greater compared to the cells cultured on non-treated PLGA on days 3 and 7 (Fig. 6). More specifically, GAG content on NaOH-treated compared to non-treated PLGA scaffolds was 16 times greater on day 3 and 11 times greater on day 7. The amount of GAG synthesized intracellularly by chondrocytes doubled from day 3 to 7 on both NaOH-treated and non-treated PLGA ($p < 0.05$), which followed a similar trend as that previously reported in the collagen data (Fig. 5). In addition, GAG amount on non-treated PLGA increased significantly ($p < 0.05$), by almost five times, from day 1 to 7.

4. Discussion

Results of this study demonstrated that NaOH treatment of PLGA scaffolds greatly influenced chondrocyte behavior. Not only did the cell numbers increase on NaOH-treated PLGA scaffolds, but also subsequent functions of chondrocytes (such as collagen, GAG, and overall total protein synthesis) were enhanced as well. The basis of these phenomena can easily be attributed to the altered surface properties of such scaffolds due to NaOH treatment [16].

Clearly, surface properties of a scaffold material have significant influence over initial protein interactions that
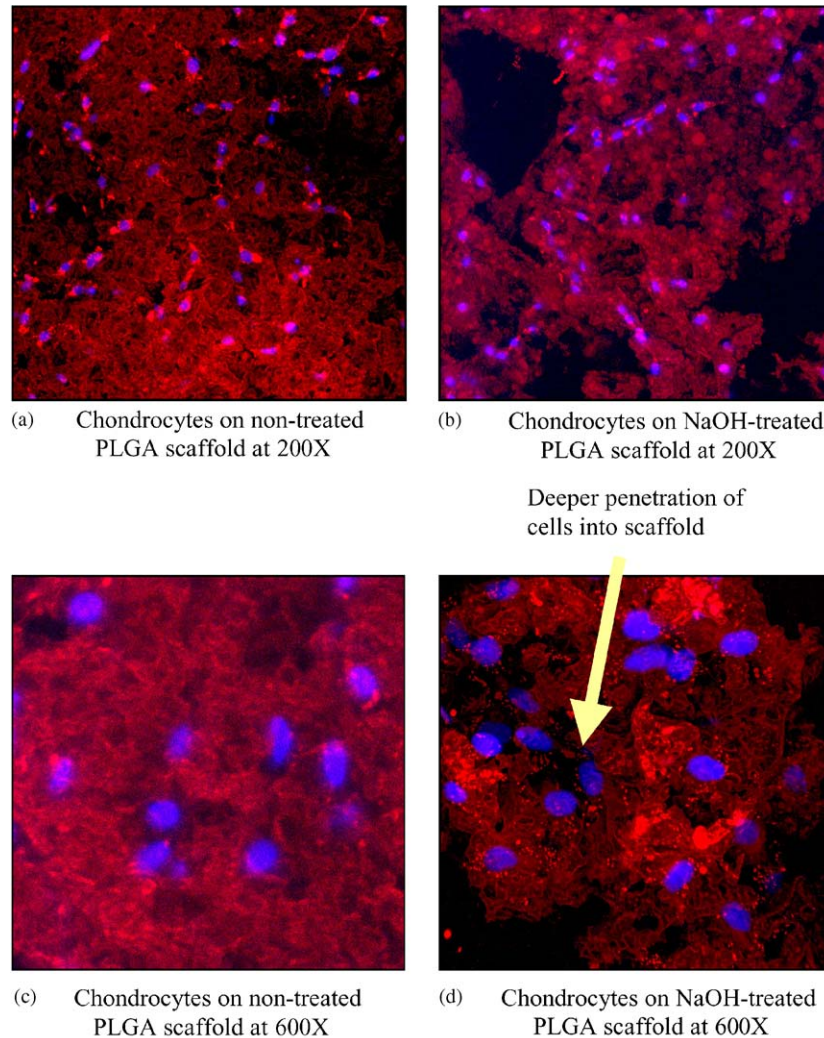
(a) Chondrocytes on non-treated PLGA scaffold at 200X

(b) Chondrocytes on NaOH-treated PLGA scaffold at 200X

Deeper penetration of cells into scaffold

(c) Chondrocytes on non-treated PLGA scaffold at 600X

(d) Chondrocytes on NaOH-treated PLGA scaffold at 600X

Fig. 3. (a–d) Confocal images at 200 × and 600 × of chondrocytes cultured for 7 days on either NaOH-treated or non-treated PLGA scaffolds. Blue color represents the chondrocyte nuclei and red color represents the scaffold and the cell body. Arrow indicates deeper penetration of chondrocytes into NaOH-treated PLGA scaffolds.

mediate subsequent cell response. Surface topography, chemistry, and wettability have all been shown to influence cell/material interactions [19,20]. For example, Gao et al. [21] have shown that NaOH treatment of polyesters, including PGA, promoted carboxylic acid exposure on the polymer surface, thus making it more hydrophilic. According to contact angle measurements, contact angles were smaller on NaOH-treated compared to non-treated PGA. XPS analysis done by Gao et al. exhibited greater percentages of oxygen content on the surface of NaOH-treated PGA.

Kay et al. [11] also observed an increase in osteoblast and chondrocyte density on NaOH-treated two-dimensional PLGA films. In this study, cell adhesion was investigated on various composites of ceramics (such as titania and alumina) and PLGA. These composites exhibited a range of surface topographies either through the use of different ceramic grain sizes (from nanometer to micron) or through PLGA NaOH treatments. When osteoblasts and chondrocytes were exposed to NaOH-treated and non-treated two-dimensional PLGA films, greater numbers of both cell lines adhered to the altered surface. The present study continued such promising results and transferred them into the three-dimensional world.

Several studies have demonstrated the effects of surface topography alone without changing the chemistry (or other polymer properties) on functions of cells; surface roughness was a major contributor to promoting cell attachment and growth in these studies [12–13,22]. For example, Thapa et al. [22] achieved nanometer PLGA surface roughness values without a change in chemistry via silastic casting techniques. Similar to this present study, NaOH treatment of PLGA was initially applied to create the desired roughness on the surface. Then, a silastic mold was fabricated using this
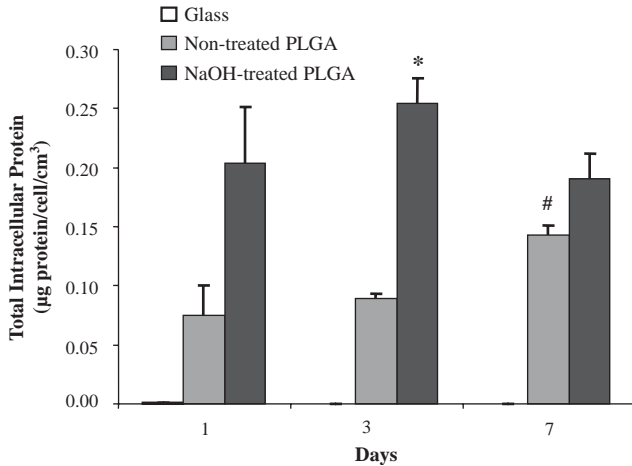
Fig. 4. Greater total intracellular protein amounts were observed when chondrocytes were cultured on NaOH-treated PLGA. Values are mean $\pm$ SEM, $n = 3$, $^*p < 0.05$ (compared to total intracellular protein on non-treated PLGA), $^{\sharp}p < 0.05$ (compared to total intracellular protein on day 3 on the respective PLGA).
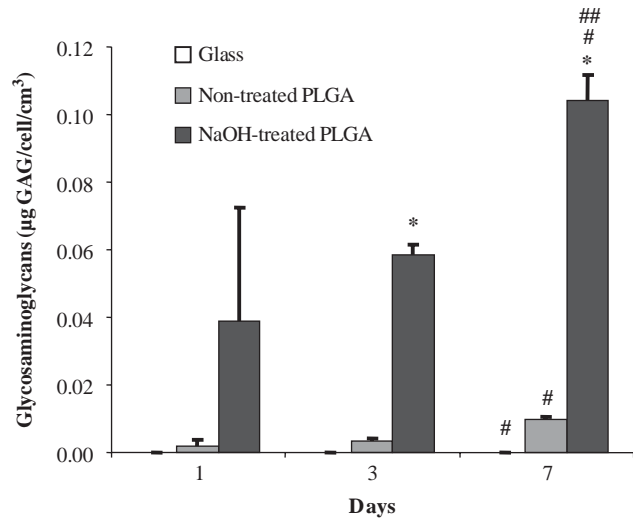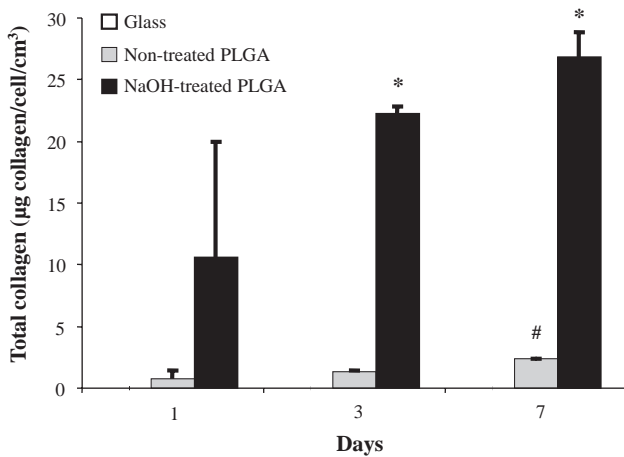

Fig. 5. Compared to non-treated PLGA, chondrocytes on NaOH-treated PLGA synthesized greater amounts of collagen. Values are mean $\pm$ SEM, $n = 3$, $^*p < 0.01$ (compared to total intracellular collagen on non-treated PLGA), $^{\sharp}p < 0.01$ (compared to total intracellular collagen on day 3 on the respective scaffold).

Fig. 6. Compared to non-treated PLGA, chondrocytes on NaOH-treated PLGA synthesized greater amounts of GAG. Values are mean $\pm$ SEM, $n = 3$, $^*p < 0.01$ (compared to GAG on non-treated PLGA on respective day), $^{\sharp}p < 0.05$ (compared to GAG on day 3 on the respective scaffold), $^{\sharp\sharp}p < 0.05$ (compared to GAG on day 1 on the respective PLGA).

In this manner, it can be speculated that the increased roughness as obtained in the present study on NaOH-treated PLGA scaffolds may be an important parameter that promoted chondrocyte function. However, clearly chemistry changes occurred through NaOH treatment of PLGA; future studies are needed to delineate which of these properties (or both) are primarily responsible for enhanced chondrocyte function on NaOH-treated PLGA.

5. Conclusion

NaOH treatment of polymers (such as PLGA) has been shown to increase bladder, vascular, and bone cell functions. Yet, studies of chondrocyte function on NaOH-treated PLGA scaffolds have been scarce. These results provided the first evidence of increased chondrocyte attachment, growth, and matrix synthesis (as indicated by intracellular collagen and glycosaminoglycan content) on PLGA scaffolds treated with NaOH. Although further in vivo studies would be needed to substantiate this in vitro evidence, these findings strongly demonstrate the potential use of NaOH-treated PLGA for enhanced articular cartilage repair.

Acknowledgements

NaOH-treated PLGA surface. The silastic mold was used to cast PLGA films with nanometer roughness features without chemical changes. Most importantly, this process eliminated the chemistry differences on the PLGA substrates to allow investigation of surface roughness on cellular functions. Results indicated that bladder smooth muscle cell density increased on PLGA films with nanometer compared to traditional micron surface roughness. Similarly, Miller et al. [12] observed enhanced vascular smooth muscle cell functions on substrates created using the same technique as Thapa et al. [13].

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