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Viscoelasticity Can Be Tuned Through Covalent Incorporation of Chondroitin Sulphate in Allylated Gelatin Hydrogels

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Cartilage is a slow-remodeling tissue with limited healing capacity. This has led to decades of tissue engineering efforts where the goal is biomaterials with regenerative capacity to restore functional integrity. Achieving full functional and mechanical integrity has proven difficult as cartilage has distinct mechanical properties. Glycosaminoglycans (GAGs) play a crucial role in cartilage mechanics due to their swelling behavior, contributing to viscoelasticity. The aims of this study are to covalently incorporate thiolated chondroitin sulphate (CSSH) in allylated gelatin (gelAGE) hydrogels at different concentrations to mimic GAG-rich regions in cartilage and create platforms to study subsequent cellular behavior. Hydrogels are evaluated for soluble fraction, swelling ratio, chondroitin sulphate (CS) retention, mechanical and viscoelastic properties, and cytocompatibility. ≈80% of CSSH is retained, and samples containing CSSH has an increased swelling ratio, indicating the incorporation of GAGs. Samples containing CSSH has an increased relaxation amplitude compared to gelAGE controls with a more elastic response. The addition of CSSH has no adverse effects on cytocompatibility. In conclusion, this study demonstrates the incorporation of thiolated CS in gelAGE hydrogels at different concentrations with no adverse effects on cytocompatibility. This allows for viscoelastic tuning which is important to consider when engineering new biomaterials.

1. Introduction

Articular cartilage has essential functions in absorbing shock and reducing friction in synovial joints. Its structural integrity is vital for healthy and pain-free aging. However, it is a slow-remodeling tissue and has therefore limited healing capacities.[1–3] This has led to decades of trying to replicate cartilage tissue and engineer replacements. The goal is to design biomaterials with the regenerative capacity to restore functional integrity. However, to this date achieving full functional and mechanical integrity of these biomaterials has proven difficult as cartilage tissue has distinct mechanical properties.

Articular cartilage is made up of a solid phase and a liquid phase which consists of up to 80% water. The solid phase is mainly composed of collagen type II (15–22%) and proteoglycans (PGs) (4–7%). Proteoglycans are made up of sulphated glycosaminoglycan (GAG) chains that are attached to a core protein. Their negative charge leads to an influx of cations into the tissue creating osmotic pressure. The collagen fibers physically limit the swelling of the PGs which then gives cartilage tissue its ability to withstand high loads.[1,3–6] Due to the two phases, cartilage as well as other living tissues, is viscoelastic meaning it exhibits mechanical properties of elastic (recoverable stress-strain) and viscous (resistance to rate of strain) materials. Purely elastic materials maintain a constant deformation under a constant load and viscous materials dissipate energy as they flow. Viscoelastic materials however exhibit

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an instantaneous elastic response upon an external load. Over time under load, these forces are dissipated as fluid flows through and out of the tissue giving it a time-dependent mechanical response.[7] As part of the tissue, GAGs play a crucial role in this time-dependent response of cartilage under load. Their swelling behavior leads to a high water content in the tissue. This is responsible for the distinct viscoelastic properties of cartilage as it promotes high initial stiffness and viscous damping.[8–10] Therefore, the mechanical properties of biomaterials play a crucial role as they determine their suitability in cartilage tissue engineering and regeneration.

The goals, therefore, are to either structurally match that of native articular cartilage and withstand high loads or to tease out responses from mechanosensitive cells to initiate tissue regeneration and desired cellular responses. In native cartilage tissue, the extracellular matrix (ECM) provides key signals that determine cellular behavior. These signals can be biochemical, such as growth factors, and also mechanical stimuli. Previous tissue engineering studies have primarily focused on the elasticity (stiffness) of the biomaterials without acknowledging the viscous components. In recent years, however, the viscoelastic properties, rather than purely elastic have come into focus in the development and engineering of biomaterials for cartilage repair and regeneration.[11,12,13,14] It has been shown that viscoelastic substrates influence cell spreading, migration, proliferation, differentiation, and ECM deposition. Fibroblasts and mesenchymal stem cells in 3D cultures exhibit greater spreading and proliferation in faster relaxing alginate hydrogels (≈1 min) versus slow (≈1 h).[11,12] Additionally, laprine chondrocytes in viscoelastic poly(L-lactide-co-ϵ-caprolactone) hydrogels with stress-relaxation times similar to native cartilage showed increased proliferation and the deposition of native cartilage-like ECM such as collagen type II and aggrecan was increased.[13] Furthermore, fibroblasts react to increased stress relaxation amplitude in alginate hydrogels.[14] Increased spreading, faster migration, and higher cell proliferation were reported with higher stress relaxation amplitude.[14] These findings indicate the importance of evaluating the viscoelastic properties of biomaterials rather than just stiffness.

As one of these biomaterials, allylated gelatin (gelAGE) is a promising hydrogel biomaterial due to its favorable biological properties and its ability to form homogenous networks by thiol-ene crosslinking.[15,16] In order to increase biological properties and bioactivity, and to mimic native tissues, different types of GAGs have been incorporated in gelatin-based hydrogels.[17–21] However, GAG chains have been functionalized through methacrylation or thiolation leading to multiple functional groups alongside their polysaccharide chains. This does not mimic the native presentation of GAGs as part of proteoglycans where they are covalently attached to the core protein at one end and thus are free of swelling. Furthermore, methacrylates are not natively present in the body and affect the bioactivity of the GAGs themselves.[17–21]

Therefore, we use a thiolation approach via reductive amination on the reducing end to induce only a single thiol group on chondroitin sulphate (CS), the most abundant type of GAG in articular cartilage. We then incorporate these CS chains in gelAGE hydrogels at different concentrations. This would allow for the fabrication of tunable tissue models that can mimic GAG-rich regions and can be used as platforms to study cellular response. The aims of this study were to covalently incorporate thiolated CS in gelAGE hydrogels at different concentrations to develop viscoelastic hydrogels that can better support cartilage regeneration by mimicking GAG-rich regions in cartilage. The hydrogels were characterized in terms of sol fraction, swelling ratio, and CS retention as well as mechanical and viscoelastic properties. Additionally, cytocompatibility was evaluated.

2. Methods

2.1. GelAGE Synthesis

Gelatin was functionalized to contain alkene groups (**Figure 1A**). Briefly, gelatin type A from porcine skin (Sigma, G1890) was dissolved in deionized water (10% w/v) at 65 °C. Once the gelatine was dissolved 2 mmol NaOH and 12 mmol allyl glycidyl ether (AGE, Sigma, A32608) per g gelatin was added. The solution was left to stir at 65 °C for 14 h and was dialyzed (MWCO 1 kDa) against deionized water. The pH was then adjusted to 7.4 and the solution was lyophilized and stored at -20 °C.

2.2. Degree of Functionalisation of gelAGE

Gelatin and gelAGE were dissolved at a 35 mg mL^{-1} concentration in MilliQ water at 50 °C. Different dilutions of the gelatin solution (0–35 mg mL^{-1}) were prepared in MilliQ water to obtain a standard curve. A 0.5 M citric acid solution was prepared, adjusted to pH 5.5, and then added to two parts of glycerol (Sigma, G5516) in a 1:2 ratio. Ninhydrin (2,2-dihydroxyindane-1,3-dione) (Sigma, N4576) was added to the citric acid/glycerol solution at a 2.5 mg mL^{-1} concentration. 50 µl of gelAGE or gelatine standard were added to 950 µl of ninhydrin solution. The solutions were placed in a boiling water bath protected from light for 13 min and then left to cool at room temperature for 1 h. Next, 250 µl of each sample were pipetted in triplicates into a 96-well plate and absorbance was read at 570 nm (Multiskan FC, Thermo Scientific). The degree of functionalization was calculated by finding the gradient of the gelatin solution regression line m and the average absorbance of sample A and using the following equation. For the gelAGE used for this study, it was calculated to be 84.1%.

$$\mathrm{DoF} = \left(1 - \frac{\mathrm{A}}{\mathrm{m}}\right) 100 \quad (1)$$

2.3. Thiolation of Chondroitin Sulphate

CS was thiolated to contain a single thiol group at the non-reducing end (Figure 1B). Chondroitin sulphate A from bovine trachea (Sigma, C9819, ≈20–30 kDa) was dissolved in MilliQ water. 100x equivalent cysteamine hydrochloride (Sigma, M6500) was dissolved in methanol (Chem-Supply, MA004) before it was added to the chondroitin sulphate solution. Acetic acid (Sigma, 695 092) was then added to the solution to make a 50:30:15 ratio solution, which was left to stir for 40 min at

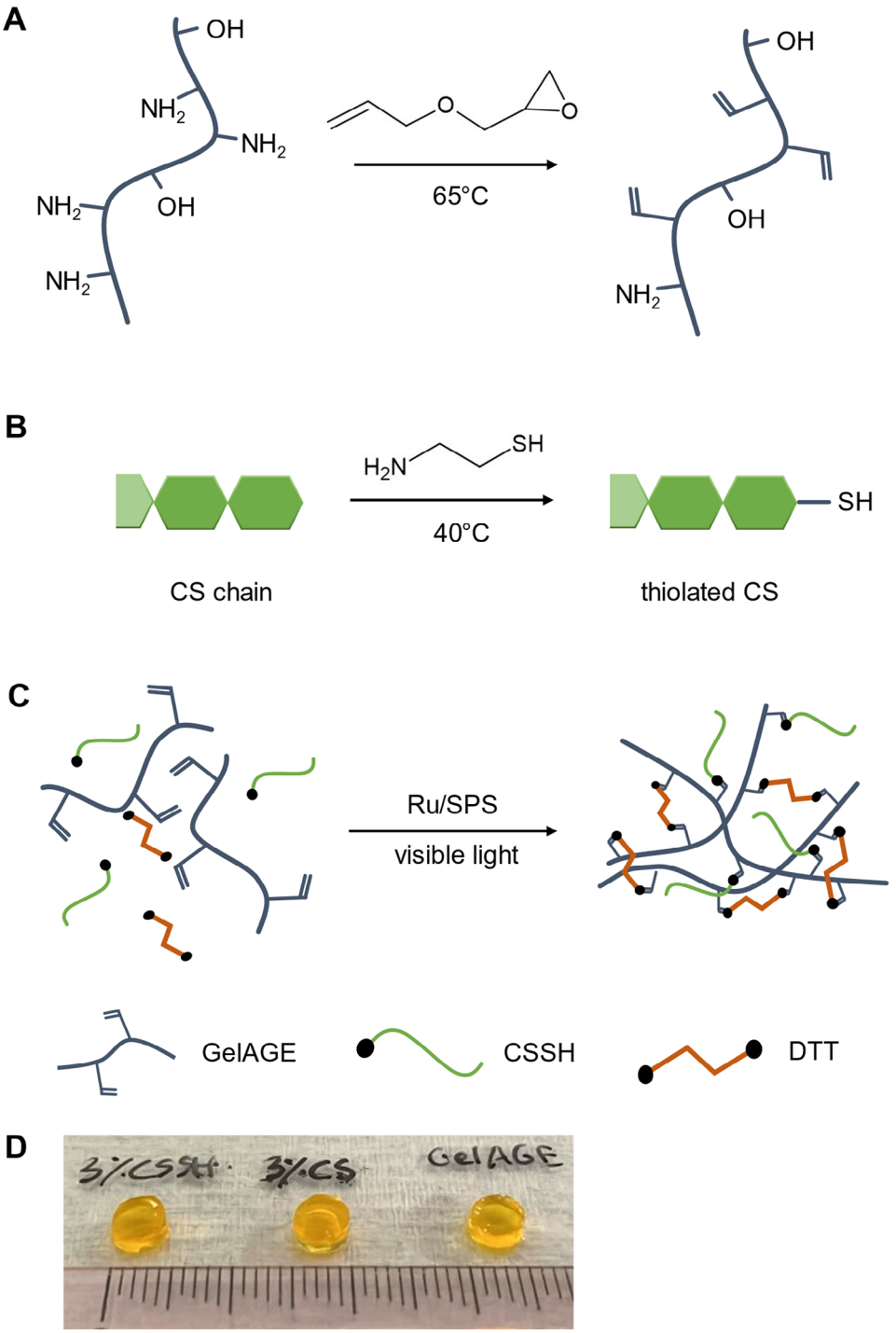

Figure 1. Schematic overview of gelAGE hydrogel formation containing thiolated chondroitin sulphate (CSSH). A) Functionalisation of gelatin with allyl glycidyl ether under alkaline conditions. B) Thiolation of chondroitin sulphate (CS) via reductive amination to contain a single thiol group, and C) formation of gelAGE hydrogel containing CSSH. Hydrogels are formed upon the addition of the visible light initiator Ru/SPS using dithiothreitol (DTT) as a crosslinker. D) Exemplar samples after cross-linking for 3% CSSH, 3% CS, and GelAGE only.

Table 1. Different hydrogel compositions were used in this study.

Hydrogel	GelAGE [% w/v]	Thiolated Chondroitin Sulphate [% w/v]	Chondroitin Sulphate [% w/v]
GelAGE	20	0	0
1% CSSH	20	1	0
1% CS	20	0	1
3% CSSH	20	3	0
3% CS	20	0	3

40 °C. 5x equivalent 2-picoline borane (Sigma, 654 213) was dissolved in methanol and subsequently added to the solution, resulting in a final ratio of 50:35:15. It was again left to stir for 40 min at 40 °C. The solution was then dialyzed (MWCO 14 kDa) against deionized water, lyophilized, and stored at -20 °C.

Thiolation of the CS was confirmed with an Ellman assay. 0.5 mg thiolated CS, 1.1 mg cysteamine hydrochloride, and 4 mg Ellman's reagent (Sigma, D8130) were each dissolved in 1 mL 0.1 M sodium phosphate solution (Sigma, S9638) containing 1 mM EDTA (Chem-Supply, EL022). Cysteamine hydrochloride was used to make standard solutions with known concentrations. 750 µl of each standard and unknown solution were incubated with 150 µl Ellman's reagent solution for 15 min at room temperature. 200 µl of each solution was pipetted to a 96-well plate in triplicates and absorbance was measured at 405 nm (Multiskan FC, Thermo Scientific). The sulfhydryl concentration of the thiolated CS was calculated based on the standard curve.

2.4. Hydrogel Preparation

GelAGE hydrogels were prepared containing CSSH (Figure 1C). Different amounts of CSSH and CS (chondroitin sulphate A from bovine trachea (Sigma, C9819)) were added based on the different groups (see **Table 1**). In a preincubation step, CSSH was dissolved in PBS at the desired end concentration. An amount of gelAGE was added and the solutions were stirred at 37 °C until fully dissolved. The amount of gelAGE was calculated so the number of allyl groups on the gelatin to thiol groups on the CSSH was at a stoichiometric ratio of 1:1.5 for thiol-ene click chemistry to occur.[22] A visible light photoinitiator tris(2,2′-bipyridyl)dichloro-ruthenium(II) hexahydrate (Sigma, 544 981) with sodium persulfate (Sigma, 216 232) (Ru/SPS) was added at 2/20 mM. The solutions were irradiated with visible light at 30 mW cm^{-2} for 5 min for the CSSH to covalently bind to the gelAGE. The same step was performed with CS as a control. GelAGE was added to the previously made solution to reach an end concentration of 20% w/v polymer concentration. Ru/SPS was added to the solutions at a final concentration of 2/20 mM. 200 mM dithiothreitol (DTT) (Astral Scientific, C-1029) was added to all groups, and the solution was injected into custom-made moulds (Ø 5 mm, 40 µl per sample). The samples were irradiated with visible light at 30 mW cm^{-2} for 5 min to crosslink. The hydrogels were then incubated in 1 mL PBS at 37 °C for up to 14 days.

2.5. Sol Fraction

Sol fraction was calculated to measure the crosslinking of the hydrogels. Twelve hydrogel samples (Ø 5 mm, 40 µl) of each group (see Table 1) were made. All samples were weighed ($m_{0,t0}$), and 6 samples of each group were lyophilized immediately after crosslinking and weighed ($m_{d,t0}$). Based on this the macromer fraction was calculated as

$$\text{Macromer fraction} = \frac{m_{d,t0}}{m_{0,t0}} \quad (2)$$

The other 6 samples of each group were incubated in 1 mL PBS for 24 h at 37 °C and then lyophilized and weighed ($m_{d,t1}$). Their initial dry weight was calculated as

$$m_{id} = m_{0,t0} * \text{macromere fraction} \quad (3)$$

Based on the initial dry weight and the swollen dry weight the sol fraction was calculated as

$$\text{Sol fraction} = \frac{m_{id} - m_{d,\,t1}}{m_{id}} * 100\% \quad (4)$$

2.6. Mass Swelling Ratio

Hydrogel samples (Ø5 mm, 40 µl) of each hydrogel composition were made and incubated in 1 mL PBS for up to 14 days at 37 °C. At each time point, 6 samples of each group were removed from the incubation liquid. They were weighed (m_w), frozen, and lyophilized before being weighed again (m_d). These weights were used to calculate the mass swelling ratio at each time point.

$$\text{Mass swelling ratio} = \frac{m_w}{m_d} \quad (5)$$

2.7. Macromer retention

The dried samples were digested in 1 mg mL^{-1} papain from papaya latex (Sigma, 221 465) dissolved in a 20 M sodium phosphate solution, containing 5 M EDTA and 2 M DTT (Sigma, D0632), at 60 °C for 6 rs. 10 µl of each sample and its respective incubation liquid as well as a standard solution, made from chondroitin sulphate from shark cartilage (Sigma, C4384), was pipetted to a 96-well plate in triplicates. After adding 100 µl 1,9-dimethylmethylene blue (DMMB) stock solution to each well, the absorbance was immediately read at 520 nm. The GAG content of the samples and their incubation liquid were calculated based on the standard curve. The macromer retention was then calculated as follows, with m_s as GAG content in the scaffold and m_l as GAG content in the incubation liquid.

$$\text{Macromer retention } (\%) = 100\left(\frac{m_s}{m_s + m_l}\right) \quad (6)$$

2.8. Mechanical Characterization

Six samples (Ø5 mm, 1.7 ± 0.4 mm thick) of each hydrogel group were tested after incubation in PBS at 37 °C for 3 days. The discs

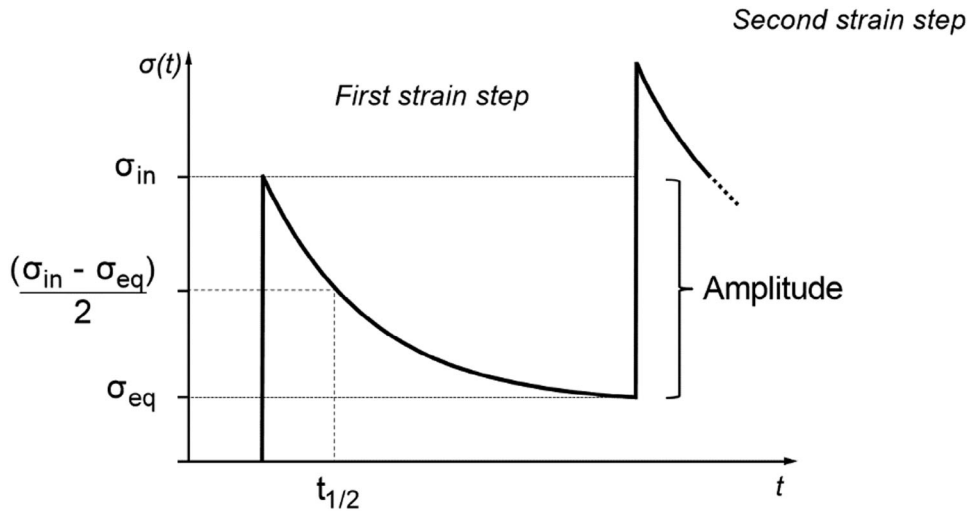

Figure 2. Schematic representation of a stress-time curve measured during stress-relaxation indentation. The relaxation amplitude refers to the difference between the initial stress (σ_{in}) and the stress at equilibrium (σ_{eq}). And the relaxation half-life time ($t_{1/2}$) is the time needed to reach half of the relaxation amplitude during the first strain step.

were placed in custom-made stainless-steel wells and immersed in phosphate buffered saline (PBS). A stress relaxation test was performed, as previously described by Nimeskern et al.[3] using a material testing machine (Zwick Z005, Ulm, Germany) with a stainless-steel indenter (Ø 0.9 mm). First, a preload of 3 mN was applied to the sample to determine its surface and measure sample thickness. After 5 min, 3 successive strain steps were applied in 5% increments of the original sample thickness. Each step was held for 20 min to allow the sample to relax. The collected data was then processed using previously developed in-house Python scripts. For every sample, an instantaneous modulus, equilibrium modulus, maximum stress, relaxation half-life time, and amplitude ratio were determined. The instantaneous modulus describes the initial stiffness of the sample upon loading, whereas the equilibrium modulus describes the stiffness of the sample after equilibrium has been reached and fluid has flown out of the sample. The relaxation amplitude refers to the difference between the initial stress (σ_{in}) and the stress at equilibrium (σ_{eq}) (**Figure 2**). Subsequently, the relaxation half-life time was determined as the time needed to reach half of the relaxation amplitude during the first strain step (Figure 2).

$$\text{Half life time (s)} = \text{time at } \frac{(\sigma_{in} - \sigma_{eq})}{2} \quad (7)$$

The amplitude ratio was calculated as the ratio between the relaxation amplitude and the maximum stress during the first strain step (σ_{in}) as shown in Figure 2.

$$\text{Amplitude ratio} = \frac{(\sigma_{in} - \sigma_{eq})}{\sigma_{in}} \quad (8)$$

One stress-relaxation curve from the first strain step of each group was normalized to visualize the shapes of the curves. The samples with values closest to the mean were chosen for visualization.

2.9. Cell Isolation and Incorporation in Hydrogel

Cells were isolated from the articular cartilage of bovine carpal joints, acquired fresh from a local butcher. No ethical permission was required since all animals were slaughtered for food purposes. The cartilage was minced and digested in High Glucose Dulbecco's Modified Eagle Medium (HG-DMEM) (Gibco, 11965-092) supplemented with 1% (v/v) Antibiotic-Antimycotic (A/A) solution (Gibco, 15240-062) and 10% (v/v) fetal bovine serum (FBS) (SAFC, 12003C) containing 1.5 mg mL^{-1} collagenase B (Sigma, 11 088 807 001) overnight at 37 °C. The digest was filtered through a cell strainer and the isolated cells were seeded at 7500 cells cm^{-2} and cultured in HG-DMEM media, (supplemented with 1% (v/v) A/A and 10% (v/v) FBS) at 37 °C and 5% CO_2. Once confluent, the cells were washed with PBS and detached with a trypsin-PBS solution. The harvested cells were resuspended in the respective hydrogel solution at a density of 12 × 10^6 cells mL^{-1}. The cell-laden hydrogels were then injected in molds (Ø 5 mm, 40 µl) and crosslinked under visible light for 5 min at 30 mW cm^{-2}. The hydrogels were cultured in HG-DMEM media, supplemented with 1% (v/v) A/A, 4% (v/v) FBS, 40 µg mL^{-1} L-proline (Gibco, 15 240 062), 1 mM sodium pyruvate (Gibco, 11 360 070), 10^{-4} mM Dexamethasone (Sigma, D4902), 1x ITS (Gibco, 41400-045), and 25 µg mL^{-1} ascorbic acid (Sigma, A8960), at 37 °C and 5% CO_2 for up to 2 weeks. At each time point, 8 samples per group were removed, 6 of which were prepared for analysis, and 2 were used for histology.

2.10. Cell Viability

A Live/Dead assay was performed after 1 and 14 days to measure cell viability, using a LIVE/DEAD Viability/Cytotoxicity Assay Kit (Thermo Fisher, L3224). A thin cross section of each sample was incubated with a previously prepared 2 µM Calcein AM and 4 µM EthD-1 solution at 37 °C and 5% CO_2 for 30 min. The stained samples were then imaged with a fluorescence microscope (Axio

Vert. A1, Zeiss, Germany). The images were further analyzed with ImageJ (Fiji). Live and dead cells were counted, and viability was calculated as

$$\text{Cell viability} \quad (\%) = \frac{n_{live}}{n_{live} + n_{dead}} * 100 \tag{9}$$

2.11. DMMB Assay for GAG Content

GAG content per dry weight was quantified with a DMMB assay in 6 samples per hydrogel composition after 1 and 14 days. Samples were frozen and lyophilized overnight. The dry weight was measured before digesting them in a papain digestion solution as previously described for 6 h. 10 µl of each sample as well as a standard solution, made from chondroitin sulphate from shark cartilage, was added to a 96-well plate in triplicates. 100 µl of a DMMB stock solution was added and subsequently, the absorbance was immediately read at 520 nm (Multiskan FC, Thermo Scientific). GAG content per dry weight was calculated based on the standard curve generated from the standard solution.

2.12. Histology

Two samples of each hydrogel group were prepared for histological analysis after 1 and 14 days. Hydrogel without cells were used as controls. The samples were fixed in neutral buffered formalin (AMBER Scientific, NBF-5L) for 1 h at room temperature and transferred to 70% ethanol. Sections were processed overnight using an automated tissue processor (Sakura Tissue-TekVIP6, Olympus, Australia). Subsequently, they were dehydrated in a series of ethanol solutions; 90% v/v for 1 h, twice in 100% v/v for 2 h each, and finally in 100% v/v for another hour before a clearing step (two exchanges in xylene, 2 h each). Infiltration of wax was done at 60 °C: three exchanges of 1 h each and one exchange of 30 min. The samples were embedded in paraffin and tissue blocks sectioned at 6 µm. For the Safranin-O staining, tissue sections were dewaxed twice in xylene, for 3 min each. Slides were then rehydrated in a series of ethanol solutions for 2 min each; twice in 100% v/v, once in 95% v/v, and once in 70% v/v, followed by two exchanges in water for 2 min each. Tissue sections were incubated in Wiegert's iron hematoxylin working solution (Sigma, HT1079) for 10 min and washed in running tap water for another 10 min. They were then stained with 0.1% Safranin-O solution for 5 min. Finally, sections were dehydrated again in a series of ethanol for 30 s each; 70% v/v, 95% v/v, twice in 100% v/v, before 2 exchanges in xylene, for 2 min each, and cover slipped.

2.13. Statistical Analysis

All results were tested for normality using the Shapiro–Wilk test. In the case of normal distribution, mechanical parameters as well as results from sol fraction and cell viability were assessed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. For results not following normal distribution (equilibrium modulus, maximum stress, and relaxation half-life time), the Kruskal–Wallis test was performed. The GAG content was compared using two-way ANOVA and Tukey's multiple comparisons test. All differences between groups were considered significant at $p < 0.05$. All results are displayed as mean ± standard deviation, where * indicates significance ($p < 0.05$). Statistical analysis was performed with GraphPad Prism 9.5.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. CSSH was Successfully Incorporated in gelAGE Hydrogels at Different Concentrations

Hydrogels were characterized in terms of CS retention, swelling ratio, and sol fraction (**Figure 3**). CSSH retention in the hydrogels remained high after 14 days of incubation for both 1% w/v CSSH and 3% w/v CSSH (Figure 3A). After 14 days 1% CSSH had 84% left of the initial amount, and 3% CSSH had 80% left. Both control groups release almost all CS after 4 days indicating no retention. The mass swelling ratio was the lowest, with a ratio of ≈5.8, for the hydrogel composed of only gelAGE (Figure 3B). For the hydrogels containing CSSH the mass swelling ratio was higher, at ≈8.1 and 8.7, respectively. Free CS was lower on average 5.95 and 5.6 respectively. The addition of CSSH and CS to the gelAGE hydrogel had a significant impact on the mass loss after 24 h (Figure 3C). The gelAGE hydrogels had very little mass loss, with an average of merely 9.3%. The more CS was added the greater the mass loss became, for both thiolated and non-thiolated CS. However, no significant difference was measured between the hydrogels with free and thiolated chondroitin sulphate. The 1% (w/v) CSSH hydrogels showed a mass loss of 16.9%, whilst the 3% (w/v) CSSH hydrogels showed a mass loss of ≈17.9%. The sol fraction of the CS groups was slightly higher, although not significantly different. The 1% CS group had a sol fraction of 19.1% and the 3% CS group had the highest sol fraction of 21.8%. These results show successful covalent incorporation of CSSH in gelAGE hydrogels at different concentrations.

3.2. CSSH-Enriched gelAGE Hydrogels Show Increased Viscoelasticity

To assess the influence of covalent incorporation of CSSH in gelAGE on mechanical properties, instantaneous modulus, equilibrium modulus, maximum stress, relaxation half-life time, and relaxation amplitude ratio were determined (**Figure 4**). The measured thickness of all samples was not significantly different, ruling out the influence of sample thickness.

The mean instantaneous modulus, which measures initial tissue stiffness upon loading was highest for the gelAGE only hydrogels with 103.6 ± 47.0 kPa (Figure 4A). However, there was no significant difference to any other group except between the control and 3% CS. This instantaneous modulus was lowest with a value of 28.0 ± 9.4 kPa. The group containing 1% CSSH was second highest 88.7 ± 27.6 kPa and was significantly different from the 3% CS control group as well. 3% CSSH was slightly lower with 73.3 ± 31.8 kPa. 1% CS was 77.9 ± 29.3 kPa. Both the samples containing 3% CS and CSSH had a lower mean instantaneous modulus than the samples containing 1% CS and CSSH.

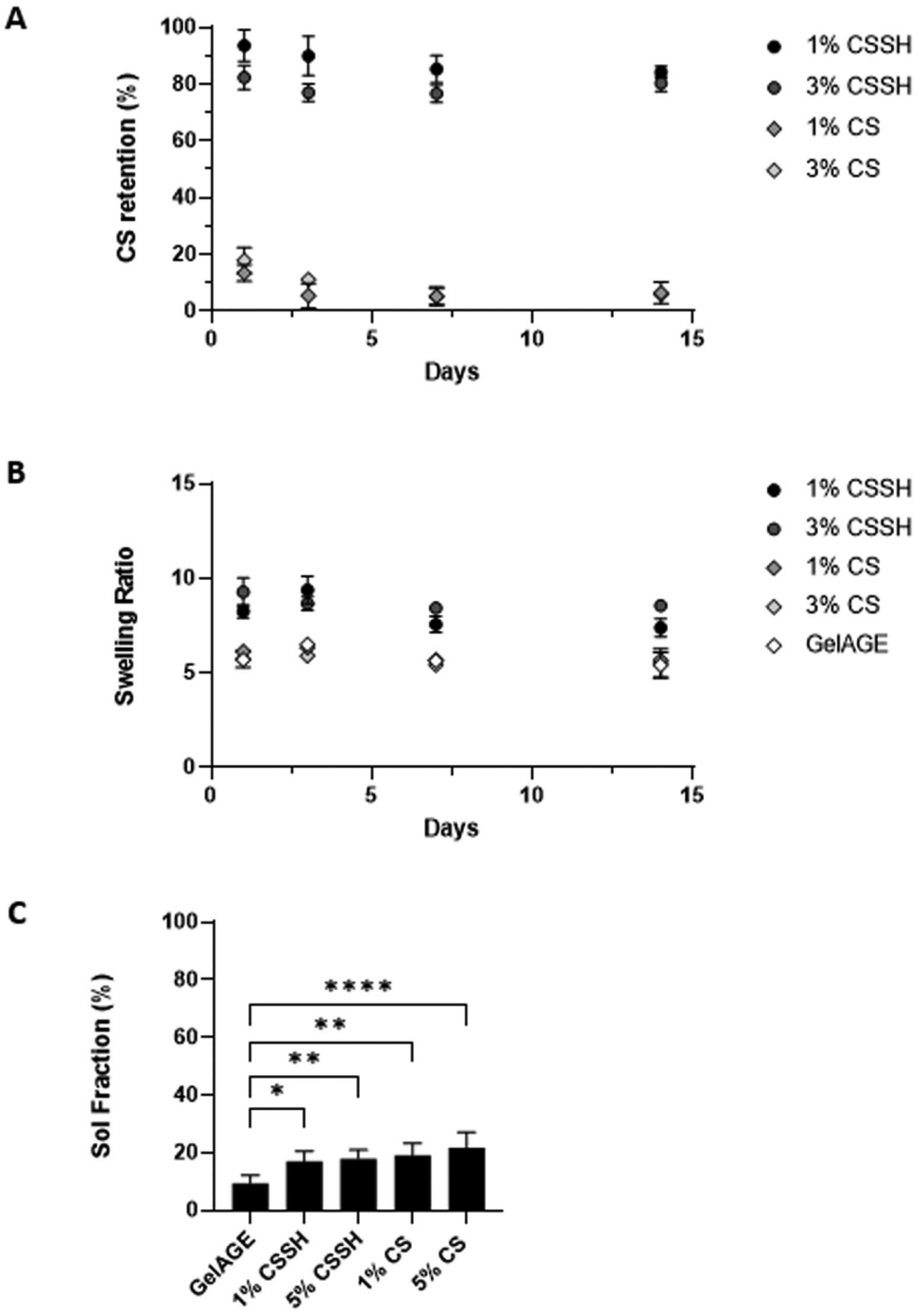

Figure 3. Characterization of CSSH hydrogels (n = 6/group). A) Retention of CSSH and CS after 14 days of incubation in PBS at 37°C. B) Swelling ratio at different time points up to 14 days of incubation in PBS at 37°C and C) Sol-fraction. Data are represented as mean ± SD. * Represents statistically significant difference ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$).

Similar to the instantaneous modulus, the equilibrium modulus measured in gelAGE control samples was the highest (53.9 ± 24.6 kPa) (Figure 4B). The samples containing 3% CSSH and 3% CS were both lower (23.4 ± 17.9 kPa and 22.0 ± 5.6 kPa). 1% CSSH 31.1 ± 12.2 kPa and 1% CS 39.3 ± 13.7 kPa. There was no significant difference between any of the groups containing CSSH or CS.

Matching the results for the instantaneous moduli, the maximum stress was highest in gelAGE hydrogels (15.4 ± 6.2 kPa) (Figure 4C), and similar to the results of the equilibrium

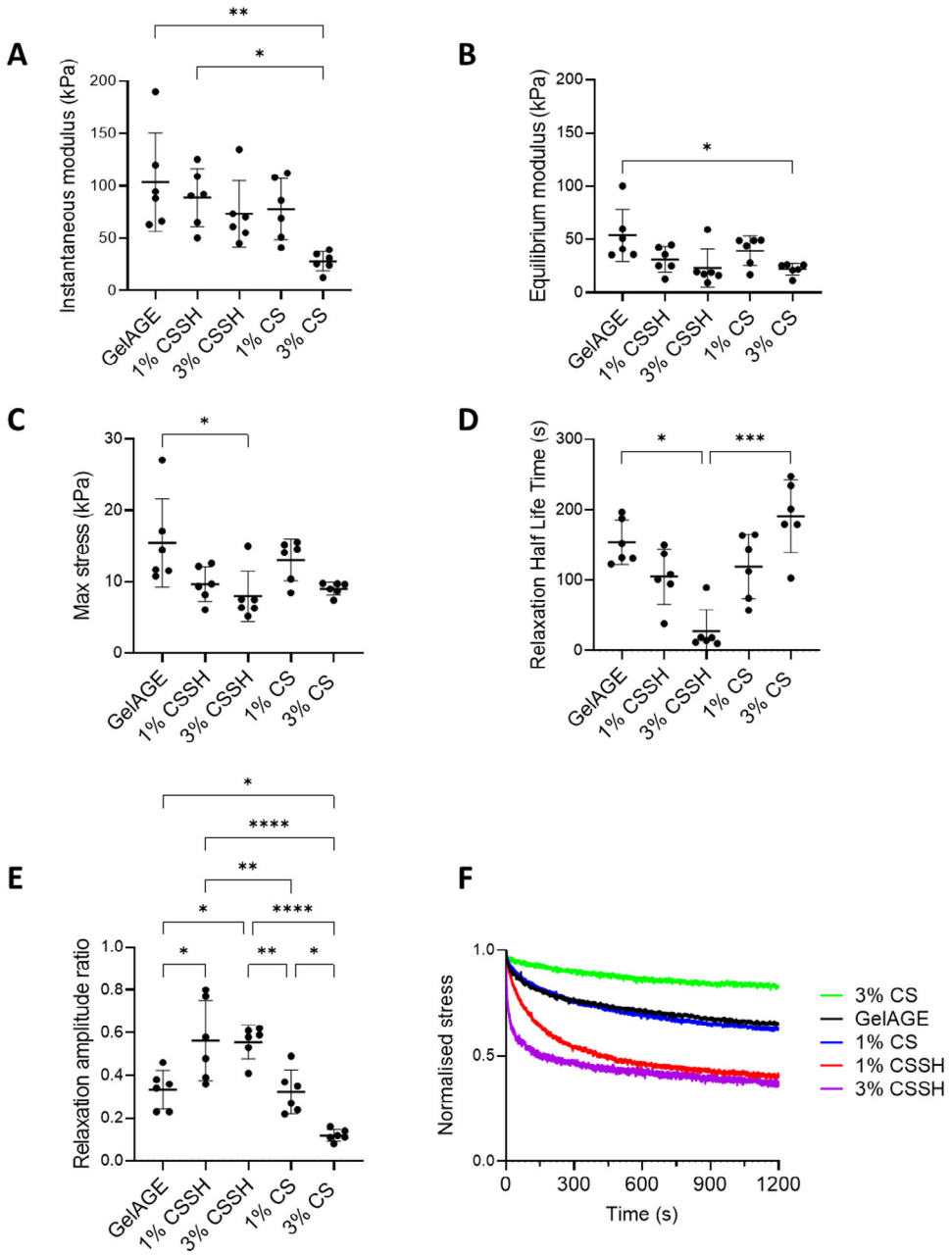

Figure 4. Mechanical testing of CSSH hydrogels using a stress-relaxation test (n = 6/group). A) Instantaneous modulus (kPa), B) equilibrium modulus (kPa), C) maximums stress (kPa), D) relaxation half-life time (s), E) relaxation amplitude ration, and F) normalized stress. (A-E) Data are represented as individual data points. * Represents statistically significant difference ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

modulus, there was no significant difference between the mean maximum stress between any of the groups containing CSSH or CS. The group with 1% CSSH was 9.7 ± 2.5 kPa, 1% CS was 13.0 ± 2.9 kPa, 3% CSSH was 8.0 ± 3.6 kPa, and 3% CS was 9.0 ± 0.9 kPa.

The relaxation half-life time is the time needed to reach half of the relaxation amplitude during the first strain step. This was highest in the control group containing 3% CS (190.7 ± 51.4 s) (Figure 4D). This result was significantly higher compared to the 3% CSSH group (27.3 ± 30.7 s), but not to any other group. There was no significant difference in half-life time between gelAGE (153.8 ± 31.3 s), 1% CSSH (105.1 ± 39.1 s), and 1% CS (119.4 ± 45.9 s).

The relaxation amplitude ratio was calculated as the ratio of the relaxation amplitude and the maximum stress at strain step one (Figure 4E). A higher ratio indicates a more viscous response and a lower ratio indicates a more elastic response. The groups containing 1% CSSH and 3% CSSH showed the highest amplitude ratio (0.56 ± 0.19 and 0.56 ± 0.08, respectively). There was no significant difference between these two groups. There was also

no significant difference between the gelAGE only group (0.33 ± 0.09) and the group containing 1% CS (0.32 ± 0.10). The group containing 3% CS was significantly different from all groups and had the lowest amplitude ratio at 0.12 ± 0.03 indicating the smallest relaxation amplitude of all the groups.

The different hydrogel groups show distinct relaxation curves at strain step one (Figure 4F). The stress in samples containing 1% and 3% CSSH decreased to ≈40% and 36% of their initial stress. Whereas samples containing CS and gelAGE only did not decrease to more than 63% of their initial stress. These results indicate an increased viscoelastic response in hydrogels upon the addition of CSSH.

3.3. CSSH-Enriched gelAGE Hydrogels Show High Cytocompatibility

Bovine chondrocytes were encapsulated in CSSH-enriched gelAGE to assess their cytocompatibility after 14 days (**Figure 5**). There was no significant difference in cell viability after 14 days between gelAGE and CSSH-gelAGE (Figure 5A). High cell viability of more than 85% in all groups indicates no cytotoxic effect upon the addition of CSSH. GAG content measured by DMMB assay showed significant differences between the groups (Figure 5B). GAG content at day 1 of culture was 24.9 ug mg^{-1} for 1% CSSH and 84.1 ug mg^{-1} for 3% accordingly, due to the initial CSSH in the hydrogels. After 14 days in culture, the GAG content in the gelAGE only group was 7.5 ug mg^{-1}. The GAG content increased to 34.0 ug mg^{-1} for 1% CSSH and to 91.5 ug/mg for 3% CSSH, indicating GAG production by the chondrocytes in addition to CSSH added to hydrogel. Safranin-O histology was assessed for cell laden hydrogels as well as cell free controls (Figure 5C). Both CSSH groups showed red staining on day 1 of culture as well as the unseeded control. After 14 days in culture, the hydrogels containing CSSH showed a slight increase in staining intensity. Furthermore, the group only containing gelAGE showed staining around the chondrocytes. Overall, all hydrogel groups showed a homogeneous tissue structure. These histology results are in agreement with DMMB results and confirm GAG production.

4. Discussion

The current study shows the successful incorporation of thiolated CS in gelAGE hydrogels and the production of heterogeneous samples. This allows for the fabrication of tunable tissue models that can mimic GAG-rich regions and can be used as platforms to study cellular response.

The hydrogels were characterized in terms of sol fraction, swelling ratio, and CSSH retention. There was an almost complete release of CS whereas ≈80% of CSSH was retained. With a hydrogel polymer concentration of 20% this leads to an amount of GAG per dry weight in the range of healthy human articular cartilage which is ≈50–150 ug/mg dry weight.[3,23] This allows for mimicking varying GAG concentrations and GAG-rich domains in cartilage.

For the samples containing gelAGE only the swelling ratio corresponds to previous studies using the same amount of macromer and crosslinker.[15] The swelling ratio was however increased in hydrogels containing CSSH. The addition of CSSH would introduce a negative charge to the hydrogels and increase the capacity of the hydrogels to retain water like in native cartilage tissue.[7,24] However, the increased swelling ratio may not solely be caused by the CSSH but also due to less crosslinking of these hydrogels. GelAGE is functionalized with allyl groups to form networks through thiol-ene crosslinking with DTT upon the addition of the visible light initiator Ru/SPS.[15,25,26] CSSH would covalently bind to allyl groups on gelAGE through a thiol-ene reaction as well. Therefore, thiol groups from both CSSH and DTT would be competing potentially leading to a lower crosslinking density compared to hydrogels without CSSH. CS is furthermore an antioxidant, which can quench the radicals needed for the crosslinking, thus impairing the crosslinking and leading to a higher swelling ratio.

Contrary to the swelling ratio, the sol fraction for hydrogels both containing CS and CSSH was significantly higher compared to gelAGE alone. For the gelAGE group, this is due to non-crosslinked polymer and correlates well with literature.[15] In the case of CS, this is likely due to the almost complete release of CS out of the hydrogels. However, given the high retention rate of CSSH, in these groups, it is likely due to a lower crosslinking efficiency due to introduced thiol groups on CSSH attaching to allyl groups on the gelAGE.

These results show successful incorporation of 1% and 3% w/v CSSH. Further studies should be conducted using additional GAG concentrations. Due to the limited availability of allyl groups, it is suspected that there would be an upper limit of CSSH percentage that can be incorporated while still forming a hydrogel. Different polymer percentages and varying the degrees of functionalization (DoF) of the gelatin could be explored.

Viscoelasticity has become an important consideration in the engineering of biomaterials. There is increasing evidence that cells respond favorably to the viscoelastic properties of hydrogels. They have been shown to display increased migration, proliferation, and matrix deposition in hydrogels with increased stress-relaxation amplitude and lower stress-relaxation time, making these parameters interesting aspects to consider when engineering biomaterials.[11–13,27,28] Hydrogel viscoelasticity can be tuned through different crosslinking densities, changing the length of polymer chains, and varying polymer concentrations.[29]

In the current study, the hydrogels containing CSSH show increased viscoelastic properties compared to the controls. The relaxation amplitude almost doubled upon the addition of CSSH, whereas there was no significant difference in moduli or the maximum stress. Similar to cartilage, hydrogels exhibit a time-dependent mechanical response due to the movement of fluid inside the hydrogels which causes energy dissipation.[30,31] The GAGs in cartilage play an important role and are responsible for the high water content. The collagen fibers are under tension and as fluid flows out of the tissue this leads to time dependent response under an external load.[3] The incorporation of CSSH in gelAGE hydrogels did increase water content in hydrogels as shown in a higher swelling ratio likely due to increased negative charges and a lower crosslinking density. This higher water content would likely lead to a more viscous response. In healthy cartilage, the relaxation curves in stress relaxation tests vary and have been shown to decrease to ≈60–10% of the initial stress.[32–34] The

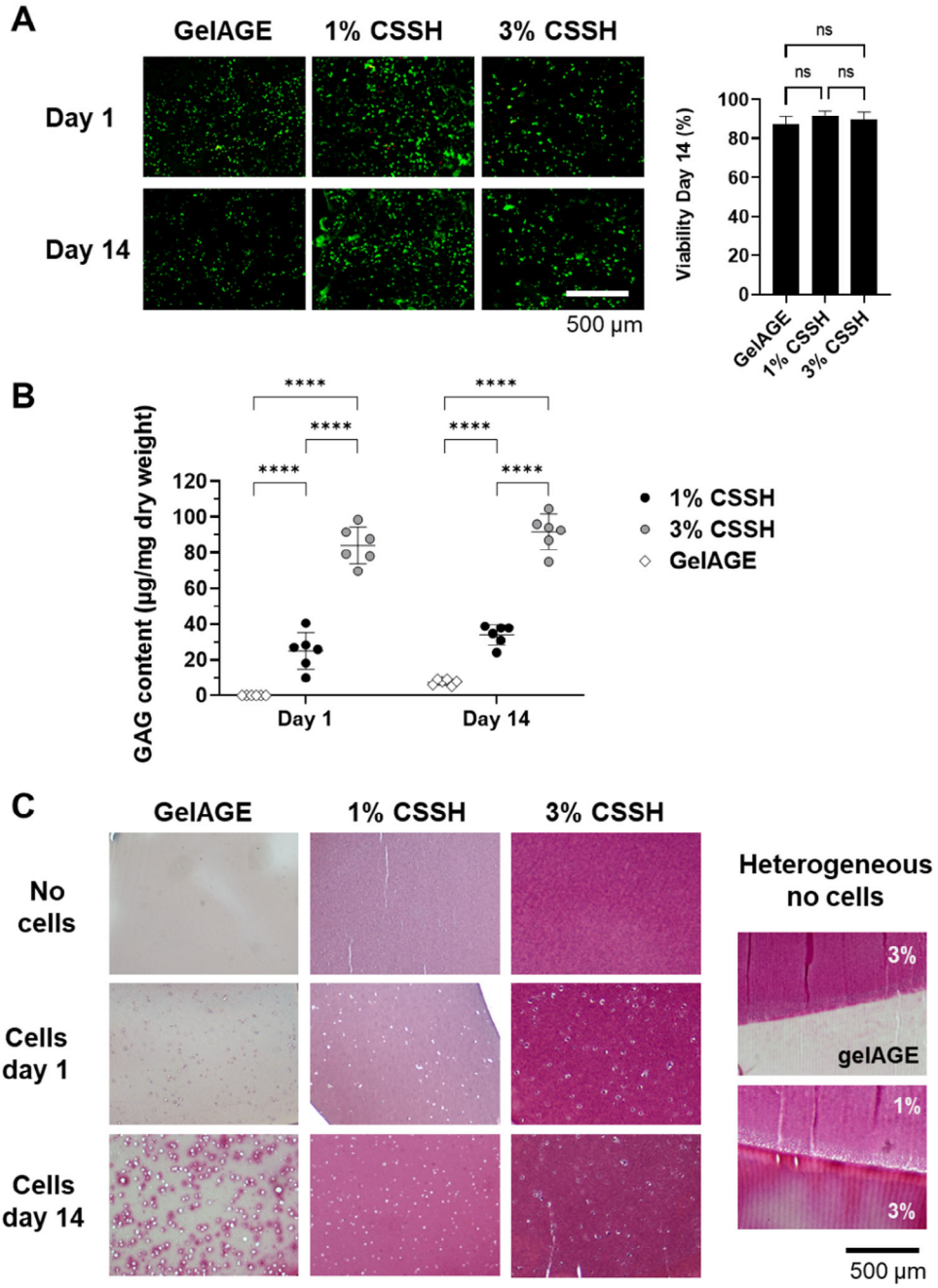

Figure 5. Assessment of cytocompatibility of CSSH hydrogels. A) Live/dead images of bovine chondrocytes encapsulated in gelAGE and CSSH-gelAGE. Cell viability of gelAGE hydrogels and CSSH-gelAGE after 14 days in culture. (Cross sections from n = 6/group). B) Glycosaminoglycan (GAG) content was measured by the 1,9-dimethylmethylene blue assay in six samples per hydrogel composition after 1 and 14 days in culture. C) Safranin-O staining of hydrogels with different CSSH concentrations. Heterogeneous samples show the successful preparation of samples containing two different GAG concentrations. Scale bars = 500 µm. * Represents statistically significant difference (**** $p < 0.0001$).

addition of 1% and 3% w/v% CSSH in this study has led to a decrease of more than 50%. Different hydrogel and CSSH compositions could be explored to achieve variable viscoelastic properties.

The hydrogels in this study were significantly softer than native articular cartilage which has an initial stiffness of ≈10–30 MPa, depending on composition.[8] The goal of this study was to replicate the viscoelastic behavior, rather than match tissue stiffness. However, further experiments with varying hydrogel properties could potentially explore the influence of stiffness in addition to variable viscoelasticity and study its effect on cells.

Another aspect that influences the mechanical properties of gelAGE hydrogels is the functionalization of gelatin with allyl groups, which occurs in alkaline conditions. Studies have shown that the higher the DoF, the shorter the gelatin chains and the

lower their molecular weight due to degradation of gelatin under these conditions. The higher crosslinking density due to higher DoF does likely compensated for the shorter chains and there were no changes in stiffness.[15] In this study, the DoF of the gelAGE used was 84.1% which is within the upper range of gelAGE used.[15,16,35] A decrease in DoF would potentially alter the amount of CSSH retained as there are fewer allyl groups available. Further studies could explore the effects of varying DoFs on CSSH incorporation.

Furthermore, the polymer percentage of gelAGE and amount of DTT used in this study were 20% w/v and 200 mM, respectively. Previous studies have shown that different polymer concentrations and DTT concentrations have an influence on the swelling, sol fraction, and stiffness of hydrogels.[15,16] Additionally, crosslinker concentration as well as the presence and timing of light exposure has been shown to be factors influencing the stiffness of gelAGE hydrogels.[16] Therefore, further studies and a more complete characterization with different polymer, crosslinker, and CSSH concentrations, as well as crosslinking and culture conditions could be conducted to explore the influence of different combinations of these parameters on mechanical properties. Furthermore, it is important to look at the relaxation amplitude in addition to other mechanical parameter to cover all aspects. This study shows that despite similar moduli and maximum stresses, the relaxation amplitudes and relaxation curves differed significantly. Given that this has an influence on the cellular response, reporting elastic modulus alone is likely not sufficient. It is lacking critical information on the characterization of biomaterials.

GAG enriched hydrogels have previously been shown to have positive effects on cells and to improve chondrogenesis.[17,18,20] As expected in this study, the addition of CSSH to gelAGE did not decrease the cytocompatibility, making it a suitable biomaterial for future studies.

The incorporation of varying concentrations of CSSH enables the introduction of spatial patterning of GAG-rich regions within hydrogels. This presents an opportunity to better replicate the zonal organization of native cartilage, where GAG concentration and mechanical properties vary across the different zones. Additionally, it would allow for studying the influence of microscale GAG heterogeneity, which is thought to play a critical role in cartilage function.[1,3,6,23,34]

Beyond structural mimicry, the interplay between the solid collagen network and fluid-phase is crucial for cartilage biomechanics.[3,5,6,8,30,31] Patterned hydrogels with CSSH-rich regions could modulate fluid flow, stress relaxation, and poroelastic behavior. These are key factors in mechanotransduction and chondrocyte phenotype maintenance.[7,9,12,13,27,28,32,34] Additionally, spatial control of CSSH incorporation may guide zonal-specific cell behavior and ECM remodeling.[17,18,20,25,26,45] Future studies should explore different fabrication methods to develop hydrogels that more accurately replicate cartilage structure and function.

Further experiments should also be conducted to investigate the influence of CSSH on long-term cell cultures and its effect on tissue formation and remodeling. Furthermore, this hydrogel could be used as a suitable model for mechanobiology studies on viscoelasticity and varying GAG concentrations and give vital insights into future tissue engineering approaches.

5. Conclusion

In conclusion, this study demonstrates the successful incorporation of thiolated CS in gelAGE hydrogels at different concentrations with no adverse effects on their cytocompatibility. This allows for tuning of the viscoelastic properties of the hydrogels. These parameters are important to consider when engineering new biomaterials in addition to stiffness because this has been shown to positively influence cell behavior and ECM production. Further studies should be conducted using different hydrogel parameters to study the cellular response to different viscoelastic properties and GAG contents.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

The conceptualization of the study was carried out by M A B. and K S S. The formal analysis was conducted by M A B, and the investigation and methodology were also developed by M A B, K S L, S R L, and K S S. Resources were provided by K S S, and the supervision of the project was managed by K S S and S R L. The original draft of the writing was created by M A B, while all authors contributed to the review and editing of the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

biomaterials, cartilage, glycosaminoglycans, mechanics, stiffness, tissue engineering, viscoelastic

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