Published in final edited form as:

J Biomech. 2015 January 2; 48(1): 122–129. doi:10.1016/j.jbiomech.2014.10.023.

Resurfacing Damaged Articular Cartilage to Restore Compressive Properties

Stephanie Grenier, PhD^{*}, Patrick E. Donnelly, PhD, Jamila Gittens, MS, and Peter A. Torzilli, PhD

Laboratory for Soft Tissue Research Tissue Engineering, Regeneration and Repair Program The Hospital for Special Surgery New York, NY 10021

Abstract

Surface damage to articular cartilage is recognized as the initial underlying process causing the loss of mechanical function in early-stage osteoarthritis. In this study, we developed structure-modifying treatments to potentially prevent, stabilize or reverse the loss in mechanical function. Various polymers (chondroitin sulfate, carboxymethylcellulose, sodium hyaluronate) and photoinitiators (riboflavin, irgacure 2959) were applied to the surface of collagenase-degraded cartilage and crosslinked *in situ* using UV light irradiation. While matrix permeability and deformation significantly increased following collagenase-induced degradation of the superficial zone, resurfacing using tyramine-substituted sodium hyaluronate and riboflavin decreased both values to a level comparable to that of intact cartilage. Repetitive loading of resurfaced cartilage showed minimal variation in the mechanical response over a 7 day period. Cartilage resurfaced using a low concentration of riboflavin had viable cells in all zones while a higher concentration resulted in a thin layer of cell death in the uppermost superficial zone. Our approach to repair surface damage initiates a new therapeutic advance in the treatment of injured articular cartilage with potential benefits that include enhanced mechanical properties, reduced susceptibility to enzymatic degradation and reduced adhesion of macrophages.

Keywords

osteoarthritis; articular cartilage; resurfacing; photocrosslinking; mechanical properties

1. INTRODUCTION

Osteoarthritis (OA) is a pathology resulting in the progressive degeneration of articular cartilage. It has been described as a process of simultaneous extracellular matrix (ECM)

© 2014 Elsevier Ltd. All rights reserved.

^{*}Corresponding address: Dr. Stephanie Grenier, National Research Council of Canada, 75 boul. de Mortagne, Boucherville, QC, J4B 6Y4, Telephone: (450) 641-5342, Stephanie.Grenier@cnrc-nrc.gc.ca.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to disclose.

degradation resulting from mechanical damage such as trauma and wear-and-tear (von Porat et al., 2004), as well as enzymatic catabolism by metalloproteinases (MMPs) and aggrecanases (Hedbom and Hauselmann, 2002). Tissue breakdown is initiated at the articular surface, notably within the superficial zone (SZ), and is characterized by surface fibrillation, loss in proteoglycan (PG) content, and the irreversible rupture and disorientation of the collagen fiber network (McDevitt et al., 1977; Hwang et al., 1992; Panula et al., 1998; Appleyard et al., 1999). While PG loss can be replaced by increased chondrocyte synthesis, collagen damage is irreversible and the chondrocyte's ability to synthesize new collagen is limited (Lorenzo et al., 2004; Stubendorff et al., 2012; Tran-Khanh et al., 2005). A serious consequence of collagen surface damage is the loss of tensile strength in the SZ, leading to increases in ECM swelling, surface porosity, permeability, water flux, and PG loss, all of which result in loss of mechanical function (resistance to compression) (Lotke and Granda, 1971; Setton et al., 1993; Owen and Wayne, 2006).

Of importance to preserving the health and function of articular cartilage is the maintenance or restoration of the SZ and mechanical properties. In healthy mature cartilage, PGs (MW~200 000 kDa) and non-aggregating aggrecans (MW~200-300 kDa) fill about 90% of the total interfibrillar volume of the ECM (Roughley and Lee, 1994; Schumacher et al., 1994). More than 50% of the dry weight of the ECM is made up of fibrillar type II collagen, which comprises 90-95% of all collagen types in cartilage. Two regions define the microstructural arrangement in the SZ (100-200 μm thick or ~10-20% of the ECM). The uppermost region (lamina splendens, LS, 5-10 μm) is composed of fine, randomly woven collagen type II fibrils, a small amount of superficial zone protein (SZP, MW~345 kDa), and no cells, while below the LS, the superficial tangential zone (STZ) contains SZP, densely packed collagen type II fibrils, and flattened, ellipsoidal shaped chondrocytes aligned parallel to the articular surface (Aydelotte and Kuettner 1988; Clark, 1990; Teshima et al., 1995; Flannery et al., 1999). Water occupies the non-solid interstitial space, comprising 80-85% of the SZ's total wet weight (Torzilli et al., 1990). The percentage of water and collagen are higher, and PG lower, in the SZ relative to the rest of the ECM (Maroudas et al., 1980; Franzen et al., 1981). The densely packed collagen fibrils in the SZ affect transport of interstitial fluid, solutes and macromolecules through the surface (Torzilli et al., 1997; Setton et al., 1993), and provide tensile strength to resist normal and shear forces imposed during physiological loading (Akizuki et al., 1987).

Our long-term objective is to mechanically stabilize the ECM of damaged articular cartilage by creating a barrier against excessive water movement and PG loss, and to promote normal metabolic function and ECM remodeling by shielding chondrocytes from abnormal stresses and strains. In a previous study, we developed an *in vitro* cartilage degradation model that emulated the SZ damage seen in early-stage OA (Grenier et al., 2014). Using our model, we treated the damaged SZ with structure-modifying treatments to regain the loss in mechanical function. To this end, a polymer solution containing a photoinitiator was deposited onto, and allowed to diffuse into, the damaged articular surface, and later exposed to ultraviolet (UV) light irradiation *in situ*. Our goal was to restore mechanical function by creating crosslinks between adjacent collagen fibrils (interfibrillar bonds), entrapping the polymer molecules within the crosslinked collagen network, and photocrosslinking the polymer molecules with

themselves and to the collagen fibrils. Using appropriate treatments, we were able to restore the compressive properties of degraded cartilage. Our approach is fundamentally different from other clinical approaches which attempt to smooth or seal the damaged surface (i.e., shaving or radio-frequency ablation, respectively), replace damaged cartilage with cells and/or scaffolds, inhibit damage progression by intra-articular injections of lubricants, or block the action of the MMPs using inhibitors. All these methods have either failed or have had limited success at repairing the damage and inhibiting further progression of the disease process (Stuart and Lubowitz, 2006; Siparsky et al., 2007). Our structure-modifying approach initiates a new therapeutic advance in the treatment of damaged cartilage, with potential benefits that include enhanced mechanical properties, reduced susceptibility to enzymatic degradation, and reduced macrophage adhesion.

2 MATERIALS AND METHODS

2.1 Tissue Preparation

Eight-millimeter diameter cylindrical plugs of articular cartilage were harvested from mature bovine knee joints. Plugs intended for mechanical testing and histology were incubated in proteinase inhibitors (Complete Mini, Roche Diagnostics, Indianapolis, IN) and stored frozen. At the time of testing, plugs were sectioned through the deep zone, parallel to the articular surface and rinsed in phosphate buffered saline (PBS) to remove inhibitors. Plugs intended for cell viability studies were incubated first in wash culture media (Dulbecco's modified eagle medium (DMEM) with 4% FBS, 1% kanamycin solution, 0.5% fungizone amphotericin and 0.1% gentamicin) for 3 hrs and then serum-rich media (DMEM with 10% FBS and 1% antibiotic-antimycotic) for 48 hrs to detect any contamination. Prior to resurfacing or mechanical testing, the plug diameters were reduced to 5 mm and the explants mounted in a confined chamber with the surface facing upward. The outer rings were used as controls (intact state) in cell viability studies.

2.2 Collagenase Degradation

The articular surface was treated with 0.1% type II bacterial collagenase (*Clostridium histolyticum* collagenase, Worthington Biochemical Company, Freehold, NJ) in serum-free DMEM for 1.5 hrs at 37 °C. To end the collagenase digestion, the explants were rinsed in PBS for 5 min, washed in proteinase inhibitors for 1.5 hrs and rinsed again in PBS for 30 min.

2.3 Resurfacing Treatments

Several different resurfacing techniques were applied to the degraded articular surface using specific polymers and photoinitiators known to either crosslink the polymer to collagen or to itself, or become entrapped within the crosslinked collagen network. Polymers tested included chondroitin sulfate (CS, 6.3 kDa) (Sigma-Aldrich, St. Louis, MO), methacrylated carboxymethylcellulose (CMC, 700 kDa) (City College of New York), sodium hyaluronate (HA, 1000 kDa) (Lifecore Biomedical, Chaska, MN), and tyramine-substituted sodium hyaluronate (TS-HA, 1000 kDa) (Corgel 5% substitution, Lifecore Biomedical, Chaska, MN). Two photoinitiators were tested, riboflavin (RF) (Sigma-Aldrich, St. Louis, MO) and irgacure 2959 (IRG) (Sigma-Aldrich, St. Louis, MO). Two concentrations of RF were

tested, 0.1% and 0.0012%, based on use in the treatment of keratoconus (Wollensak et al., 2003a) and chondrocyte encapsulation in hydrogels (Hu et al., 2012, Ibusuki et al., 2007). The 0.05% IRG concentration was based on its cytocompatibility when used with bovine chondrocytes (Williams et al., 2005). The polymer/photoinitiator combinations tested are given in Table 1.

A water-based solution containing a polymer and photoinitiator was deposited onto the articular surface and allowed to permeate into the ECM for 30 min. Thereafter, excess solution was aspirated and the surface was exposed to UV light (wavelength = 365 nm, surface intensity = 5 mW/cm²) for 12 min (dosage = 3.6 J/cm²). Exposure parameters were selected to remain within reported safety thresholds for intensity (< 6 mW/cm²), wavelength (330-400 nm) and dosage (< 40 J/cm²) (Bryant et al., 2000).

2.4 Model System for Riboflavin Catalyzed Dityramine Synthesis

Since we are the first, to our knowledge, to report photocrosslinking of TS-HA with RF, we characterized the TS-HA/RF gel formation process. Solutions of tyramine and RF were prepared in ultrapure water (resistivity = 18.2 M · cm) at concentrations of 27 mM and 0.17 mM, respectively. RF solution (8 ml) was added to the tyramine solution (2ml), and the pH was adjusted to 3 using 6 M HCl. The reaction mixture was irradiated by UV light (365 nm, 4 W) for 12, 30 and 60 min. A control reaction was performed in the absence of RF under 60 min of UV exposure. Aliquots from the reaction conditions were taken and analyzed by liquid chromatography (LC) and mass spectroscopy (MS). A HP 1100 series LC with a DAD detector (monitoring at 274 nm with a reference at 450 nm) was used with an Agilent Eclipse XDB-C18 5 µm particle size, 150 mm x 4.6 mm column and isocratic flow of 95% A (97% water/3% acetonitrile with 0.1% formic acid) and 5% B (90% acetonitrile/10% water with 0.1% formic acid) with a flow rate of 0.1 ml/min for 35 min with an injection volume of 2.5 µl. An Agilent 6210 TOF MS operating in positive mode was used with internal standards betaine, hexamethoxyphosphazene, and hexakis(1H, 1H, 3H-tetrafluoropropoxy)phosphazene.

2.5 Mechanical Testing

As previously described, intact cartilage explants were subjected to a 15 min confined compression creep test (intact state), followed by degradation of the articular surface by collagenase and another 15 min compression creep test (degraded state) (Grenier et al., 2014). The degraded surface was then resurfaced, washed in PBS, and subjected to another 15 min creep test (repaired state). For assessment of long-term mechanical properties, one resurfaced explant was stored in PBS at 4°C and retested after 12, 24, 36, 48, 60 hrs and 7 days post-treatment.

Mechanical properties were determined from the loading and creep responses for each explant state (intact, degraded, repaired) (Grenier et al., 2014). The instantaneous modulus was calculated by fitting an exponential function to the stress-strain response during the loading phase and calculating the slope at a strain of 5%. The instantaneous strain was determined at the end of the loading phase. The creep deformation response was numerically fitted to the analytical solution for linear biphasic creep of articular cartilage in confined

compression (Mow et al., 1980). The equilibrium aggregate modulus was calculated using the entire 15 min creep deformation while the apparent permeability and apparent modulus were measured using only the first 100 sec of creep deformation.

2.6 Histology

Intact, degraded and repaired explants were fixed in 10% formalin at 4°C for 24 hrs, dehydrated, and embedded in paraffin for histological analysis. Six-micrometer-thick sections were deparaffinized and rehydrated for Safranin-O/Fast Green staining using a standard protocol.

2.7 Chondrocyte Viability

Explants used for cell viability evaluation were not degraded with collagenase prior to resurfacing in order to isolate potential cytotoxicity of the resurfacing treatments. The articular surfaces of intact explants were exposed to the three TS-HA treatments or UV light only. Control (intact) and treated specimens were incubated in wash media for 3 hrs, and then serum-free culture media (Advanced DMEM/F12 supplemented with 1000× dexamethasone, 50 mg/ml ascorbate-2-phosphate, 1% antibiotics-antimycotic and 10× L-Glutamine) for up to 7 days. After 0, 3 and 7 days in culture, chondrocyte viability was assessed using the LIVE/DEAD Cell Viability/Cytotoxicity Kit. A 1.5 mm thick cross-section was removed from the center of the explant and incubated in 2 M Calcein AM and 2 M EthD-1 for 40 min. After washing in PBS, images were captured at several penetration depths using a confocal microscope (Leica TC-SSP5) and overlaid to produce a composite image.

2.8 Statistical Analysis

For mechanical testing, ten independent experiments were performed for each TS-HA resurfacing treatment and two-way repeated measures ANOVA with Bonferroni post tests were used for statistical analysis ($\alpha = 0.05$). Four independent chondrocyte viability experiments were performed in duplicates per time point and condition.

3 RESULTS

3.1 Dityramine Synthesis

Fig. 1 shows a representative chromatogram for a 60 min reaction time. There were three major peaks at 274 nm, corresponding to two products and the reactant. The peak at retention time 17.2 min was assigned to C-C bonded dityramine, the peak at retention time 18.4 min to C-O bonded dityramine, and the peak at 21.3 min to tyramine. These results were also supported by mass spectroscopy data (see supplementary Fig. S1-S4 showing the three major peaks and a representative control chromatogram). We calculated the dityramine product total peak area fraction, defined as C-C bonded dityramine peak area plus C-O bonded dityramine peak area divided by the sum of all three peak areas, to evaluate dityramine product formation. A linear trend was observed for dityramine formation with respect to time (Fig. 2). Finally, no dityramine formation was found in the control (no RF was present during reaction).

3.2 Mechanical properties

As previously reported (Grenier et al., 2014), degrading the articular surface with bacterial collagenase significantly increased ECM deformation when explants were loaded in confined creep compression (Fig. 3). Preliminary mechanical testing of all resurfacing treatments showed a noticeable variation in the restoration of the creep response. Resurfacing the collagenase-treated explants with photocrosslinking treatments using CS, CMC and HA with RF or IRG, or no polymer (Φ), resulted in less than 50% restoration in the creep response (Fig. 3), and were therefore not selected for further testing. When TS-HA was used in conjunction with 0.05% IRG or 0.0012% RF, only partial restoration (50%) was achieved. However, treatment with TS-HA and 0.1% RF fully restored the creep response to that of the intact.

While permeability significantly increased from intact to degraded states, all three TSHA resurfacing treatments resulted in a statistically significant decrease in the permeability of collagenase-degraded explants (Fig 4A). Permeability for 0.0012% RF and 0.05% IRG were restored to a level comparable to that of intact while the 0.1% RF treatment resulted in a statistically lower permeability than that of intact. Collagenase degradation caused a statistically significant decrease in the aggregate modulus (Fig. 4B). Resurfacing using RF as a photoinitiator partially restored the aggregate modulus while the use of IRG did not. The instantaneous strain remained the same, or was slightly lower after degradation but experienced a statistically significant decrease after resurfacing (Fig. 4C). The instantaneous modulus did not change after degradation but significantly increased after resurfacing, especially when using RF (Fig 4D). Collagenase degradation caused a significant decrease in explant thickness, which was further decreased after resurfacing (Fig. 4E). Finally, the apparent modulus significantly decreased after collagenase degradation, and then increased after resurfacing, with a full restoration to that of intact for the 0.1% RF treatment (Fig. 4F). No statistically significant differences in any properties (permeability, moduli, strain and thickness) were observed between TSHA treatment types for the intact, degraded or repaired state.

Long-term assessment found minimal variation in the creep response of resurfaced cartilage over a 7 day period (Fig. 5). Mechanical properties remained fairly constant after repetitive mechanical testing (see supplementary Fig. S5).

3.3 Histological findings

Histological analysis of cartilage explants using Safranin-O staining revealed a loss in PG content in the superficial and intermediate zones of collagenase-degraded specimens (Fig. 6). Similar to PG, the hyaluronate in TS-HA is negatively charged and will bind to the positively charged Safranin-O dye. All TS-HA resurfaced explants had a thin layer (~50 Pm) of hyaluronate attached to the articular surface, similar to the TS-HA/RF 0.1% treatment shown in Figure 6.

3.4 Chondrocyte Viability

Potential cytotoxicity generated from the exposure to UV light and the three TS-HA resurfacing treatments was evaluated after 0, 3 and 7 days in culture (Fig. 7, data not shown

for day 3). At all time points, intact control explants had viable cells in all zones, except for a few dead cells located at the articular surface. Samples treated with UV light only and 0.0012% RF both displayed a similar cell viability pattern to that of intact, regardless of time in culture. However, explants treated with 0.1% RF or 0.05% IRG displayed a thin layer of cell death in the SZ. Prolonged time in culture did not affect the thickness of this layer.

4 DISCUSSION

The objective of this study was to develop a treatment that could prevent, stabilize or reverse the loss in mechanical function of articular cartilage associated with early-stage OA. Techniques to bind, crosslink and entrap molecules, polymers and hydrogels within the ECM have been used to modify the ultrastructure and mechanical properties of cartilage (Ibusuki et al., 2007, Teixeira et al., 2012, Rowland et al., 2013, Oungoulain et al., 2014). Numerous chemicals such as glutaraldehyde, carbodiimide and tannic acid have been used to directly crosslink collagen, however their cytotoxicity and potential harmful side effects exclude most from *in vivo* use. On the other hand, photocrosslinking collagen using UV irradiation (Hamed and Rodriguez, 1975; Weadock et al. 1995), photo-oxidative dyes and initiators (RF, IRG, hematoporphyrin) (Wollensak et al. 2003a; Moore et al. 1994), and a variety of biopolymers (CS, HA, alginate, polyethylene glycol, fibrin, fibronectin) (Lee et al. 2001a; Stalling et al. 2009; Erickson et al. 2012) which can be entrapped or crosslinked after incorporating functional groups such as methacrylate, thiolate or tyramine have been shown to be efficacious and biocompatible with controlled cytotoxicity levels when used in cell culture, animal and human studies (Akens et al., 2002; von Rechenberg et al., 2004; Bryant et al., 2000; Sharma et al., 2013). The properties of crosslinked collagen have been extensively investigated and benefits include enhanced mechanical properties (Wollensak et al., 2003b; Weadock et al., 1995; Koide and Daito, 1997), reduced susceptibility to enzymatic degradation (Spoerl et al., 2004; Stanescu and Stanescu, 1988; Lee et al., 2001b), and reduced adhesion of macrophages (Van Luyn et al., 1998; Bailey, 2000; Watanabe and Koyama, 1999).

The use of TS-HA has been investigated for applications including injectable delivery systems for controlled release of proteins and drugs (Lee et al., 2009; Kim et al., 2011), bone cement augmented with nanocrystalline apatite for repairing bone defects (Pek et al., 2009), and scaffolds to modulate mesenchymal stem cell chondrogenesis (Toh et al., 2012). Some of these studies used a small TS-HA molecule (90-100 kDa) while others used a larger molecule (~1000 kDa) but all chemically crosslinked TS-HA using horseradish peroxidase and hydrogen peroxide. For this study, we used a 1000 kDa TSHA but crosslinked it using a photoinitiator (RF) and UV light. To the best of our knowledge, this is the first time that photocrosslinking techniques have been used to gel or solidify TS-HA.

Dimerization of tyramine and tyrosine was reported as far back as 1959 using horseradish peroxidase and hydrogen peroxide (Gross and Sizer, 1959). The authors suggested a possible mechanism for dityramine formation involving the formation of a phenoxy radical, which dimerizes and then enolizes to form the dityramine dimer. RF photochemistry has been well studied and we know it can form reactive oxygen species when photoexcited (Bradley et al., 2006; Yettella and Min, 2008). The formation of C-C bonded dityramine and

C-O bonded dityramine has also been reported under oxidizing conditions, as well as the oxidation of phenols in general (Lee et al., 2008; Musso, 1963). Our results suggest that RF catalyzes the formation of a phenoxy radical on tyramine, allowing it to dimerize and form dityramines. This appears to effectively crosslink the TS-HA through simple exposure to UV light, rather than the chemical (hydrogen peroxide and horseradish peroxidase) catalyzed process (Darr and Calabro, 2009).

Our model reaction system found that RF was required to form dityramine products and showed a positive association between reaction time and product formation. The reaction was performed at pH 3 to ensure protonation of the phenol and free amine to minimize potential side reactions occurring at the amine. It should be noted that this reaction was not optimized for product formation but was performed to demonstrate the ability of RF to dimerize tyramine. Further, our analysis is an underestimation for the amount of product formed as the dityramine products and tyramine do not have the same molecular extinction coefficient at 274 nm. Nevertheless, to our knowledge, this is the first report of RF photochemistry being utilized to dimerize tyramine, although RF has been used extensively to crosslink collagen, which is thought to occur through aromatic amino acids (Wollensak et al., 2003a). This simple model system provides proof-of-principle that RF can be used to drive the crosslinking of tyramine-substituted polymers, such as hyaluronate described herein.

Photocrosslinking TS-HA with RF provided us with increased spatial and temporal control as chemical crosslinking with hydrogen peroxide and horseradish peroxidase results in fast gelation times but much longer times to fully set (Lee et al., 2008; Jin et al., 2010; Wang et al., 2014). The low percent substitution of carboxyl groups for tyramine required to produce stable gels allowed the preservation of the majority of the native hyaluronate structure and negative charge (Darr and Calabro, 2009). Hyaluronate provides an ideal environment as it is a major constituent of cartilage, possesses excellent biocompatibility and is well known for its anti-inflammatory properties (Tobetto et al., 1993; Goldberg and Toole, 1987). Its negative charge attracts water and provides resistance to mechanical compression (Nishimura et al., 1998). When chemically crosslinked, TS-HA has improved mechanical properties (Lee et al., 2008; Toh et al., 2012), in vivo stability (Chan et al., 2005) and resistance to digestion by hyaluronidase (Lee et al., 2009; Darr and Calabro, 2009).

Previous data from our OA model showed that collagenase degradation of cartilage explants resulted in the cleavage of the collagen fiber network, which increased surface porosity and permeability, thus allowing PG to leach out from the SZ (Grenier et al., 2014). The loss in PG content and the cleaved collagen fiber network increased interstitial fluid exudation and lowered the compressive moduli. Mechanical testing indicate that our photocrosslinking treatments using TS-HA effectively decreased the permeability by reducing surface porosity, created a barrier against excessive water efflux from the ECM and potentially stopped further PG loss. The diminished water movement through the SZ also resulted in a decrease in the instantaneous strain. The photocrosslinked TS-HA molecules reinforced the injured ECM which provided higher resistance to compressive loads as evidenced by the increase in aggregate, apparent and instantaneous moduli. Resurfacing further reduced the thickness of

collagenase-treated specimens likely due to the crosslinking itself and the compaction of degraded specimens from loading (Grenier et al., 2014).

Safranin-O staining of resurfaced cartilage explants revealed the presence of a thin layer (~50 µm thick) of TS-HA attached to the articular surface. This layer did not appear to infiltrate into the STZ to fully integrate within the host tissue (Fig. 6). This may be due to the large size and negative charge of the TS-HA, both of which may have limited the diffusion distance into the ECM. Based on a previous study on solute diffusion in intact cartilage (Torzilli et al., 1987), we estimate that less than 20% of the TS-HA infiltrated to a depth of 20 µm in the 30 min allowed for diffusion prior to photocrosslinking. However, collagen cleavage and PG loss would most likely allow the TS-HA to reach greater depths but even with limited integration, the mechanical properties of the degraded explants were significantly enhanced by resurfacing, with little change in these properties over the 7 days of repetitive loading. This suggests that the TS-HA layer remained intact and functional after being subjected to a porous load platen that created interstitial water efflux, and compressive, shear and frictional forces. Interestingly, repetitive testing of resurfaced explants did not affect the specimen's thickness, indicating that resurfacing prevented the gradual collapse of the SZ, a phenomenon previously observed after repetitive loading of collagenase-degraded explants (Grenier et al., 2014).

Chondrocyte viability was evaluated to determine any potential cytotoxic effects of the TS-HA treatments. Intact control explants had viable cells except for a few dead cells located at the articular surface. This pattern is commonly observed after isolation of mature bovine cartilage (Dumont et al., 1999). The use of UV light only or 0.0012% RF did not affect chondrocyte viability. Although we carefully selected photoinitiator concentrations and UV exposure parameters to remain within reported safety thresholds, TS-HA photocrosslinked with 0.1% RF and 0.05% IRG caused some cell death at the articular surface. Taken alone, the limit of each parameter is higher but when parameters are combined together, the cytotoxic irradiance level can be ten-fold lower (Wollensak et al., 2004). In addition, these limiting values are highly dependent on tissue type (UV light absorbance coefficient). Comparison between TS-HA/RF 0.1% and TS-HA/RF 0.0012% indicates that RF concentration was an aggravating factor. Photoinitiators are free radical donors which enhance the crosslinking process; however, they can also damage chondrocytes (Hu et al., 2012). A fine balance in photoinitiator concentration is therefore required to maximize mechanical properties while maintaining cell viability.

Several photocrosslinking techniques have been investigated for tissue repair. Wollensak et al. (2003a) demonstrated the clinical usefulness of combining RF and UV light to crosslink collagen and stop the progression of keratoconus. Sitterle et al. (2009) developed photochemical collagen crosslinking methods to bond adjacent cartilage tissue across a repair interface to improve integration. With appropriate parameters, bond strength of treated tissue was on the order of that achieved through several weeks of culture. Other investigators focused on *in situ* photocrosslinking for the filling of focal cartilage defects. For instance, Erickson et al. (2012) directly photocrosslinked a metacrylated HA hydrogel containing chondrocytes and mesenchymal stem cells within an *in vitro* cartilage defect. Sharma et al. (2013) conducted a pilot clinical study where a poly(ethylene glycol)

diacrylate hydrogel was physically immobilized to a debrided cartilage defect using a CS adhesive and photocrosslinked in conjunction with standard microfracture surgery. Augmenting microfracture with the adhesive-hydrogel technology resulted in greater tissue filling of the defect, decreased tissue water content, increased tissue organization and significant improvements in the severity and frequency of pain, as compared to microfractured-only cases. Finally, Schlichting et al. (2011) developed an injectable nanocomposite photocrosslinkable co-polymer with attached mineralization peptides to restore the structural integrity of acutely injured cartilage following impact.

Our study provides a starting point for the development of structure-modifying approaches that can potentially prevent, stabilize or reverse the loss in mechanical function of articular cartilage in early-stage OA. Future work is needed to investigate the use of other chemical and photocrosslinkable biopolymers and photoinitiators to improve polymer transport and integration within the SZ, and their ability to resist the compressive, tensile and shear stresses at the articular surface during joint motion. Fatigue testing will also be required to assess the wear properties of the resurfacing treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Steven B. Nicoll (City College of New York) for kindly providing the methacrylated CMC used in this study and Ann Mularz (Princeton University) for technical assistance. Support for this study was made possible by Grant Number R21-AR059203 (PAT) and AR007281 (PED) from the National Institutes of Health - National Institute of Arthritis and Musculoskeletal and Skin Diseases. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH-NIAMS. This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06-RR12538-01 from the National Center for Research Resources, National Institutes of Health.

REFERENCES

- Akens MK, von RB, Bittmann P, Nadler D, Zlinszky K, Auer JA. In vitro studies of a photo-oxidized bovine articular cartilage. *J Vet Med A Physiol Pathol Clin Med*. 2002; 49:39–45. [PubMed: 11913825]
- Akizuki S, Mow VC, Muller F, Pita JC, Howell DS. Tensile properties of human knee joint cartilage. II. Correlations between weight bearing and tissue pathology and the kinetics of swelling. *J Orthop Res*. 1987; 5:173–186. [PubMed: 3572588]
- Appleyard RC, Ghosh P, Swain MV. Biomechanical, histological and immunohistological studies of patellar cartilage in an ovine model of osteoarthritis induced by lateral meniscectomy. *Osteoarthritis Cartilage*. 1999; 7:281–294. [PubMed: 10329303]
- Aydelotte MB, Kuettner KE. Differences between sub-populations of cultured bovine articular chondrocytes. I. Morphology and cartilage matrix production. *Connect Tissue Res*. 1988; 18:205–222. [PubMed: 3219850]
- Bailey AJ. Perspective article: the fate of collagen implants in tissue defects. *Wound Repair Regen*. 2000; 8:5–12. [PubMed: 10760210]
- Bradley DG, Kim HJ, Min DB. Effects, quenching mechanisms, and kinetics of water soluble compounds in riboflavin photosensitized oxidation of milk. *Journal of agricultural and food chemistry*. 2006; 54:6016–6020. [PubMed: 16881710]

- Bryant SJ, Nuttelman CR, Anseth KS. Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts in vitro. *J Biomater Sci Polym Ed.* 2000; 11:439–457. [PubMed: 10896041]
- Chan J, Darr A, Alam D, Calabro A. Investigation of a novel cross-linked hyaluronan hydrogel for use as a soft-tissue filler. *AM. J. Cosmet. Surg.* 2005; 22:105–108.
- Clark JM. The organisation of collagen fibrils in the superficial zones of articular cartilage. *J Anat.* 1990; 171:117–130. [PubMed: 2081698]
- Darr A, Calabro A. Synthesis and characterization of tyramine-based hyaluronan hydrogels. *J Mater Sci Mater Med.* 2009; 20:33–44. [PubMed: 18668211]
- Dumont J, Ionescu M, Reiner A, Poole AR, Tran-Khanh N, Hoemann CD, McKee MD, Buschmann MD. Mature full-thickness articular cartilage explants attached to bone are physiologically stable over long-term culture in serum-free media. *Connect Tissue Res.* 1999; 40:259–272. [PubMed: 10757114]
- Erickson IE, Kestle SR, Zellars KH, Dodge GR, Burdick JA, Mauck RL. Improved cartilage repair via in vitro pre-maturation of MSC-seeded hyaluronic acid hydrogels. *Biomed Mater.* 2012; 7:1–10.
- Flannery CR, Hughes CE, Schumacher BL, Tudor D, Aydelotte MB, Kuettner KE, Caterson B. Articular cartilage superficial zone protein (SZP) is homologous to megakaryocyte stimulating factor precursor and is a multifunctional proteoglycan with potential growth-promoting, cytoprotective, and lubricating properties in cartilage metabolism. *Biochem Biophys Res Commun.* 1999; 254:535–541. [PubMed: 9920774]
- Franzen A, Inerot S, Hejderup SO, Heinegard D. Variations in the composition of bovine hip articular cartilage with distance from the articular surface. *Biochem J.* 1981; 195:535–543. [PubMed: 7316972]
- Goldberg RL, Toole BP. Hyaluronate inhibition of cell proliferation. *Arthritis Rheum.* 1987; 30:769–778. [PubMed: 3619961]
- Grenier S, Bhargava MM, Torzilli PA. An in vitro model for the pathological degradation of articular cartilage in osteoarthritis. *J Biomech.* 2014; 47:645–652. [PubMed: 24360770]
- Gross AJ, Sizer IW. The oxidation of tyramine, tyrosine, and related compounds by peroxidase. *The Journal of biological chemistry.* 1959; 234:1611–1614. [PubMed: 13654426]
- Hamed G, Rodriguez F. Gelation of dilute collagen solutions by ultraviolet light. *J Appl Polymer Sci.* 1975; 19:3299–3313.
- Hedbom E, Hauselmann HJ. Molecular aspects of pathogenesis in osteoarthritis: the role of inflammation. *Cell Mol Life Sci.* 2002; 59:45–53. [PubMed: 11846032]
- Hu J, Hou Y, Park H, Choi B, Hou S, Chung A, Lee M. Visible light crosslinkable chitosan hydrogels for tissue engineering. *Acta Biomater.* 2012; 8:1730–1738. [PubMed: 22330279]
- Hwang WS, Li B, Jin LH, Ngo K, Schachar NS, Hughes GN. Collagen fibril structure of normal, aging, and osteoarthritic cartilage. *J Pathol.* 1992; 167:425–433. [PubMed: 1403362]
- Ibusuki S, Halbesma GJ, Randolph MA, Redmond RW, Kochevar IE, Gill TJ. Photochemically cross-linked collagen gels as three-dimensional scaffolds for tissue engineering. *Tissue Eng.* 2007; 13:1995–2001. [PubMed: 17518705]
- Jin R, Teixeira LS, Dijkstra PJ, van Blitterswijk CA, Karperien M, Feijen J. Enzymatically-crosslinked injectable hydrogels based on biomimetic dextran hyaluronic acid conjugates for cartilage tissue engineering. *Biomaterials.* 2010; 31:3103–3113. [PubMed: 20116847]
- Kim KS, Park SJ, Yang JA, Jeon JH, Bhang SH, Kim BS, Hahn SK. Injectable hyaluronic acid-tyramine hydrogels for the treatment of rheumatoid arthritis. *Acta Biomater.* 2011; 7:666–674. [PubMed: 20883838]
- Koide T, Daito M. Effects of various collagen crosslinking techniques on mechanical properties of collagen film. *Dent Mater J.* 1997; 16:1–9. [PubMed: 9549996]
- Lee CR, Grodzinsky AJ, Spector M. The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. *Biomater.* 2001a; 22:3145–3154.
- Lee F, Chung JE, Kurisawa M. An injectable enzymatically crosslinked hyaluronic acid-tyramine hydrogel system with independent tuning of mechanical strength and gelation rate. *Soft Matter.* 2008; 4:880–887.

- Lee F, Chung JE, Kurisawa M. An injectable hyaluronic acid-tyramine hydrogel system for protein delivery. *J Control Release*. 2009; 134:186–193. [PubMed: 19121348]
- Lee JE, Park JC, Hwang YS, Kim JK, Kim JG, Sub H. Characterization of UV-irradiated dense/porous collagen membranes: morphology, enzymatic degradation, and mechanical properties. *Yonsei Med J*. 2001b; 42:172–179. [PubMed: 11371103]
- Lorenzo P, Bayliss MT, Heinegard D. Altered patterns and synthesis of extracellular matrix macromolecules in early osteoarthritis. *Matrix Biol*. 2004; 23:381–391. [PubMed: 15533759]
- Lotke PA, Granda JL. Changes in the permeability of human articular cartilage in early degenerative osteoarthritis. *Surg Forum*. 1971; 22:449–450. [PubMed: 5121435]
- Maroudas A, Bayliss MT, Venn MF. Further studies on the composition of human femoral head cartilage. *Ann Rheum Dis*. 1980; 39:514–523. [PubMed: 7436585]
- McDevitt C, Gilbertson E, Muir H. An experimental model of osteoarthritis; early morphological and biochemical changes. *J Bone Joint Surg Br*. 1977; 59:24–35. [PubMed: 576611]
- Moore MA, Bohachevsky IK, Cheung DT, Boyan BD, Chen WM, Bickers RR, McIlroy BK. Stabilization of pericardial tissue by dye-mediated photooxidation. *J Biomed Mater Res*. 1994; 28:611–618. [PubMed: 8027101]
- Mow VC, Kuei SC, Lai WM, Armstrong CG. Biphasic creep and stress relaxation of articular cartilage in compression? Theory and experiments. *J Biomech Eng*. 1980; 102:73–84. [PubMed: 7382457]
- Musso H. Phenol oxidation reactions. *Angewandte Chemie International Edition in English*. 1963; 2(12):723–735.
- Nishimura M, Yan W, Mukudai Y, Nakamura S, Nakamasu K, Kawata M, Kawamoto T, Noshiro M, Hamada T, Kato Y. Role of chondroitin sulfate hyaluronan interactions in the viscoelastic properties of extracellular matrices and fluids. *Biochim Biophys Acta*. 1998; 1380:1–9. [PubMed: 9545510]
- Oungoulian SR, Hehir KE, Zhu K, Willis CE, Marinescu AG, Merali N, Ahmad CS, Hung CT, Ateshian GA. Effect of glutaraldehyde fixation on the frictional response of immature bovine articular cartilage explants. *J Biomech*. 2014; 47:694–701. [PubMed: 24332617]
- Owen JR, Wayne JS. Influence of a superficial tangential zone over repairing cartilage defects: implications for tissue engineering. *Biomech Model Mechanobiol*. 2006; 5:102–110. [PubMed: 16506018]
- Panula HE, Hyttinen MM, Arokoski JP, Langsjo TK, Peltari A, Kiviranta I, Helminen HJ. Articular cartilage superficial zone collagen birefringence reduced and cartilage thickness increased before surface fibrillation in experimental osteoarthritis. *Ann Rheum Dis*. 1998; 57:237–245. [PubMed: 9709181]
- Pek YS, Kurisawa M, Gao S, Chung JE, Ying JY. The development of a nanocrystalline apatite reinforced crosslinked hyaluronic acid-tyramine composite as an injectable bone cement. *Biomaterials*. 2009; 30:822–828. [PubMed: 19027947]
- Roughley PJ, Lee ER. Cartilage proteoglycans: structure and potential functions. *Microsc Res Tech*. 1994; 28:385–397. [PubMed: 7919526]
- Rowland CR, Lennon DP, Caplan AI, Guilak F. The effects of crosslinking of scaffolds engineered from cartilage ECM on the chondrogenic differentiation of MSCs. *Biomaterials*. 2013; 34:5802–5812. [PubMed: 23642532]
- Schlichting KE, Copeland-Johnson TM, Goodman M, Lipert RJ, Prozorov T, Liu X, McKinley TO, Lin Z, Martin JA, Mallapragada SK. Synthesis of a novel photopolymerized nanocomposite hydrogel for treatment of acute mechanical damage to cartilage. *Acta Biomater*. 2011; 7:3094–3100. [PubMed: 21530694]
- Schumacher BL, Block JA, Schmid TM, Aydelotte MB, Kuettner KE. A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. *Arch Biochem Biophys*. 1994; 311:144–152. [PubMed: 8185311]
- Setton LA, Zhu W, Mow VC. The biphasic poroviscoelastic behavior of articular cartilage: role of the surface zone in governing the compressive behavior. *J Biomech*. 1993; 26:581–592. [PubMed: 8478359]

- Sharma B, Fermanian S, Gibson M, Unterman S, Herzka DA, Cascio B, Coburn J, Hui AY, Marcus N, Gold GE, Elisseeff JH. Human cartilage repair with a photoreactive adhesive-hydrogel composite. *Sci Transl Med*. 2013; 5:1–9.
- Siparsky P, Ryzewicz M, Peterson B, Bartz R. Arthroscopic treatment of osteoarthritis of the knee: are there any evidence-based indications? *Clin Orthop Relat Res*. 2007; 455:107–112. [PubMed: 17279040]
- Sitterle VB, Nishimuta JF, Levenston ME. Photochemical approaches for bonding of cartilage tissues. *Osteoarthritis Cartilage*. 2009; 17:1649–1656. [PubMed: 19591798]
- Spoerl E, Wollensak G, Seiler T. Increased resistance of crosslinked cornea against enzymatic digestion. *Curr Eye Res*. 2004; 29:35–40. [PubMed: 15370365]
- Stanescu R, Stanescu V. In vitro protection of the articular surface by cross-linking agents. *J Rheumatol*. 1988; 15:1677–1682. [PubMed: 2853226]
- Stalling SS, Akintoye SO, Nicoll SB. Development of photocrosslinked methylcellulose hydrogels for soft tissue reconstruction. *Acta Biomater*. 2009; 5:1911–1918. [PubMed: 19303378]
- Stuart MJ, Lubowitz JH. What, if any, are the indications for arthroscopic debridement of the osteoarthritic knee? *Arthroscopy*. 2006; 22:238–239. [PubMed: 16523583]
- Stubendorff JJ, Lammintausta E, Struglics A, Lindberg L, Heinegard D, Dahlberg LE. Is cartilage sGAG content related to early changes in cartilage disease? Implications for interpretation of dGEMRIC. *Osteoarthritis Cartilage*. 2012; 20:396–404. [PubMed: 22334095]
- Teixeira LS, Feijen J, van Blitterswijk CA, Dijkstra PJ, Karperien M. Enzyme-catalyzed crosslinkable hydrogels: emerging strategies for tissue engineering. *Biomaterials*. 2012; 33:1281–1290. [PubMed: 22118821]
- Teshima R, Otsuka T, Takasu N, Yamagata N, Yamamoto K. Structure of the most superficial layer of articular cartilage. *J Bone Joint Surg Br*. 1995; 77:460–464. [PubMed: 7744937]
- Tobetto K, Nakai K, Akatsuka M, Yasui T, Ando T, Hirano S. Inhibitory effects of hyaluronan on neutrophil-mediated cartilage degradation. *Connect Tissue Res*. 1993; 29:181–190. [PubMed: 8222645]
- Toh WS, Lim TC, Kurisawa M, Spector M. Modulation of mesenchymal stem cell chondrogenesis in a tunable hyaluronic acid hydrogel microenvironment. *Biomaterials*. 2012; 33:3835–3845. [PubMed: 22369963]
- Torzilli PA, Adams TC, Mis RJ. Transient solute diffusion in articular cartilage. *J Biomech*. 1987; 20:203–214. [PubMed: 2437125]
- Torzilli PA, Arduino JM, Gregory JD, Bansal M. Effect of proteoglycan removal on solute mobility in articular cartilage. *J Biomech*. 1997; 30:895–902. [PubMed: 9302612]
- Torzilli, PA.; Askari, E.; Jenkins, JT. Water content and solute diffusion properties of articular cartilage.. In: Mow, VC.; Ratcliffe, A.; Woo, SLY., editors. *Biomechanics of Diarthroidial Joints*. Springer-Verlag; New York: 1990. p. 363-390.
- Tran-Khanh N, Hoemann CD, McKee MD, Henderson JE, Buschmann MD. Aged bovine chondrocytes display a diminished capacity to produce a collagen-rich, mechanically functional cartilage extracellular matrix. *J Orthop Res*. 2005; 23:1354–1362. [PubMed: 16048738]
- van Luyn MJ, Khouw IM, van Wachem PB, Blaauw EH, Werkmeister JA. Modulation of the tissue reaction to biomaterials. II. The function of T cells in the inflammatory reaction to crosslinked collagen implanted in T-cell-deficient rats. *J Biomed Mater Res*. 1998; 39:398–406. [PubMed: 9468048]
- von Porat A, Roos EM, Roos H. High prevalence of osteoarthritis 14 years after an anterior cruciate ligament tear in male soccer players: a study of radiographic and patient relevant outcomes. *Ann Rheum Dis*. 2004; 63:269–273. [PubMed: 14962961]
- von Rechenberg B, Akens MK, Nadler D, Bittmann P, Zlinszky K, Neges K, Auer JA. The use of photooxidized, mushroom-structured osteochondral grafts for cartilage resurfacing--a comparison to photooxidized cylindrical grafts in an experimental study in sheep. *Osteoarthritis Cartilage*. 2004; 12:201–216. [PubMed: 14972337]
- Wang LS, Lee F, Lim J, Du C, Wan AC, Lee SS, Kurisawa M. Enzymatic conjugation of a bioactive peptide into an injectable hyaluronic acid-tyramine hydrogel system to promote the formation of functional vasculature. *Acta Biomater*. 2014; 10:2539–2550. [PubMed: 24561710]

- Watanabe K, Koyama Y. Adhesion of macrophages on collagen irradiated with ultraviolet light. *J Biomater Sci Polym Ed.* 1999; 10:351–361. [PubMed: 10189103]
- Weadock KS, Miller EJ, Bellincampi LD, Zawadsky JP, Dunn MG. Physical crosslinking of collagen fibers: comparison of ultraviolet irradiation and dehydrothermal treatment. *J Biomed Mater Res.* 1995; 29:1373–1379. [PubMed: 8582905]
- Williams CG, Malik AN, Kim TK, Manson PN, Elisseff JH. Variable cytocompatibility of six cell lines with photoinitiators used for polymerizing hydrogels and cell encapsulation. *Biomaterials.* 2005; 26:1211–1218. [PubMed: 15475050]
- Wollensak G, Spoerl E, Reber F, Seiler T. Keratocyte cytotoxicity of riboflavin/UVA-treatment in vitro. *Eye (Lond).* 2004; 18:718–722. [PubMed: 14739922]
- Wollensak G, Spoerl E, Seiler T. Riboflavin/ultraviolet-a-induced collagen crosslinking for the treatment of keratoconus. *Am J Ophthalmol.* 2003a; 135:620–627. [PubMed: 12719068]
- Wollensak G, Spoerl E, Seiler T. Stress-strain measurements of human and porcine corneas after riboflavin-ultraviolet-A-induced cross-linking. *J Cataract Refract Surg.* 2003b; 29:1780–1785. [PubMed: 14522301]
- Yettella RR, Min DB. Quenching mechanisms and kinetics of Trolox and ascorbic acid on the riboflavin-photosensitized oxidation of tryptophan and tyrosine. *Journal of agricultural and food chemistry.* 2008; 56(22):10887–10892. [PubMed: 18975971]

Figure 1. Representative chromatogram from a 60 min reaction of tyramine in the presence of RF monitored at 274 nm. Tyramine retention time at 21.3 min, C-O bonded dityramine at 18.4 min and C-C bonded dityramine at 17.2 min.

Figure 2.

Dityramine product fraction of total peak area dependence on reaction time. Dityramine product fraction defined as the ratio of C-C bonded dityramine and C-O bonded dityramine peak areas to total peak area (products and reactant). Note the positive correlation. Control reaction was exposed to UV for 60 min in the absence of RF. Data reported as average with standard deviation error bars, $n = 2$.

Figure 3.

Predicted theoretical creep deformations for intact, degraded and repaired cartilage explants generated from the average experimental values for permeability, instantaneous strain, and aggregate modulus using the biphasic theory.

Figure 4.

Cartilage explant properties (y-axis, mean $\pm$ SEM) of intact, degraded and repaired states for the TS-HA resurfacing treatments (x-axis). The instantaneous modulus was calculated by fitting an exponential function to the stress-strain response during the loading phase and the slope calculated at a strain of 5%. The instantaneous strain was determined at the end of the loading phase. The aggregate modulus was approximated at the end of the 15 min creep test data. The apparent permeability and modulus were both measured after 100 sec. Two-way repeated measures ANOVA with Bonferroni post tests were used for statistical analysis ($\alpha=0.05$, N = 10). * indicates statistically significant difference with intact state. ° indicates statistically significant difference with degraded state.

Figure 5.

Experimental creep responses for intact, degraded and repaired (TS-HA / RF 0.1%) cartilage explant immediately (0 hr), and 12, 24, 36, 48, 60 hrs and 7 days after resurfacing treatment.

Figure 6.

Representative micrographs (10X) of Safranin-O/Fast Green staining of intact, degraded and repaired (TS-HA / RF 0.1%) cartilage explants. In the repaired explant, a thin layer of hyaluronate (red) is attached to the articular surface as indicated by the arrows. Scale bar = 250 μ m.

Figure 7.

Representative micrographs (20X) of intact (control) and treated (TS-HA) cartilage explants depicting chondrocyte viability. The articular surface is located at the top of each image and resurfacing treatments include the use of UV light only, and TS HA in conjunction with either 0.0012% RF, 0.1% RF or 0.05% IRG. The LIVE/DEAD stain provides a two-color fluorescence with green corresponding to live cells and red representing dead cells. A predominance of viable cells was seen in all images, independent of time in culture and treatment type. A thin layer of dead cells (red) was observed at the articular surface when using 0.1% RF or 0.05% IRG.

Table 1

List of resurfacing treatments tested with corresponding polymer/photoinitiator combinations.

Abbreviation	Polymer	Photoinitiator
Φ/RF 0.1%	ΦD (none)	0.1 % Riboflavin
CS/RF 0.1%	1.5 % Chondroitin Sulfate	0.1 % Riboflavin
CMC/IRG0.05%	2 % Methacrylated Carboxymethylcellulose	0.05 % Irgacure
HA/RF0.1%	1.5 % Sodium Hyaluronate	0.1 % Riboflavin
TS-HA/RF 0.1%	1.5 % Tyramine-Substituted Sodium Hyaluronate	0.1 % Riboflavin
TS-HA/RF 0.0012%	1.5 % Tyramine-Substituted Sodium Hyaluronate	0.0012 % Riboflavin
TS-HA/IRG 0.05%	1.5 % Tyramine-Substituted Sodium Hyaluronate	0.05 % Irgacure