Published in final edited form as:

Acta Biomater. 2011 May ; 7(5): 2080–2090. doi:10.1016/j.actbio.2011.01.012.

The effects of fixed electrical charge on chondrocyte behavior

Mahrokh Dadsetan^{a,*}, Matthias Pumberger^{a,b,*}, Michelle E. Casper^a, Kristin Shogren^a, Melissa Giuliani^{a,c}, Terry Ruesink^a, Theresa E. Hefferan^a, Bradford L. Currier^a, and Michael J. Yaszemski^{a,+}

^aDepartment of Orthopedic Surgery, Mayo Clinic College of Medicine, 200 First Street SW, Rochester MN 55905, USA

^bParacelsus Medical University, Strubergasse 21, 5020 Salzburg, Austria

^cAntioquia School of Engineering, Medellin, Colombia

Abstract

In this study, we have compared the effects of negative and positive fixed charge on chondrocyte behavior *in vitro*. Electrical charges have been incorporated into oligo(poly(ethylene glycol) fumarate) (OPF) using small charged monomers such as sodium methacrylate (SMA) and (2-(methacryloyloxy) ethyl)-trimethyl ammonium chloride (MAETAC) to produce negatively and positively charged hydrogels, respectively. The hydrogel physical and electrical properties were characterized through measuring and calculating the swelling ratio and zeta potential, respectively. Our results revealed that the properties of these OPF modified hydrogels varied according to the concentration of charged monomers. Zeta potential measurements demonstrated that the electrical property of the OPF hydrogel surfaces changed due to incorporation of SMA and MAETAC and that this change in electrical property was dose-dependent. Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy was used to determine the hydrogel surface composition. To assess the effects of surface properties on chondrocyte behavior, primary chondrocytes isolated from rabbit ears were seeded as a monolayer on top of the hydrogels. We demonstrated that the cells remained viable over 7 days and began to proliferate while seeded on top of the hydrogels. Collagen type II staining was positive in all samples; however, the intensity of the stain was higher on negatively charged hydrogels. Similarly, GAG production was significantly higher on negatively charged hydrogels compared to neutral hydrogel. Reverse transcription polymerase chain reaction showed up-regulation of collagen type II and down-regulation of collagen type I on the negatively charged hydrogels. These findings indicate that charge plays an important role in establishing an appropriate environment for chondrocytes and hence in the engineering of cartilage. Thus, further investigation into charged hydrogels for cartilage tissue engineering is merited.

Keywords

hydrogel; cartilage tissue engineering; OPF; scaffold

⁺Correspondence to: Michael J. Yaszemski, M.D., Ph.D., 200 First Street SW, MS 3-69, Rochester MN 55905, Phone: 507-284-3747, Fax: 507-284-5075, yaszemski.michael@mayo.edu.

^{*}These authors contributed equally to this work.

Conflicts of Interest

A non provisional patent has been filed for photocrosslinkable oligo(poly(ethylene glycol) fumarate used in this research, and this technology has been licensed to BonWrx.

1. Introduction

Even though articular cartilage is known for its durability and ability to resist high stresses and strains, once injured it has a very poor intrinsic capacity to heal itself. Unlike most tissue, cartilage is composed of one cell type, chondrocytes, and an extracellular matrix (ECM) which is avascular and lacks a neuronal supply. Damaged cartilage progressively deteriorates, often ending in joint destruction and even complete loss of function. Surgical treatments available today are microfracture, drilling, abrasion chondroplasty, mosaicplasty and autologous chondrocyte implantation (ACI). The concept behind microfracture, drilling, and abrasion chondroplasty is to form a connection between the defect and its subchondral bone by inducing blood clot formation. Although new cartilage is formed, it is inferior in its composition and biomechanical properties when compared to native articular cartilage [1]. Relief from pain is what justifies these procedures to be used as standard surgical intervention strategies for repairing damaged cartilage. ACI is a newer surgical intervention strategy in which chondrocytes are harvested arthroscopically, expanded *in vitro* and implanted in the debrided area, where they are secured in place by a periosteal flap [2, 3]. In order to reduce the amount of tissue hypertrophy associated with ACI, a second generation of ACI techniques was developed in which scaffolds were used instead of periosteum to secure the cells into the defect area [2, 4]. These improved results have led to an explosion of interest in developing an ideal scaffold for the next generation of ACI [4]. In general, each of these surgical interventions works by creating a functional tissue that reduces some of the pain associated with damaged cartilage. However, they each are associated with their own specific shortcomings that include donor site morbidity, extensive surgical procedures, cost, host pain and availability of cells.

In theory, cartilage tissue engineering combines a scaffold, cells and growth factors to create a tissue mimicking native healthy cartilage biochemically and structurally. This discipline, however, is still in its infancy. There is no consensus on an optimal scaffold, best cell type or combination of growth factors. Moreover, since there are numerous diseased and damaged cartilage states, an individualistic approach to cartilage repair may be required, using different combinations for optimal repair of each state of damage or disease. With the lack of intrinsic repair modalities, repairing damaged cartilage with engineered cartilage tissue constructs is one possible solution.

In the present study, oligo(poly(ethylene glycol) fumarate) (OPF), a derivative of poly(ethylene glycol) (PEG) was used to create hydrogels with different charges. PEG itself has been under investigation in cartilage tissue engineering for years [5–7]. For example, it has been shown that the cross-linking density of PEG has an effect on chondrocyte morphology [7], and loading has an effect on chondrocyte metabolism [6]. In 2001, Jo et al. were able to synthesize OPF by connecting PEG and fumaric acid through ester bonds [8]. OPF was chosen in this study because it has a high degree of swelling in aqueous environments (>95%) and other properties which mimic native cartilage [9]. Cross-linking density, water content, modulus and surface tension can be modified in this hydrogel in order to optimize cell survival, proliferation and extracellular matrix secretion [10]. Also, it has been previously shown that OPF is biodegradable, biocompatible and degraded through hydrolysis of the ester bonds [9].

Recent research efforts have been directed towards the effect of incorporating charged molecules into PEG-based hydrogels [11]. When chondroitin was incorporated into PEG-hydrogels there was a positive effect versus pure chondroitin sulfate gels [11]. Total collagen content and collagen type II gene expression increased, but the aggrecan content remained unchanged. In 2010, the same group demonstrated an increase of collagen and proteoglycan content of bovine chondrocytes in charged PEG hydrogels under dynamic

loading conditions [12]. However, the effect of incorporating charge into OPF hydrogels has yet to be determined. From our laboratory, Dadsetan et al. recently demonstrated that neuron attachment and differentiation of dorsal root ganglia improved, and neurite extension was significantly greater when the neurons were cultured on OPF hydrogels with small charged monomers [13]. Although OPF had been shown to be a promising candidate for cartilage tissue engineering [9], the impact of charge in the OPF hydrogels on chondrocyte behavior is still unknown. Since previous studies using PEG hydrogels with incorporated charge demonstrated a positive effect in other cell lineages and chondrocytes in particular, it seems logical to deduce that charge might also affect the cartilage tissue quality when incorporated into OPF hydrogels.

Aggrecan is the most abundant protein expressed by chondrocytes. These huge molecules have a high anionic charge from the numerous branches of charged anionic sulfate (SO_3^-) and carboxyl (COO^-) that they contain. Given that fixed charge density of the ECM plays a key role in maintaining healthy cartilage [12, 14], the charge status of engineered cartilage matrix is also likely to have an effect on the regenerated cartilage tissue.

In this study, we compared the effects of negative and positive fixed charge on chondrocyte behavior *in vitro* to test the hypothesis that engineered cartilage incorporating negatively charged molecules in the matrix would more closely resemble the structure and function of native cartilage than engineered cartilage with positively charged matrix. Small negatively charged molecules of sodium methacrylate (SMA) were copolymerized with the OPF hydrogel to produce a negatively charged hydrogel. [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC), which is a positively charged monomer, was copolymerized with the OPF for comparison of the charge effects. The resulting polymers were characterized by assessing the swelling ratio, zeta potential, ion conductivity and surface composition. After hydrogel characterization, chondrocytes were seeded on top of the hydrogels and stained for viability and collagen type II. For protein expression, the normalized GAG production was assessed. These results were then compared with those of the neutrally charged hydrogels.

2. Materials and Methods

2.1. OPF synthesis

OPF was synthesized using polyethylene glycol (PEG) with an initial molecular weight of 10,000 according to a previously described method [8]. Briefly, 50g (polyethylene) glycol (PEG) was azeotropically distilled in toluene to remove residual water and then dissolved in 500ml of distilled methylene chloride. The resulting PEG was placed in an ice bath and purged with nitrogen for 10 minutes. Then, 0.9 mol triethylamine (TEA: Aldrich, Milwaukee, WI) per mol PEG, and 1.8 mol distilled fumaryl chloride (Acros, Pittsburgh, PA) per mol PEG were added dropwise. The reaction vessel was removed from the ice bath and stirred at room temperature for 48 h. Methylene chloride was removed by a rotary evaporator, followed by dissolving the OPF in ethyl acetate and filtering to remove the salt from the reaction of TEA and chloride. OPF was recrystallized in ethyl acetate and vacuum dried overnight.

2.2. Hydrogel preparation

Hydrogels were made by dissolving OPF macromer with a final concentration of 33% (w/w) in deionized water containing 0.05% (w/w) Irgacure 2959 (Ciba-Specialty Chemicals, Tarrytown, NY) and 0.33% (w/w) N-vinyl pyrrolidinone. Sodium methacrylate (SMA) and [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC) were added to the hydrogel solution to obtain negatively and positively charged hydrogels, respectively (Table

1). The hydrogels were cross-linked using 365 nm UV light at intensity of $\sim 8 \text{ mW/cm}^2$ (black-Ray Model 100AP, Upland, CA) for 30 minutes.

2.3. Attenuated Total Reflectance (ATR) Fourier Transform Infrared (FTIR) Spectroscopy

Using an ATR-FTIR spectroscope (Nicolet 8700), coupled to a Continuum microscope (Thermo Electron Corp., Madison, WI), we analyzed the surface of the modified and unmodified hydrogels. The microscope used an ATR slide on a germanium crystal. Spectra were collected at a resolution of 4 cm^{-1} for 128 scans with a sampling area of $150 \times 150 \mu\text{m}$.

2.4. Mechanical testing

After crosslinking, hydrogels were cut with a cork borer into 10 mm diameter disks, and were swollen in phosphate-buffered saline (PBS, pH 7.0) for 24 hours. The compressive modulus of the hydrogels was determined using a dynamic mechanical analyzer (DMA-2980, TA Instruments, New Castle, DE). Mechanical testing was performed under load control, where load was applied at a rate of 4 N/min. Stress and strain data collected during testing were plotted, and the storage modulus was determined as the slope of the linear region of the stress versus strain curve. Ultimate strength was defined as the maximum stress before failure.

2.5. Swelling ratio

Hydrogel disks were vacuum dried after fabrication, weighed (W_i , initial weight) and swelled for 24 hours at 37°C in different solutions including: KOH (pH 13), HCl (pH 1), PBS (pH 7), and deionized water. Swollen samples were blotted dry and weighed (W_s , swollen weight) followed by drying in a vacuum oven at reduced pressure, and weighed again (W_d , dried weight). The swelling ratios of the hydrogels in different solutions were calculated using the following equation:

$$\text{Swelling ratio} = (W_s - W_d) / W_d$$

Sol fraction was measured in deionized water to determine the amount of uncrosslinked polymer using the following equation:

$$\text{Sol Fraction} = [(W_i - W_d) / W_i] \times 100$$

2.6. Zeta potential and conductivity

Zeta potential and conductivity were measured by a Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd., Worcestershire, United Kingdom). Samples were swollen in deionized water to the equilibrium state and ground into small particles. After drying in a vacuum oven for 24 hours, 5 mg of the particles were weighed, diluted in 1 mL deionized water and measured. The pH of hydrogel dispersions in deionized water was measured using a pH meter.

2.7. Chondrocyte isolation, culture and characterization

All animal surgeries in this study were performed according to a protocol approved by Mayo Clinic Institutional Animal Care and Use Committee (IACUC). Chondrocytes were isolated from the ears of three to five month old New Zealand white rabbits as reported by Klagsbrun [15]. Briefly, the cartilage was separated from the rabbit ears, chopped into small pieces and digested with sterile 0.2% collagenase (Worthington, NJ, USA) for 15 hours. The resulting cell suspension was filtered twice, and isolated chondrocytes were seeded in ventilated 75

cm² polystyrene monolayer cell culture flasks (Costar, NY, USA) at an initial density of 10⁴ cells/cm². The flasks were cultured at 37° C, 5% CO₂ and 100% humidity. The cells were supplied with 10ml DMEM Ham's F12 (Sigma, St. Louis, MO), 10% FBS (Hyclone, UT, USA) and streptomycin/penicillin (Gibco, Carlsbad, CA). They were enzymatically detached (0.25% trypsin; Worthington, NJ) when confluent, and used in subsequent experiments.

Swollen hydrogel disks in triplicate were disinfected with 70% ethanol and washed several times with PBS under sterile conditions. Hydrogel films were placed into 24-well tissue culture plates, secured with sterile silicone rubber rings (Cole-Parmer, Vernon Hills, IL) and incubated in DMEM media for 24 hours prior to cell culture. Suspended chondrocytes were seeded onto the hydrogels at a density of 20,000 cells/cm² and incubated at 37°C. Medium was replaced every two to three days.

2.8. Viability

Viability of the seeded cells after 1, 3 and 7 days was examined using the Live/Dead Kit (Molecular Probes, Eugene, Oregon) according to the kit instructions. This technique stains living cells green and dead cells red. After staining, the cells were visualized using confocal scanning microscopy.

2.9. Cell count

At desired time points, the total number of adherent cells was determined using an MTS CellTiter96 kit (Promega, Fitchburg, WI), which measures the metabolic activity of viable cells.

2.10. Biochemical assay for GAG production

Hydrogel samples in triplicate were used for the biochemical analysis of GAG expression after 7 days. Media was removed, and the disks were rinsed several times with PBS, homogenized with a pellet grinder, and digested in a solution of 50 µg/ml proteinase K in 100 mM K₂HPO₄ (pH 8.0) at 60°C for 16 hours. Proteinase K was inactivated by heating to 90°C for 10 minutes. The digest solution was then used for DNA and GAG quantifications. Total DNA content was measured with a Picogreen Cell Proliferation Kit (Molecular Probes, Eugene OR) according to the manufacturer's instructions. Total sulfated glycosaminoglycan content was quantified using Blyscan Glycosaminoglycan Assay Kit (Biocolor, Newtonabbey, Northern Ireland) according to the manufacturer's instructions.

2.11. Immunofluorescence staining

Samples were fixed in 2% paraformaldehyde for 20 minutes and permeabilized in 0.2% Triton X-100 for 2 minutes. Nonspecific sites were blocked by incubation in 1% bovine serum albumin for 90 minutes, followed by incubation with mouse anti-chicken collagen type II monoclonal antibody (Chemicon, International Inc., Billerica, MA) at room temperature for 1 hour. Samples were rinsed with PBS and incubated in Cy5 conjugated secondary antibody (Chemicon, International Inc.) for 1 hour at 37° C. All samples were mounted with mounting gel, including DAPI for counterstaining of nuclei, and imaged using confocal microscopy with settings adjusted to blacken any residual background fluorescence from the corresponding nonspecific control antibody.

2.12. Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated using the SV Total RNA Isolation System from Promega. Fifty ng of total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California). PCR amplification was carried out in 25 µL reaction mixtures

containing 5 μ L of cDNA, 2.5 μ L 10 $\times$ PCR buffer without magnesium (Promega, Madison, Wisconsin), 1.5 μ L of 25 mM magnesium chloride (Promega, Madison, Wisconsin), 0.25 μ L AmpliTaq Gold[®] DNA Polymerase (Applied Biosystems, Foster City, California), 2.5 pmol of each primer (Mayo Clinic laboratories, Rochester, Minnesota), 0.5 μ L of 10 mM dTTPs (Invitrogen). The PCR conditions were set as follows: 94 $^{\circ}$ C for 2 minutes, followed by 35 cycles of denaturing at 94 $^{\circ}$ C for 1 minute, annealing at 55 $^{\circ}$ C for 1 minute, and extension at 68 $^{\circ}$ C for 1 minute. The following primers were used: rabbit collagen II forward: 5'-GACCTGCGTCTACCCCAAC-3' and collagen II reverse: 5'-GCTGCTTCTGGCTCTTGC-3'. Rabbit collagen I forward: 5'-TCAAGGTTTCCAAGGACCTG-3' and collagen I reverse: 5'-GTCCTTTCAATCCGTCCAGA-3'. Rabbit GAPDH forward: 5'-GCGAGAGCACCAGAGGAG-3' and reverse 5'-TCTCAGCGTGGTGGGACT-3'. The products from the PCR reaction were run on a 2% agarose gel and the densitometry was performed on the PDQuest software from Bio-Rad. We excluded the hydrogels with 30% positive charge from our experiment because we were unable to extract RNA from these specimens.

2.13. Statistical analysis

All data are reported as means $\pm$ standard deviations (SD) for $n=3$ except for GAG and DNA measurements where $n=6$. Single factor analysis of variance (ANOVA) was performed with JMP8 (SAS Institute Inc., Cary, NC, USA) to assess the statistical significance across the groups ($p < 0.05$). Significant differences between neutrally charged hydrogels and the different experimental groups were evaluated using Least Means Difference Student's T-test.

3. Results

3.1. ATR-FTIR

The ATR-FTIR spectra of OPF hydrogels unmodified and those incorporating different concentrations of SMA and MAETAC are compared in Fig. 2a and 2b. As shown, after copolymerization of OPF with SMA, a new peak emerged at 1550 cm^{-1} that is characteristic of the carboxylic acid from SMA. Bands at 1650 and 1045 cm^{-1} are assigned to the carbonyl and carbon-oxygen bonds (C-O-C) of OPF, respectively (Fig. 2a). Copolymerization of the hydrogel with MAETAC resulted in a new peak at 1725 cm^{-1} that is assigned to the methacryl carbonyl from MAETAC (Fig. 2b).

3.2. Swelling behavior

The swelling ratio of the hydrogels varied with changes in both the pH and the ionic nature of the surrounding fluid, as well as the charge type and density (Fig. 3 a, b). Figure 3a shows that the hydrogel swelling ratio increased in deionized water with increasing concentration of SMA in the hydrogel formulation. Similarly, there was an increase in swelling ratios of SMA-modified hydrogels in PBS, HCl and KOH as compared to the unmodified hydrogel. However, these changes were not as profound as in water. In all charge densities except neutral, the hydrogels immersed in dH_2O had higher swelling ratios as compared to those in PBS, HCl (which were intermittent), and KOH (which were the lowest). These differences were all significant with the exception of the 30% hydrogel submersed in HCl. Of note, hydrogel swelling ratios in basic conditions (KOH) were minimal for all of the hydrogel formulations regardless of their charge density, but still significantly higher than that of the neutral hydrogel. Figure 3b shows that the swelling ratios of all hydrogels increased in deionized water with incorporation of MAETAC in the hydrogel formulations. Even though these changes are significant when compared to neutral hydrogels ($p < 0.05$), the swelling ratios of positively charged hydrogels in water were less than those of negatively charged

hydrogels. In PBS, it appears that the swelling ratio slightly decreased with the addition of MAETAC. The swelling ratios of hydrogels in KOH and HCl did not change with incorporation of MAETAC. This indicates that unlike SMA, MAETAC is not sensitive to the pH of the environment.

3.3. Zeta potential and conductivity

Table 1 shows the Zeta potential, pH, and conductivity of both positively and negatively charged hydrogels in deionized water. Sol fraction has been also reported in this table. The Zeta potentials of the neutral hydrogels were initially -1.75 ± 0.7 mV and these changed with incorporation of MAETAC and SMA. As seen in Table 1, Zeta potentials of the hydrogels became more negative as the SMA concentration increased, with the 30% SMA being significantly more negative than the 20%, 10% and 5% all of which were significantly more negative than the neutral hydrogels ($p < 0.05$). The same trend was observed when MAETAC was added to the hydrogel formulations. The Zeta potentials of hydrogels became more positive with incorporation of MAETAC and increased as the concentration of MAETAC increased. This demonstrated that positive and negative fixed charges were successfully incorporated into the hydrogels. As shown in Table 1, the hydrogel solutions became more conductive with incorporation of the charged moieties into their formulations. There was a trend toward increasing conductivity of the hydrogel solutions as the concentration of the charged monomer increased. Thus, a greater percentage of fixed charge density resulted in a higher conductivity of the material. Our results in Table 1 showed the changes in pH of hydrogel solutions with incorporation of MAETAC and SMA. The pH of aqueous solutions decreased with incorporation of MAETAC in OPF hydrogel ranging from 6.22 ± 0.07 to 3.97 ± 0.05 . However, there was an increase in the pH of solutions with incorporation of SMA into the hydrogel ranging from 8.58 ± 0.08 to 9.12 ± 0.05 . The addition of MAETAC to the hydrogels resulted in their sol fraction decreasing from $8.51 \pm 2.69\%$ to $3.56 \pm 1.34\%$ after swelling in water. It appears that the hydrogel crosslinking density increased with increasing MAETAC concentration. However, with addition of SMA to the hydrogels, the sol fraction increased to 34.48 ± 1.92 and remained unchanged up to an SMA concentration of 30%.

3.4. Mechanical properties

Figure 4a shows that ultimate strength of the OPF hydrogel initially increased from 0.22 ± 0.03 MPa to 0.26 ± 0.03 MPa with the addition of 5% SMA and then increased further to 0.38 ± 0.07 MPa with the addition of 10% SMA. This was a significantly higher strength than that of the unmodified hydrogels ($p = 0.0129$). However, the increase in ultimate strength of the hydrogels with 20% and 30% SMA was not significant when compared to unmodified hydrogels. The compressive modulus of the hydrogels also increased from 0.03 ± 0.01 MPa to 0.05 ± 0.02 MPa with the addition of 5% SMA and remained the same thereafter, however the differences were not statistically significant in most experimental groups. Figure 4b shows that the ultimate strength of the hydrogel formulations increased significantly from 0.23 ± 0.04 MPa to 0.38 ± 0.07 MPa with addition of MAETAC in amounts of 10% and higher ($p < 0.05$). As seen in this figure, the compressive modulus was also significantly increased from 0.03 ± 0.01 MPa to 0.08 ± 0.02 MPa with addition of 10% MAETAC. However compressive moduli of hydrogels with 20% and 30% MAETAC were not significantly different from those of unmodified hydrogels.

3.5. Viability

Cell staining using a Live/Dead kit demonstrated that cells remained viable during all culture periods on hydrogels modified with both MAETAC and SMA. The micrograph of the cells as seen in Figure 5 shows that chondrocytes cultured on negatively charged hydrogels maintained a round morphology at days 1 and 3. As time increased, the cells began to aggregate. Unlike their behavior on negatively charged hydrogels, the cells had a

more fibroblastic, spindle shaped morphology on hydrogels modified with 20% and 30% MAETAC at days 1 and 3. By day 7, these cells tended to aggregate on all hydrogels (Fig. 6).

3.6. Cell count

Cell counts were performed after 1, 3, and 7 days in culture. Cell counts on negatively charged hydrogels are shown in Figure 7a. Similar cell counts on all hydrogels were observed on day 1. Cell counts on all hydrogels increased significantly as time increased. At day 7, the cell counts on all hydrogels were significantly higher than those at day 1 ($p < 0.05$). Figure 7b shows cell counts on the positively charged hydrogels. There was no significant difference in cell counts among any of the charge groups at day 1. However, there was an increase in cell count for all charge density groups as the number of days in culture increased. This increase became significant by day 7 when compared to day 1, with the exception of hydrogels with 30% MAETAC for which cell counts on day 7 were less than they were on day 1. The cell counts on the 30% MAETAC hydrogels were significantly less than those on the neutral hydrogels at day 7.

3.7. Glycosaminoglycan (GAG)

The total GAG content of the cells for the different samples ($n=6$) was measured using a Blyscan kit (Figure 8a). After 7 days of culture, there was increasing trend in GAG content on the negatively charged hydrogels with increasing charge density, although the lowest value occurred in the 5% hydrogels as opposed to the neutral hydrogels. As seen in Figure 8a, there was significantly more GAG produced on negatively charged hydrogels with 30% charge incorporation as compared to neutral hydrogels. Figure 8b demonstrates that the DNA content on negatively charged hydrogels with 20% and 30% SMA was significantly greater than that on hydrogels with positive and neutral charge.

3.8. PCR

Figure 9 illustrates expression of collagen type II and I in the hydrogel formulations used in Table 1. The expression level of collagen type II and I for a given amount of cDNA appears to be different based on hydrogel formulations. The expression of collagen type II on negatively charged hydrogels was higher than that on neutral and positively charged hydrogels. However, up-regulation of collagen type I was seen on positively charged hydrogels.

3.9. Immuno-fluorescence

Figure 10 shows collagen type II staining on hydrogels of different groups after 7 days in culture. Collagen type II staining was positive on all samples, including neutral, positively and negatively charged hydrogels. However, fluorescence intensity appeared to be higher on the negatively charged hydrogels.

4. Discussion

The biological activities of chondrocyte populations are regulated by genetic and other biological and biochemical factors, as well as environmental factors. Physical environmental factors such as stress, flow, and electric field are important in regulating cellular activities [16]. Several researchers have investigated the effect of material mechanical properties on chondrocyte differentiation and production of cartilage-specific proteins, such as collagen type II and aggrecan [17–19]. The electrical events in cartilage have been known for more than three decades. However, only a few studies have focused on the details of electrical potential within the ECM where chondrocytes reside [16, 20, 21]. It has been reported that

in the intervertebral disc a charged hydrated soft tissue exhibits electromechanical transduction phenomena such as streaming potential [14]. The electrical conductivity of intervertebral discs and its correlation to tissue porosity and fixed charge density has been investigated by Gu et al. [14]. A recent report by Yasuda et al. has also shown that negatively charged polymer-based double network gel effectively induces cartilage regeneration in an in vivo rabbit model [22]. In this study, we have used oligo(poly(ethylene glycol) fumarate) (OPF) as a matrix for cell attachment and differentiation. Small molecules with electrical charge have been incorporated within the hydrogel to investigate their effects on the differentiation and function of the primary chondrocytes.

The specific characteristics of charged OPF hydrogels have been analyzed by various techniques to compare them with neutral OPF hydrogels. To show the sensitivity of the materials to the nature of their surrounding environment, the swelling ratio was measured in different solutions. Major factors that influence the degree of swelling of ionic polymers include the properties of the polymer (charge, concentration and pKa of the ionizable group, degree of ionization, crosslink density and hydrophilicity or hydrophobicity). The properties of swelling medium such as pH, ionic strength and counterion and its valency are also important factor in swelling of the charged hydrogels [23]. We demonstrated that the SMA-modified hydrogels reacted differently in each environment, with the swelling ratio being least when the hydrogels were submerged in KOH. This could be due to the pH and ionic strength of the solution. This difference was significant when compared to those submerged in dH₂O, which had the highest swelling ratios regardless of charge group or immersing solution. When analyzing the data within each solution, negatively charged hydrogels had significantly higher swelling ratios than their neutral hydrogel counterparts regardless of the magnitude of their charge. Swelling ratios of positively charged hydrogels in dH₂O also increased with increasing MAETAC concentrations. However, in PBS, the swelling ratio decreased and then reached a plateau. Unlike negatively charged hydrogels, swelling ratios of positively charged hydrogels did not change in either acidic or basic solution. The loss of fixed charge density was shown in recent studies to be an important factor in tissue degradation and tissue creep response [21] during the early stages of cartilage damage. Zeta potential measurements confirmed the electrically charged nature of SMA and MAETAC modified hydrogels, and that the amount of charge was dependent upon the SMA and MAETAC concentration in the polymerization formulation. We demonstrated that incorporation of small molecules containing positive (MAETAC) and negative (SMA) charge into the OPF hydrogel conferred in electrolyte properties and ion conductivity on the hydrogels. Our data revealed that both negatively and positively charged hydrogel dispersions were conductive in deionized water. This conductivity was associated with ionization of the charged groups on the hydrogels generating electrostatic repulsive forces responsible for osmotic swelling of the hydrogels [23]. The change in pH of the hydrogel solutions could be also due to ionization of the charged group on the hydrogels. These data showed an excellent correlation between the charged monomer concentrations and electrical properties of hydrogel. The conductivity of the polymer solutions increased with increases in both SMA and MAETAC concentration in the hydrogel formulations. Attenuated total reflectance FT-IR spectroscopy demonstrated successful incorporation of SMA and MAETAC into the OPF hydrogels. It appeared that the intensity of the peaks corresponding to MAETAC (at 1725cm⁻¹) and SMA (at 1550 cm⁻¹) modified hydrogels linearly increased as their concentration in the hydrogel precursor solution increased.

Chondrocytes were seeded on top of these charged hydrogels in order to ascertain whether they would remain viable and produce healthy cartilage more effectively than chondrocytes seeded on neutral hydrogels. We demonstrated that cells not only remained viable on negatively charged hydrogels, but also proliferated on them. Cell number was not significantly different at any time point between charged and neutral hydrogels. However, in

positively charged hydrogels, cell number decreased at day 7 as charge incorporation increased to 30%. GAG content as a marker of chondrogenic differentiation was measured in all hydrogel groups. Our data showed a significant increase in GAG content in cells cultured on hydrogels with 20% SMA as compared to those cultured on the neutral hydrogels. In addition, the GAG content of the cells on negatively charged hydrogels with 30% was significantly higher than that on all other charge density groups, including neutral and positively charged hydrogels. PCR data revealed that there was an increasing trend in expression of collagen type II on all negatively charged hydrogels as compared to TCPS control and positively charged hydrogels. Reduced expression of collagen type II on positively charged hydrogels could be due to the chondrocyte dedifferentiation on those hydrogels to a fibroblastic morphology shown in Fig. 5b. Intense collagen type II staining on hydrogels with 30% SMA confirms our results from PCR. A recent study has shown that incorporation of chondroitin sulfate into PEG-based hydrogels improved differentiation of chondrocytes when compared to that of chondrocytes seeded on pure chondroitin sulfate gels [11].

Bryant et al. [11] showed that although both total collagen content and collagen type II gene expression increased for chondrocytes seeded on charged hydrogels compared to those seeded on neutral hydrogels, the aggrecan content remained unchanged. Another study by the same group has investigated the effect of negative charge in PEG hydrogels under dynamic loading [12]. In that study, bovine chondrocytes were cultured on PEG hydrogels of different charge densities. The 20% negatively charged PEG hydrogels showed an increase in collagen and proteoglycan content of 565% and 162%, respectively when compared to the neutral PEG hydrogels. However, the cells did not retain their enhanced activity after dynamic loading was removed. Overall, the data from this study show that negatively charged OPF hydrogels increase the extent of chondrocyte differentiation compared to that on the neutral or positively charged hydrogel scaffolds. We performed this study in a two-dimensional system to initially screen the effects of surface chemistry and charge on chondrocyte behaviors. Cell behavior in a two-dimensional system is more influenced by surface charge and chemistry rather than swelling and water content of the hydrogels. However, further investigation in three dimensional cultures is required to more completely understand the effect of charge on chondrocyte differentiation.

5. Conclusions

This study shows that modification of OPF with negatively and positively charged monomers changed the characteristics of scaffolds fabricated from OPF. Hydrogels became sensitive to pH and the ionic strength of their environment. Zeta-potential measurements confirmed that the hydrogels contained electrical charge in their structure and that they were conductive. Furthermore, cell attachment was higher on charged hydrogels and the expression of chondrocyte specific proteins was significantly different from that of the control groups. Scaffolds fabricated from the negatively charged hydrogels had superior cell attachment and matrix expression properties compared to those fabricated from the neutral and positively charged OPF hydrogels. Our data suggest that the incorporation of charge into scaffolds may play an important role in cartilage tissue engineering. However, further investigations are needed to determine whether charge also plays a role in a three-dimensional in vitro system and animal model.

Acknowledgments

This work was supported by funding from the Mayo Division of Orthopedic Research and NIH grants R01 AR 454871, and R01 EB 003060.

References

1. Furukawa T, Eyre DR, Koide S, Glimcher MJ. Biochemical studies on repair cartilage resurfacing experimental defects in the rabbit knee. *J Bone Joint Surg Am.* 1980 Jan; 62(1):79–89. [PubMed: 7351420]
2. Brittberg M. Autologous chondrocyte implantation--technique and long-term follow-up. *Injury.* 2008 Apr;39 Suppl 1:S40–S49. [PubMed: 18313471]
3. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med.* 1994 Oct 6; 331(14):889–895. [PubMed: 8078550]
4. Tuan RS. A second-generation autologous chondrocyte implantation approach to the treatment of focal articular cartilage defects. *Arthritis Res Ther.* 2007; 9(5):109. [PubMed: 18021426]
5. Bryant SJ, Anseth KS. The effects of scaffold thickness on tissue engineered cartilage in photocrosslinked poly(ethylene oxide) hydrogels. *Biomaterials.* 2001 Mar; 22(6):619–626. [PubMed: 11219727]
6. Bryant SJ, Chowdhury TT, Lee DA, Bader DL, Anseth KS. Crosslinking density influences chondrocyte metabolism in dynamically loaded photocrosslinked poly(ethylene glycol) hydrogels. *Annals of biomedical engineering.* 2004 Mar; 32(3):407–417. [PubMed: 15095815]
7. Bryant SJ, Nuttelman CR, Anseth KS. The effects of crosslinking density on cartilage formation in photocrosslinkable hydrogels. *Biomedical sciences instrumentation.* 1999; 35:309–314. [PubMed: 11143369]
8. Jo S, Shin H, Mikos AG. Modification of oligo(poly(ethylene glycol) fumarate) macromer with a GRGD peptide for the preparation of functionalized polymer networks. *Biomacromolecules.* 2001 Spring;2(1):255–261. [PubMed: 11749181]
9. Dadsetan M, Szatkowski JP, Yaszemski MJ, Lu L. Characterization of photo-cross-linked oligo[poly(ethylene glycol) fumarate] hydrogels for cartilage tissue engineering. *Biomacromolecules.* 2007 May; 8(5):1702–1709. [PubMed: 17419584]
10. Temenoff JS, Athanasiou KA, LeBaron RG, Mikos AG. Effect of poly(ethylene glycol) molecular weight on tensile and swelling properties of oligo(poly(ethylene glycol) fumarate) hydrogels for cartilage tissue engineering. *J Biomed Mater Res.* 2002 Mar 5; 59(3):429–437. [PubMed: 11774300]
11. Bryant SJ, Arthur JA, Anseth KS. Incorporation of tissue-specific molecules alters chondrocyte metabolism and gene expression in photocrosslinked hydrogels. *Acta biomaterialia.* 2005 Mar; 1(2):243–252. [PubMed: 16701801]
12. Villanueva I, Gladem SK, Kessler J, Bryant SJ. Dynamic loading stimulates chondrocyte biosynthesis when encapsulated in charged hydrogels prepared from poly(ethylene glycol) and chondroitin sulfate. *Matrix Biol.* 2010 Jan; 29(1):51–62. [PubMed: 19720146]
13. Dadsetan M, Knight AM, Lu L, Windebank AJ, Yaszemski MJ. Stimulation of neurite outgrowth using positively charged hydrogels. *Biomaterials.* 2009 Aug; 30(23–24):3874–3881. [PubMed: 19427689]
14. Gu WY, Justiz MA, Yao H. Electrical conductivity of lumbar annulus fibrosis: effects of porosity and fixed charge density. *Spine (Phila Pa 1976).* 2002 Nov 1; 27(21):2390–2395. [PubMed: 12438989]
15. Klagsbrun M. Large-scale preparation of chondrocytes. *Methods Enzymol.* 1979; 58:560–564. [PubMed: 423791]
16. Lai WM, Sun DD, Ateshian GA, Guo XE, Mow VC. Electrical signals for chondrocytes in cartilage. *Biorheology.* 2002; 39(1–2):39–45. [PubMed: 12082265]
17. Loeser RF. Integrin-mediated attachment of articular chondrocytes to extracellular matrix proteins. *Arthritis and rheumatism.* 1993 Aug; 36(8):1103–1110. [PubMed: 8343186]
18. Guilak F, Mow VC. The mechanical environment of the chondrocyte: a biphasic finite element model of cell-matrix interactions in articular cartilage. *Journal of biomechanics.* 2000 Dec; 33(12): 1663–1673. [PubMed: 11006391]

19. Guilak F, Meyer BC, Ratcliffe A, Mow VC. The effects of matrix compression on proteoglycan metabolism in articular cartilage explants. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 1994 Jun; 2(2):91–101.
20. Bassett CA, Pawluk RJ. Electrical behavior of cartilage during loading. *Science (New York, NY)*. 1972 Dec 1; 178(64):982–983.
21. Gu WY, Yao H. Effects of hydration and fixed charge density on fluid transport in charged hydrated soft tissues. *Ann Biomed Eng*. 2003 Nov; 31(10):1162–1170. [PubMed: 14649490]
22. Yasuda K, Kitamura N, Gong JP, Arakaki K, Kwon HJ, Onodera S, et al. A novel double-network hydrogel induces spontaneous articular cartilage regeneration in vivo in a large osteochondral defect. *Macromolecular bioscience*. 2009 Apr 8; 9(4):307–316. [PubMed: 19031389]
23. Gupta P, Vermani K, Garg S. Hydrogels: from controlled release to pH-responsive drug delivery. *Drug discovery today*. 2002 May 15; 7(10):569–579. [PubMed: 12047857]

Fig. 1. Schematics of oligo-(polyethylene glycol) fumarate (OPF), charged monomers and crosslinking.

2a

2b

Fig. 2. ATR-FTIR of hydrogels after crosslinking and lyophilization. Charged hydrogels with different SMA concentrations (a), and charged hydrogels with different MAETAC concentrations (b).

3a

Fig. 3.

Swelling ratio of charged hydrogels in various solutions. Negatively charged hydrogels with different concentration of SMA (a), positively charged hydrogels with different concentration of MAETAC (b). + ($p=0.0091$ or less) indicates significant difference from the neutral hydrogel in dH₂O. * ($p < 0.0001$) indicates significant difference from neutral hydrogel in the same solution. Significance was evaluated using Least Means Squares Difference Student's t-Test.

4a

4b

Fig. 4. Compressive modulus and ultimate strength of oligo(poly(ethylene glycol) fumarate) hydrogels modified with SMA (a), or MAETAC (b) at equilibrium swelling. Data represent mean $\pm$ SD ($n = 3$). * ($p < 0.05$) denotes significant difference as compared to neutral hydrogel samples.

Fig. 5. Chondrocyte viability on hydrogels with different concentrations of SMA in their formulations at days 1, 3 and 7: 0% (a, f, k), 5% (b, g, l), 10% (c, h, m), 20% (d, i, n), and 30% (e, j, o). Viable cells are stained green and dead cells are stained red. All magnifications are $\times 10$. Scale bar=20 μm .

Fig. 6. Chondrocyte viability on hydrogels with different concentrations of MAETAC in their formulations at days 1, 3 and 7: 0% (a, f, k), 5% (b, g, l), 10% (c, h, m), 20% (d, i, n), and 30% (e, j, o). Viable cells are stained green and dead cells are stained red. All magnifications are $\times 10$. Scale bar=20 μm .

Fig. 7. Cell attachment and proliferation on OPF hydrogels modified with SMA (a), and modified with MAETAC (b). Data represent mean $\pm$ SD ($n = 3$). * ($p < 0.05$) indicates significant difference when compared to the cell number on the same sample at day 1. + ($p < 0.05$) indicates significant difference when compared to the cell number on the neutral hydrogel at the same time point.

8a

8b

Fig. 8. Total GAG (a), and total DNA (b) content of the chondrocytes on different hydrogels after 7 days in culture (b). *($p < 0.05$) indicates significant difference when compared to the neutral hydrogel at the same time point. # ($p < 0.05$) indicates significant difference when compared to the positively charged hydrogels at the same monomer concentration. Significant differences were evaluated using Least Means Difference Student's t-test.

9a

9b

Fig. 9. RT-PCR analysis for mRNA expression of collagen type II (a), and collagen type I (b). To quantify the collagen type II and I expression of chondrocytes on different hydrogels, the optical density of the dark spots on the PCR gel has been measured using densitometry image analysis software. Optical density of the collagen type II and I was normalized to GAPDH on the same gel.

Fig. 10.

Collagen type II immunofluorescence staining on neutral (a), positively charged hydrogel with 30% MAETAC (b), and negatively charged hydrogel with 30% SMA (c). All magnifications are $\times 10$. Scale bar=20 μm .

Table 1

Characteristics of charged hydrogels

Hydrogel Formulations	Zeta-Potential (mV)	Conductivity (mS/cm)	pH [#]	Sol Fraction (%)
0% (Neutral)	-1.75 ± 0.7	0.04 ± 0.00	6.77 ± 0.31	8.51 ± 2.69
MAETAC concentration in hydrogel formulation				
5%	$1.90 \pm 0.8^*$	$0.11 \pm 0.00^*$	$6.22 \pm 0.07^*$	12.12 ± 4.14
10%	$3.10 \pm 0.5^*$	$0.12 \pm 0.02^*$	$3.88 \pm 0.14^*$	11.87 ± 5.25
20%	$3.70 \pm 1.1^*$	$0.15 \pm 0.02^*$	$4.09 \pm 0.08^*$	3.78 ± 1.87
30%	$6.42 \pm 1.7^*$	$0.16 \pm 0.02^*$	$3.97 \pm 0.05^*$	3.56 ± 1.34
SMA concentration in hydrogel formulation				
5%	$-4.04 \pm 0.9^*$	$0.11 \pm 0.01^*$	$7.94 \pm 0.14^*$	$34.48 \pm 1.92^*$
10%	$-4.38 \pm 0.9^*$	$0.14 \pm 0.02^*$	$8.58 \pm 0.08^*$	$32.92 \pm 2.07^*$
20%	$-5.44 \pm 0.6^*$	$0.22 \pm 0.00^*$	$9.02 \pm 0.06^*$	$32.81 \pm 0.96^*$
30%	$-7.85 \pm 1.7^*$	$0.30 \pm 0.04^*$	$9.12 \pm 0.05^*$	$33.03 \pm 5.02^*$
dH ₂ O	—	0.01 ± 0.00	$6.54 \pm 0.02^*$	—

[#] pH of hydrogel dispersions in deionized water* Significantly different from neutral hydrogels ($p < 0.001$)