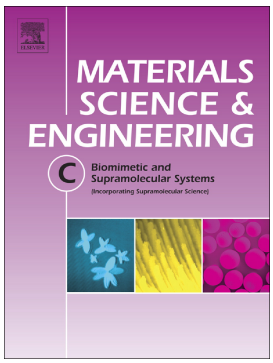

RGDSP functionalized carboxylated agarose as extrudable carriers for chondrocyte delivery

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RGDSP functionalized carboxylated agarose as extrudable carriers for chondrocyte delivery

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Abstract:

The limited potential of cartilage to regenerate itself has led to development of new strategies and biomaterials for cartilage tissue engineering and regenerative medicine. Although de novo strategies for cartilage repair have been realized, extrudable hydrogels that can be administered in minimally invasive manner while simultaneously supporting chondrogenic differentiation could lead to development of new systems to deliver cells to cartilage lesions. In this work, we explored the suitability of thermo-reversible, extrudable gels derived from carboxylated agarose for maintaining human articular chondrocyte (HAC) phenotype. Towards this objective, we have investigated the impact of hydrogel stiffness and presence of integrin-binding peptide sequence GGGGRGDSP on HAC differentiation potential. We discovered that stiffer hydrogels (5.8 kPa) are more efficient than softer counterparts (0.6 kPa) in promoting chondrogenesis. Interestingly, in GGGGRGDSP modified gels, a synergy between stiffness and RGD signaling led to enhanced expression of chondrogenic related genes (aggrecan, collagen type II and sox9). These findings were also supported by quantitative analysis of sulphated glycosaminoglycans. Since carboxylated agarose are highly suitable as bioink for 3D bioprinting, we propose that extrudable GGGGRGDSP-linked stiff carboxylated agarose as a medium for direct printing of chondrocyte into cartilage lesion.

Keywords: mechanical properties, 3D printing, hydrogel stiffness, polymer extrusion, cartilage tissue engineering

Introduction:

Cartilage lesions are associated with low self-repair potential and tissue engineering approach can address this limitation. In the past decades, several strategies for de novo cartilage formation have been realized [1-3], however to date there has been single example of truly de novo engineering of the cartilage [4]. These approaches utilize cells such as mesenchymal stem cells (MSCs) or chondrocytes harvested from patient cartilage biopsy, followed by their expansion and differentiation in vitro in order to generate a tissue mass [5, 6], which could then be potentially implanted to the patient [1, 7, 8]. Implantation of scaffolds with cells often requires open surgeries, as reported previously for the implantation of chondrocyte loaded alginate scaffolds [9]; this could be associated with patient morbidity as well as reduced patient compliance. To minimize surgical complications and facilitate patient recovery, injectable hydrogels that can be administered in a minimally invasive manner would be beneficial. In this regard, physical hydrogels that could be injected as a solution, which forms a firm hydrogel upon injection and are able to maintain the phenotype of chondrocytes in situ, could lead to advancement in therapies. Several hydrogel systems have been described as potential candidates for minimally invasive procedures. This includes alginate, which forms a hydrogel network upon mixing with a calcium ion solution [10], and agarose, a thermogelling hydrogel that crosslinks upon a change in temperature [11, 12]. Along with the advantage of being an injectable system, physical hydrogels such as alginate or agarose mimic the natural cellular mechanical environment, thereby demonstrating potential for tissue engineering applications [3, 10, 13, 14].

Our recent study has brought in to focus the importance of the dynamics between matrix stiffness and degradation in re-differentiation of expanded human articular chondrocytes and maintenance of this phenotype after implantation [15]. Key finding of the study was that the stiffness of

hydrogels could significantly assist chondrogenic differentiation and maintain the chondrogenic phenotype [15]. Since degradation is variable in this study we chose to decouple degradation from stiffness using carboxylated agarose of varying stiffness [11] in order to investigate the true impact of stiffness. Although these hydrogels have attractive mechanical properties, they do not possess any biological information. As a result, the cells dispersed within these hydrogels do not interact biologically with the material. Previously, we have extensively investigated the *in vitro* differentiation of human articular chondrocytes on planar surfaces functionalized with collagen type I [16] and RGD peptide [17]. Therefore in order to couple the cell with the mechanical attributes of the microenvironment we introduced the cell-integrin binding peptide sequence GGGGRGDSP to our hydrogel system. Towards this objective, the effect of the cell binding peptide sequence GGGGRGDSP and the stiffness of the hydrogels on the phenotype of de-differentiated HAC were investigated. In this study, stiff (5.8 kPa) and soft (0.6 kPa) hydrogels functionalized with the GGGGRGDSP integrin binding peptide sequence were compared to the stiff and soft hydrogels without any biological signals, **Schematic 1**. The chondrogenic re-differentiation of HACs was evaluated using real-time polymerase chain reaction (RT-PCR), histological staining and biochemical assay.

Materials and Methods

Materials

Agarose was purchased from Merck (Darmstadt, Germany) and GGGGRGDSP was procured from Peptides Internationals (Louisville, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), (2,2,6,6-Tetramethylpiperidin-1-yl)oxyl (TEMPO), NaCl, NaOH solution,

$NaBH_4$, NaBr and NaOCl were purchased from Sigma Aldrich (St Louis, USA) and used as received.

Synthesis of carboxylated agarose and RGD linked agarose

The hydrogels were prepared following a procedure described previously.[11] One gram of native agarose was transferred into a three-necked round bottom flask, equipped with a mechanical stirrer and pH meter. The reactor was heated to 90°C to dissolve agarose and then cooled to 0°C under continuous mechanical stirring in order to prevent the solution from gelling. The reactor was then charged with TEMPO (0.160 mmol, 20.6 mg), NaBr (0.9 mmol, 0.1 g), and NaOCl (2.5 mL, 15% vol/vol solution) under vigorous stirring. The pH of the solution was adjusted to 10.8 throughout the duration of the reaction, and the degree of carboxylation was controlled by the addition of predetermined volumes of NaOH solution (0.5 M). At the end of the reaction $NaBH_4$ (0.1 g) was added, and the solution was acidified to pH 8 and stirred for 1 h. The resulting hydrogel was precipitated by sequential addition of NaCl (0.2 mol, 12 g) and ethanol (500 mL), and the solid material was collected by vacuum filtration and extracted using ethanol. Residual ethanol was removed by extensive dialysis against water and the carboxylated hydrogel was obtained as a white solid upon freeze-drying overnight. Functionalization of the carboxylated agarose with the GGGGRGDSP peptide was performed using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) coupling chemistry. Briefly, hydrogels (30 mg, 0.25μmol) were dissolved in MES sterile buffer and an excess EDC (210 mg, 1.3 mmol) was added and the solution was stirred for 30 min and following this the peptide was added (1 mg, 1.33μmol) and the solution stirred for an additional 2 h at room temperature. The hydrogels were then sterilized overnight in 70% ethanol, and ethanol was removed by extensive dialysis against autoclaved MilliQ water. The sterile functionalized hydrogels were freeze-dried overnight to

yield a white fibrous solid. The amount of peptide incorporated in the gel was quantified by elemental analysis as previously described [11] and was determined to be identical based on the nitrogen content whose source can be uniquely attributed to the peptide.

Mechanical studies

Rheology experiments were performed with a Physica MCR 301 (Anton Paar, Austria) equipped with plate geometry PPR25. Samples were prepared by dissolving 2 % (w/v) concentration of polysaccharide in deionized water at 90 °C for 10 min until a clear solution was obtained. The liquid was then introduced into the rheometer plate and allowed to set at room temperature for 30 min to induce gelation. G′and G″values were determined at 1 Hz by increasing the rotation frequency from 0.01 rad/s up to 10 rad/s with a 1% deformation. For the viscosity experiments samples were prepared in an identical manner and the shear strain was increased from 1 to 20 rad.

Stability studies

Hydrogels (0.5 mL) at 2 % (w/v) were solubilized at 90°C and pipetted into cell strainer (diameter of 25 mm, mesh size of 40 µm, Corning, USA) and following gelation the strainer-gel ensemble were assembled in a 6 well plate (Sarstedt, Germany) and immersed in PBS and incubated at 37 °C for 14 days. At pre-determined time points, the cell strainers were removed from the well plate, excess media was wiped from the bottom of the mesh and the mass was recorded on a balance (Mettler Toledo, USA). This data was use to calculate weight loss of the hydrogel as a function of time.

Zeta potential

Carboxylated agarose was dissolved in deionized water below the concentration for gel formation (0.02 % w/v) and Zeta potential was measured in triplicate using a Delsa Nano C (Beckman Coulter Inc., Brea, CA) at room temperature in deionized water.

Scanning electron microscopy (SEM)

Samples were imaged using a Quanta 250 FEG (FEI, Inc.) environmental scanning electron microscopy equipped with a vCD (FEI, Inc.) back-scattered electron detector at an accelerating voltage of 10 kV, under low vacuum, at a working distance of 10 mm. Hydrogels at 2% (w/v) were freeze-dried and then vertically dissected to expose the interior for imaging. Images shown are representative of the entire cross-section of several batches of a given modification.

Atomic force microscopy (AFM)

Atomic Force Microscopy (AFM) pictures were obtained using a Dimension 2100 (Bruker AXS) in tapping mode with a phosphorous-doped silica RFESP cantilever (Bruker AXS). For imaging gels (2 % w/v) were directly formed on a glass slide and imaged in the wet state.

Cell culture

Healthy articular were harvested from the femoral condyle of 17 years old male donor, under informed consent and in accordance with the Local Ethical Committee of the Uniklinik Freiburg. HAC were isolated and expanded based on the previously published protocol [18]. Briefly, the harvested tissue was transferred to the laboratory under sterile conditions, and minced into smaller tangential chips and then incubated at 37 °C in 0.15% type II collagenase. The digested

cartilage was filtered through a 100 µm cell strainer to remove large debris and the collagenase was then neutralized with DMEM supplemented with 10% fetal bovine serum (FBS) followed by centrifugation, washing and resuspension in complete medium containing Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), 10% FBS (Invitrogen), 4.5 mg/mL d-glucose, 0.1 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate, 100 mM HEPES buffer (Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco), and 0.29 mg/mL l-glutamate (Gibco). HAC were plated in cell culture flasks at a density of 10^4 cells/cm2 and expanded in complete medium supplemented with 5 ng/mL of fibroblast growth factor 2 (FGF-2) (R &D systems) and 1 ng/mL of transforming growth factor β1 (TGF- β1) (R &D systems). When cells reached 70% confluency, they were detached using 0.3% type II collagenase treatment followed by 0.05% trypsin/0.53 mM EDTA treatment and re-plated at a density of 5000 cells/cm2, and cultured until sub-confluent level for further passages. For cell encapsulation studies, chondrocytes from passage 3-5 were used. The cells were detached from the culture flask by first treating with 0.3% collagenase (Invitrogen, Germany), followed by trypsin digestion. The cells were then pelleted before resuspension into agarose gels. Agarose dispersed in dissolution medium (high glucose 4.5% DMEM with glutamax and sodium pyruvate, 1% HEPES, 1% NEAA, 1% PS) was heated to 90° C and agarose-RGD gels were heated to 70°C. They were then cooled to 37°C before HAC resuspension following which 120 µl of agarose-HAC suspension (2x 10^6 cells/100 µl) was seeded onto 48 well plates. The plate was then cooled at 4°C for gel formation before the addition of 2X differentiation medium (high glucose 4.5% DMEM with glutamax and sodium pyruvate, 1% HEPES, 1% NEAA, 1% PS, 1% ITS + 1 (Insulin, transferrin, selenium) (Pan-Biotech, Germany), 1% HSA (human serum albumin) (Baxter, Germany), 0.1 mM Ascorbic acid-2 phosphate (Sigma-Aldrich, Germany), 10-7 M dexamethasone (Sigma-Aldrich, Germany)

and 10 ng/ml TGF-β1) to each well. Medium change was performed every alternate day with 1X differentiation medium.

RNA isolation and RT-PCR

After 2 weeks of incubation, RNA was isolated from HAC encapsulated in agarose gels as follows. First gel was dissolved in a buffer (Qiagen) following which RNA was isolated using RNAeasy mini kit (Qiagen) and cDNA was synthesized using 250 ng of RNA (QuantiTect RT kit, Qiagen) with the following thermal cycler conditions: 95°C for 5 minutes, followed by 40 cycles of 95°C for 5 seconds and 60°C for 10 seconds. Expression of Aggrecan, Collagen type II, Collagen type I and Sox9 was performed using real time PCR and the data was normalized to 18S rRNA. Gene expression levels of various markers in HAC encapsulated hydrogels after 14 days of culture was then normalized to 2-D HAC before encapsulation. The sequence of the primers was as follows: Aggrecan: Forward TCAGGGTTACCAGGGTTCAG Reverse: TTCAACCAAACTGGTGTCCA; Collagen I: Forward: GTGCTAAAGGTGCCAATGGT Reverse: ACCAGGTTCACCGCTGTTAC; Collagen II: Forward: CCCTGAGTGGAAGAGTGGAG Reverse: GTTTGCTGGATTGGGGTAGA; Sox9 Forward AGACAGCCCCCTATCGACTT, Reverse: ATCGAAGGTCTCGATGTTGG; 18srRNA: Forward: CCTGCGGCTTAATTTGACTC; Reverse: AACTAAGAACGGCCATGCAC.

DNA and s-GAG quantification

At the end of day 14, agarose gels (with and without RGD, with and without cells) were washed with PBS and used for further analysis. Briefly, agarose gels were freeze-dried and incubated in papain cocktail (125 μg/ml papain, 10 mM L-cysteine, 100 mM Na2HPO4, 10 mM EDTA, pH 6.3) for 16 h at 60°C. DNA was estimated using Picogreen dye (Invitrogen) according to the manufacturer's instructions. Briefly, papain digested samples were centrifuged and the 10 μL of

supernatant was transferred to 96 well plate and mixed with 90 μL of 1X TE buffer. The sample was then mixed with Quant-iT PicoGreen reagent (1:200 dilution) and the fluorescence in the wells was determined with excitation and emission wavelengths of 485 and 528 nm, respectively using a Synergy HT microplate reader (Bio-TEK Instruments INC, USA). s-GAG was measured using 1, 9-dimethylmethylene blue (DMMB) assay [6, 19] and quantified using standard curves generated with tracheal chondroitin 4-sulfate. The GAG was then normalized to DNA, which was determined using λ phage DNA a reference.

Histological analysis and cell shape factor determination

At the end of day 14, samples were fixed with paraformaldehyde for 1 hour at room temperature. The samples were then washed with PBS and incubated with OCT before cryosectioning. Safranin-O (Applichem, Germany) staining was performed on 10 μm thick sections and the sections were imaged using Zeiss Axio microscope (Observer.A1). For cell shape factor, bright field images of chondrocyte-encapsulated hydrogels were used and cell shape factor was determined by ImageJ software using the following equation:

cell shape factor = perimeter/surface area.

Statistical analysis

All quantitative results in this study were expressed as mean ± standard error/deviation. Further, statistical analysis was performed using one-way analysis of variance (ANOVA) and student's t-test. Values of $p < 0.05$ were considered to be statistically significant.

Results and Discussion

Injectable carboxylated agarose hydrogels

To investigate the impact of the stiffness on chondrocyte phenotype, two types of injectable hydrogels were prepared at 2% (w/v) concentration of polysaccharide. The carboxylated agarose (**Figure 1A)** with 28% of carboxylic acid functional group (CA28) was chosen as stiff material exhibiting a shear modulus of 5.8 kPa and the carboxylated agarose with 60% of carboxylic acid functional group (CA60) was selected as the soft hydrogel with a shear modulus of 0.6 kPa (**Figure 1B)** [11]. It is known that agarose forms hydrogel through the aggregation of α-helical domains. We have demonstrated previously that carboxylation of agarose induces a switch of α-helical domain to β-sheet-like domain leading to changes in the nature of crosslinks and as a consequence realization of hydrogels of varying stiffness independent of the polysaccharide concentration in solution [11, 20]. Since CA form nanoparticles in solution, Zeta potential can be used to assess the density of carboxylic groups and these values were found to be -16.7 ± 2.1 mV and -11.7 ± 1.8 mV for soft (60% carboxylation) and stiff (28% carboxylation) hydrogels, respectively (Figure 1C). Stability of both soft and stiff hydrogels was characterized by incubation in PBS for 14 days, and the mass variation was measured at the end of day 7 and 14 and compared to the initial mass, **Figure 1D**. Over the time course of study (14 days), both soft and stiff hydrogels were stable with a limited mass loss of 4 (±3) % and 2 (±1.5) %, respectively, suggesting that the hydrogels are stable under *in vitro* conditions.

In order for a hydrogel to be exploited as an injectable system, it has to exhibit appropriate viscosity during extrusion and this can be characterized by measuring the viscosity of the hydrogel as a function of increasing strain. Shear-thinning is a non-Newtonian phenomenon

where a material exhibits lower viscosity with increasing shear stress. It was found that inspite of the significant difference between the viscosities of the two hydrogels emerging from the difference in crosslinking characteristics,shear thinning was observed for both the stiff (CA 28) and soft (CA 60) hydrogels (**Figure 1E)**, suggesting the possibility of extruding these hydrogels. These results further confirm that CA28 and CA60 are also good candidates for printing cells as using micro-extrusion bioprinting application in addition to their demonstrated utility as bioinks for printing mesenchymal stem cells using a drop-on-demand bioprinter [21].

The macrostructure of the soft and stiff carboxylated hydrogels were characterized using scanning electronic microscopy (SEM) and atomic force microscopy (AFM). Cross-section of freeze-dried hydrogel imaged by SEM confirmed our earlier findings that CA 28 and CA 60 hydrogels have different macroscale organization (**Figure 2A, 2B**) [11]. Additionally, surface analysis of the hydrated hydrogels was characterized by AFM, wherein we observed that the softer hydrogel exhibited a smoother surface (**Figure 2 C**) as compared to the stiffer hydrogels (**Figure 2 D**). Since the gelation of CA is driven by aggregation of polysaccharide chains the differences in surface roughness can be attributed to size of these aggregating species and is also consistent with our earlier findings [11]. Although some studies have attributed changes in fate choices in cells to macroscopic variables such as pore size [22], real time PCR of various genes involved in chondrogenesis namely, aggrecan (AGG), collagen type II (COL2A1) and Sox 9 (SOX9) [23] showed no appreciable differences suggesting that the macroscopic differences in the gels are not contributing factors (**Figure 3, Left Panel**).

Hydrogel stiffness and RGD sequence modulate expression of chondrogenic genes

Since CA has a carboxylic acid moiety, it is amendable to functionalization with biological signals through peptide coupling reaction and thus represents a unique class of biomaterials for studying effects of matrix mechanics on cell function and the biological signal is mechanically coupled with the polymer network [11]. This, therefore allows for investigation of the impact of mechanical properties on signaling pathways *via* cell surface receptors, such as integrins which in addition to being involved in binding to the ECM is also associated with cell motility [24] and cell differentiation [25]. It has previously been reported that the GGGGRGDSP sequence targets the integrin receptor [26] and has been used to couple cells with synthetic hydrogels such as alginate [27] and CA [11]. It has also been shown that chondrocytes in 2D culture show higher spreading and improved chondrogenic differentiation after expansion on RGD modified surfaces [17]. Therefore, the GGGGRGDSP peptide sequence was immobilized on both hydrogels, and the impact of signaling *via* integrins in conjunction with stiffness was characterized. Since the difference in the modulus of the soft and stiff hydrogels is over an order of magnitude and previous studies have reported no appreciable effects of modification with biomolecules on the agarose gel properties [28, 29] we can reasonably assume that the observed impact of RGD and stiffness is due to a synergy between these two signals perceived by the cells. It was observed that the expression of aggrecan, Sox 9, collagen type 2 were statistically significantly upregulated in stiff GGGGRGDSP modified gels (**Figure 3, Right Panel**), while expression of collagen 1 which is associated with chondrocyte hypertrophy remained unchanged in both soft/RGD and stiff/RGD conditions. More interestingly, HACs within RGD conjugated stiff gels demonstrated statistically significantly higher collagen type II/type I ratio as compared to HACs in soft RGD-modified gels and unmodified gel environments (**Figure 4A**). Since no significant

differences in HAC chondrogenic phenotype were observed between soft and stiff hydrogels (**Figure 3, Left Panel**), the observed enhancement in the preservation of the chondrogenic phenotype in RGD modified stiff gels can be attributed to a synergistic effect involving these two variables. These results also suggest that covalent immobilization of GGGGRGDSP on agarose gels is necessary for perceiving the mechanical environment and for the upregulation of markers of the chondrocyte phenotype. This could be attributed to binding of GGGGRGDSP to integrin subunits of chondrocytes (mainly α_v and β_1) followed by activation of a cascade of signaling pathways regulating cell-matrix interaction and cell fate processes like cell anchorage, differentiation and migration [30-32]. Presence of GGGGRGDSP may also allow for better cell-substrate interaction as well as better TGF-β accessibility thereby leading to higher expression of markers of the chondrogenic phenotype, as reported previously [33, 34]. However, contradictory results have also been reported in literature wherein GGGGRGDSP linked alginate hydrogels inhibited chondrogenesis of mesenchymal stem cells (MSCs) [35]. This discrepancy may be attributed to several variables including different polymer systems (alginate v/s carboxylated agarose in our case), cell types (bovine MSCs v/s HAC in our case) and analysis time points (7 days v/s 14 days in our case).

Stiffness and RGD also impact extracellular matrix markers of chondrocyte phenotype

RT-PCR data suggested that articular chondrocytes demonstrated re-differentiation in the stiff hydrogels modified with GGGGRGDSP peptide. While quantification of DNA in the various conditions at the end of day 14 did not reveal any differences (**Figure 4B**) thereby indicating that mechanical properties of hydrogels or presence of GGGGRGDSP did not impact cell

proliferation and viability, the cell shape factor analysis however revealed that the presence of RGD promoted a round morphology irrespective of mechanical properties of the cell (**Figure 4C**). More specifically, the cell shape factor decreased from 0.83 ± 0.01 and 0.84 ± 0.01 for soft and stiff hydrogels respectively, to 0.58 ± 0.02 and 0.57 ± 0.03 for soft-RGD and stiff-RGD respectively (**Figure 4C**). This further confirmed that neither hydrogel macrostructure nor the difference in functional group concentration impacted cell shape. To further assess the chondrocyte phenotype in the different hydrogel formulations, the amount of sulfated glycosaminoglycan (s-GAG) was quantified and normalized to the amount of DNA for each formulation (**Figure 4D)**. It was found that the secretion of s-GAG was significantly higher in RGD modified gels and furthermore, the average expression levels were on average higher in stiffer gels. This quantitative finding was further verified in Safranin O stained histological sections (**Figure 4E)** confirming that stiffer CA hydrogels modified with RGD are more conducive to maintaining HAC phenotype.

Conclusion

In sum, our results demonstrate that tunable mechanical properties of carboxylated agarose combined with accessible functional group, allows the investigation of the interplay between hydrogel stiffness and integrin signaling in the maintenance of HAC phenotype. We have shown that the stiffer CA hydrogels functionalized with GGGGRGDSP, in addition to being extrudable, also stimulates the expression of chondrogenic genes i.e., collagen type II, aggrecan and sox9 in HACs. Additionally, HACs cultured in CA gels secreted s-GAG a extracellular matrix molecules distinctly associated with cartilage tissue and more importantly the secretion of s-GAG was significantly higher within the stiffer GGGGRGDSP-modified hydrogels as compared to softer

GGGGRGDSP-modified hydrogels, thereby demonstrating the potential of RGD-modified carboxylated agarose hydrogels for chondrocyte delivery in cartilage tissue engineering. The results of this study also the impetus for the development of extrudable hydrogels supporting chondrocyte phenotype with other cell adhesion peptides for 3D bioprinting applications [36]. In future studies, such 3D-printable environments can be combined with cells engineered to express near infrared proteins [37] and for visualization of protein secretion in vitro and in vivo via optical imaging techniques such as fluorescence molecular tomography [18].

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Conflicts of interest: None

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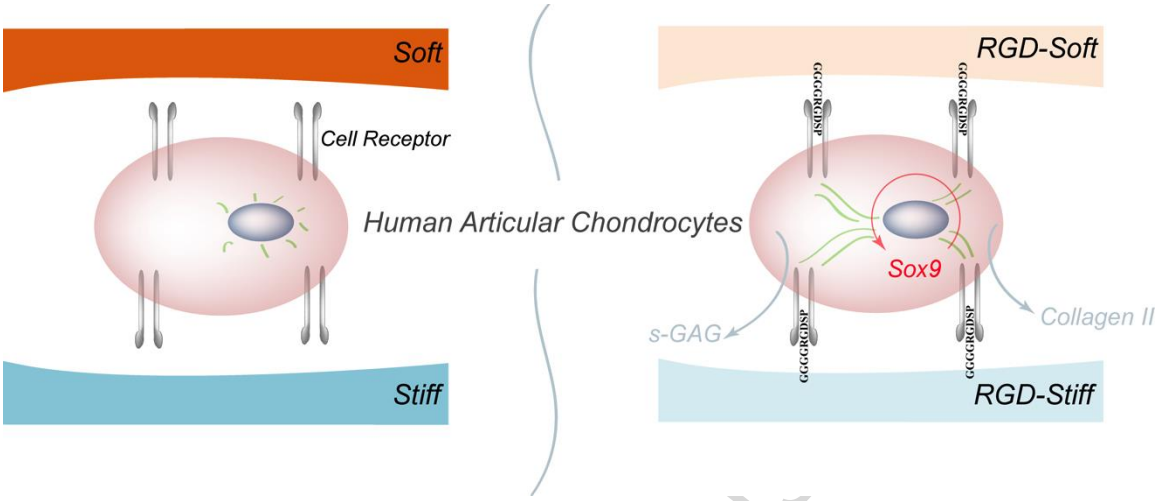
Figures



Schematic 1. Schematic representation of the experiment conducted in this study. Human articular cartilage cells are dispersed either in a soft or stiff hydrogel modified or not with RGD. The production of sulfated GAG and activation of gene pathways characteristic of the chondrocyte phenotype is assessed.

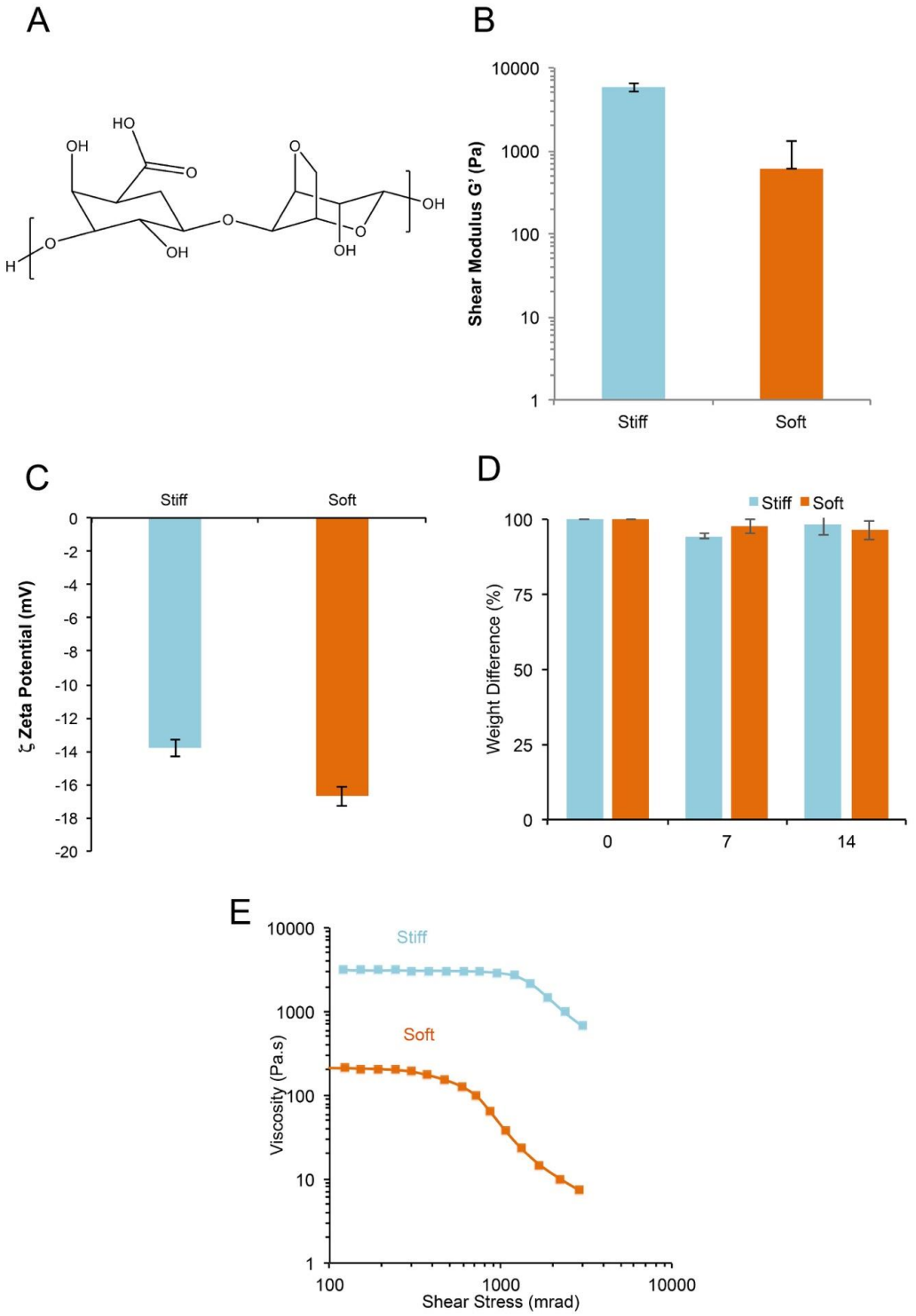


Figure 1. A: Chemical structure of carboxylated agarose hydrogel. **B:** Stability of the hydrogels under physiological conditions assessed by weight change over 14 days. **C:** Zeta potential of stiff (28% carboxylated agarose) and soft (60% carboxylated agarose) hydrogels, n = 3. D: The shear modulus of the stiff and soft carboxylated agarose hydrogels, n=3 . **E:** Shear thinning properties of the stiff and soft hydrogels used in this study as assessed by rheology.

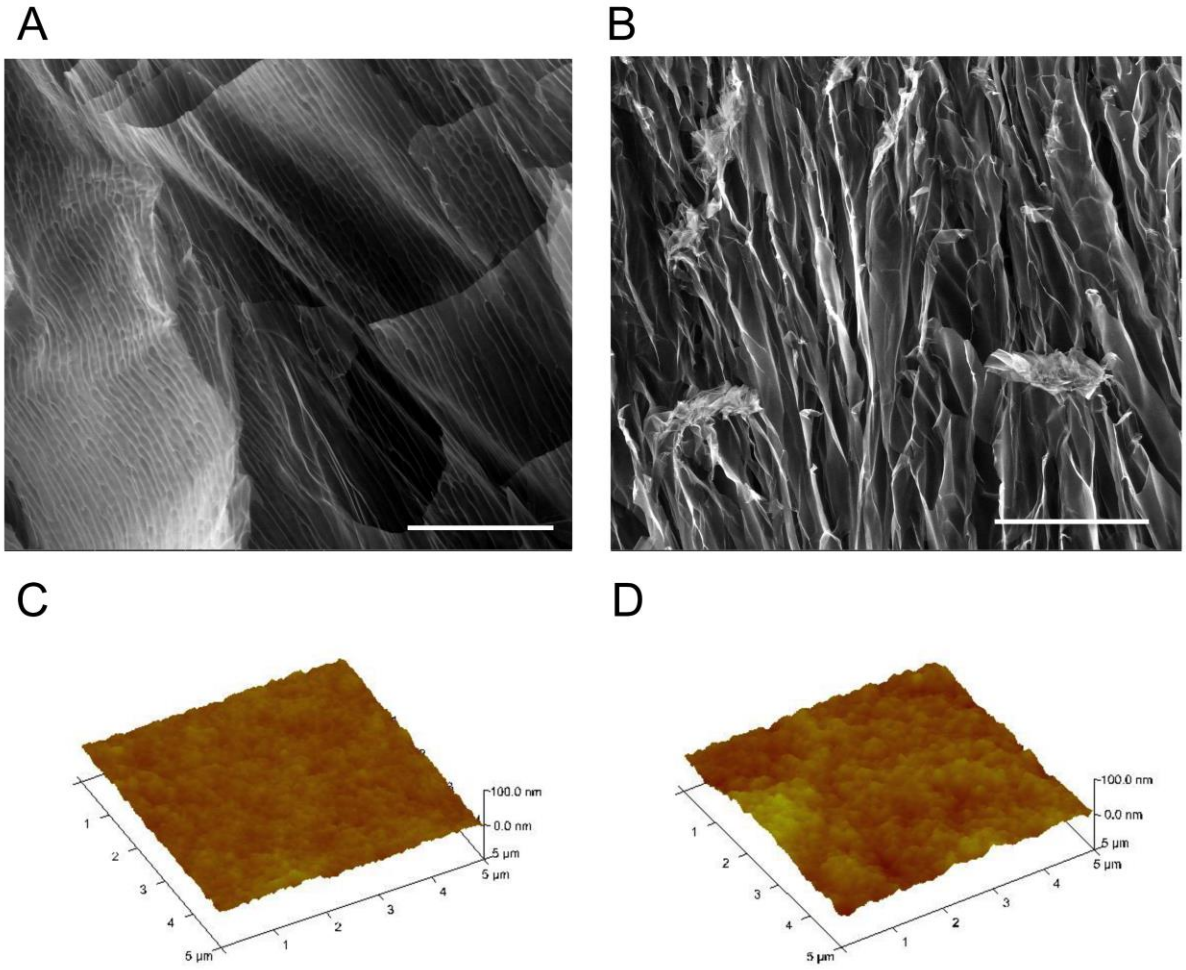


Figure 2: Scanning electron micrographs of soft (A) and stiff (B) hydrogels (Scale Bar – 100 µm). Atomic force micrographs of soft (C) and stiff (D) hydrogels

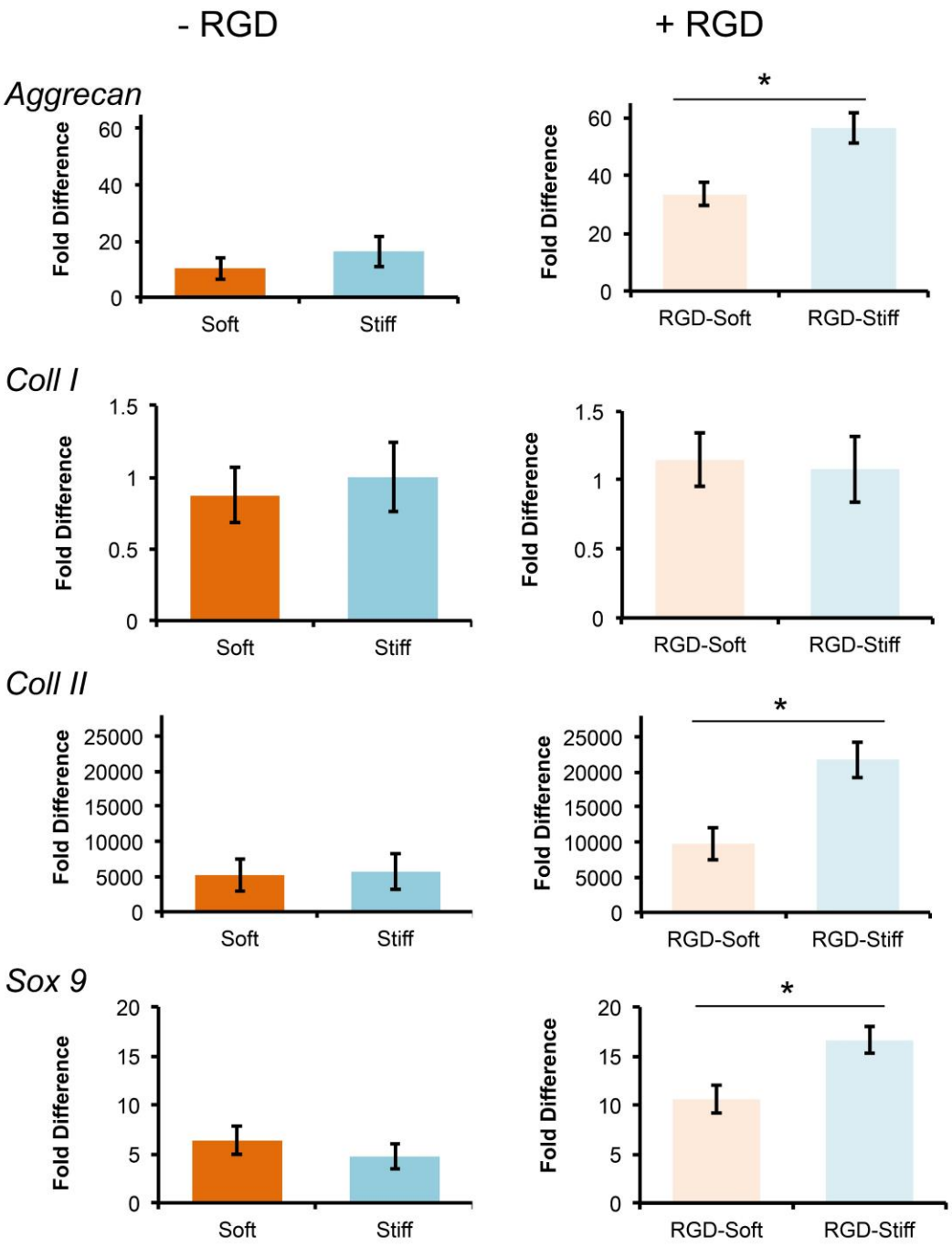


Figure 3. Gene expression of key chondrogenic markers associated with human articular chondrocytes phenotype in soft and stiff hydrogels without GGGGRGDSP functionalization (Left Panel) and with GGGGRGDSP functionalization (Right Panel) (n = 4-6, * indicates $p < 0.05$)

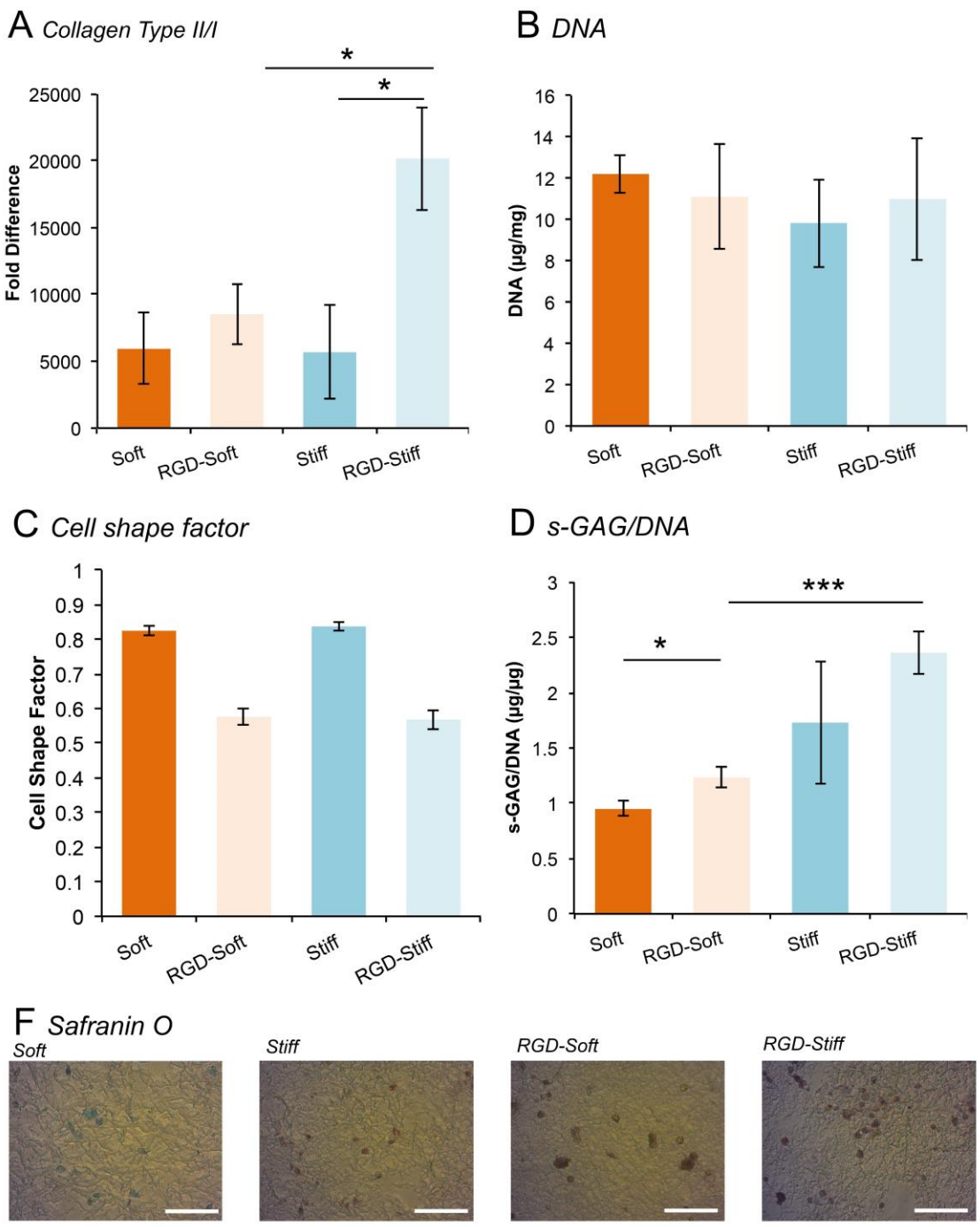
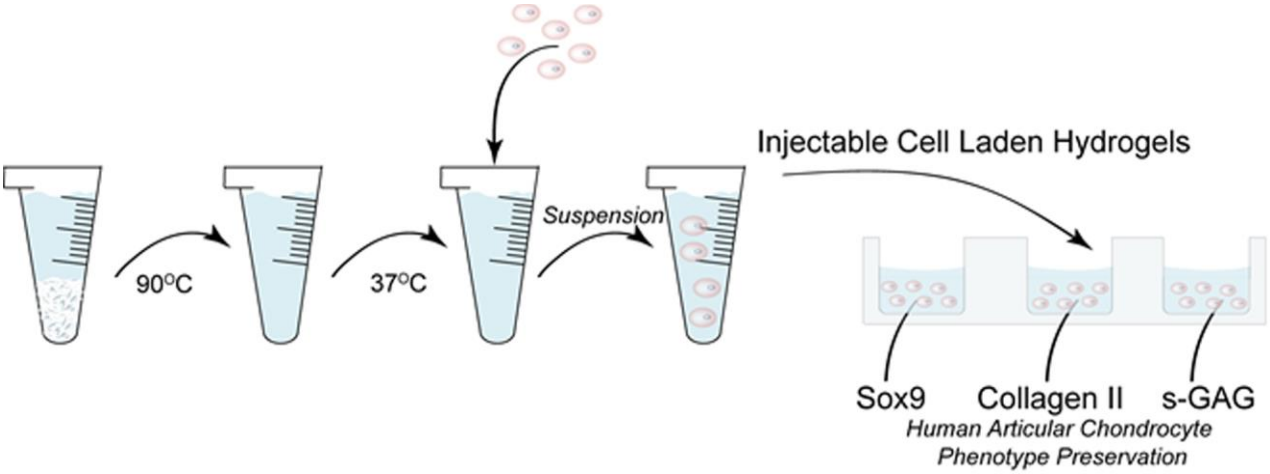


Figure 4. A: The ratio of collagen type II gene expression to collagen type I (n=4-6, * indicates $p < 0.05$). **B:** Quantification of DNA in different types of HAC encapsulated hydrogels (soft and stiff, with and without GGGGRGDSP functionalization) (n=3). **C**: Determination of cell-shape factor of chondrocytes embedded in different types of hydrogels, n= 31-65 (*** indicates $p<0.001$). C: **D**: Quantitative analysis of sGAG/DNA ratio in different types of hydrogels (n=3, * indicates $p<0.05$, *** indicates $p<0.001$). **E:** Safranin O staining showing the presence of GAG in10 µm thick sections of hydrogels encapsulated with HAC.



Graphical abstract

Highlights:

- Carboxylated agarose support human articular chondrocyte phenotype
- Stiffness of hydrogel impacts human articular chondrocyte re-differentiation
- Stiffness and RGD mediated integrin signaling enhances expression chondrogenic genes in human articular chondrocytes
- Carboxylated agarose hydrogels are suitable bioinks for bioprinting of chondrocytes