Hydrogels Based on Oxidized Cellulose Sulfates and Carboxymethyl Chitosan: Studies on Intrinsic Gel Properties, Stability, and Biocompatibility

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Cellulose and chitosan are excellent components for the fabrication of bioactive scaffolds, as they are biocompatible and abundantly available. Their derivatives O-carboxymethyl chitosan (CMChi) and oxidized cellulose sulfate (oxCS) can form in situ gelling, bioactive hydrogels, due to the formation of imine bonds for crosslinking. Here the influence of the degrees of sulfation (DS), oxidation (DO), and the molecular weight of oxCS on intrinsic and rheological properties of such hydrogels and their ability to support the survival and growth of human-adipose-derived stem cells (hADSC) is investigated. It is found that the pH of the hydrogels is generally slightly acidic, while their network density and E-modulus are found to be dependent on the DS and DO, which makes the properties of hydrogels tunable. Extensive studies show that hydrogels can be stable for up to 14 days and that their stability is largely dependent on the DO, molecular weight, and the components mixing ratio. Cytotoxicity studies of the hydrogel with hADSCs show biocompatible gels in dependence on the molecular weight and degree of oxidation with viable cells up to 14 days. These findings can help to develop specifically tailored hydrogels for tissue engineering applications to replace different types of connective tissue.

1. Introduction

Biomimetic materials in comparison to conventional synthetic biomaterials can improve the performance of scaffolds or hydrogels in tissue engineering. Biomaterials used in medical applications as implants, scaffolds, and hydrogels should provide specific chemical cues for cell attachment through integrin or other cell surface receptors and appropriate physical stimuli such as mechanical stress and E-modulus that provides the cells with desired chemical and mechanical stimuli to colonize the biomaterial and to regenerate tissues. Synthetic materials like polycaprolactone or polylactic acid are already used widely in tissue engineering due to their favorable biocompatible and mechanical properties. However, because of their chemical composition, they lack any specific biological activity and need often to be further functionalized. Therefore, in recent years, the research has focused on the development of bioactive materials based on biopolymers like collagen, elastin, or polysaccharides. Biopolymers that represent components of mammalian extracellular matrix possess an inherent bioactivity that can promote a specific cellular reaction. Others like those derived from other animal species like chitosan or plants like alginate and cellulose possess the benefit of being renewable, biocompatible, biodegradable, and are available in high abundancy. However, many of them require additional chemical modifications to achieve a certain bioactivity. Among them is chitosan, a promising biomaterial, as it is biodegradable and has shown antimicrobial properties. In
addition, the positive charge of the polymer promotes the attachment and differentiation of cells\[11\] whereas one amine group per glucosamine unit offers the possibility of further modification by chemical conversion or grafting of other groups. Chitosan and its water-soluble derivative, carboxymethyl chitosan (CMChi), are widely used in the field of drug delivery and tissue engineering.\[12,13\] Another promising biopolymer candidate is cellulose, as it is one of the most abundant nature-based polymers and widely used in biomedical materials, as it is biocompatible, cheap, and renewable.\[14,15\] A drawback is that cellulose polymers with polymerization degrees of higher than 6 show a significant decrease in their solubility in water and most organic solvents, and lack any bioactivity that supports cell adhesion and growth.\[16\] However, by functionalizing cellulose with sulfate groups, it becomes water soluble and shows a bioactivity, similar to glycosaminoglycans such as chondroitin sulfate or heparin.\[17\] Cellulose sulfates have been demonstrated to promote the chondrogenesis of mesenchymal stem cells\[18\] and show an ability to bind growth factors promoting the proliferation and differentiation of cells as sulfated polysaccharides possess an affinity to bind growth factors.\[19-23\] They also show antibacterial, antiviral, and anticoagulant properties.\[24-26\] Recently, studies have shown that aldehyde-functionalized cellulose sulfates can react with amine groups in a click reaction to form reversible imine bonds.\[27\] This mechanism can be used to couple these bioactive molecules to surfaces or to form in situ gelling hydrogels with amine-containing polymers, such as chitosan or CMChi.

Hydrogels find many applications in the field of engineering soft tissues as they can simulate a physiological environment regarding water content, transport of oxygen and nutrients, but also the mechanical properties of these tissues.\[29,30\] An additional benefit of in situ gelling hydrogels is that they are able to fill out irregular-shaped defects, when damage to the tissue is difficult to match by use of preshaped implants or scaffolds. In addition, surgical procedures can be done in a minimal invasive manner.\[31\] The use of preactivated biopolymers that crosslink directly with each other means that there is no need to use small, potentially toxic crosslinkers like glutaraldehyde, or high amounts of genipin.\[12,31\] Recent publications have already shown the usage of hydrogels, which are formed by aldehyde-functionalized polysaccharides reacting with polyamines like chitosan.\[34,35\]

In addition to the exclusion of toxic effects, a profound understanding of the mechanical and structural behaviors of such hydrogel systems is critical, as their intrinsic properties need to be specifically tuned depending on the desired medical or pharmaceutical application. For example, there are different mechanical demands for the stimulation of growth of cartilage tissue as there are for bone tissue.\[36\] Mechanical properties of hydrogels such as the formation and degradation of the network can be monitored by oscillation rheology. Storage modulus, loss modulus, and loss factor are the critical hydrogel properties to be monitored over time.\[37\] Compression studies provide the Young’s modulus and reflect the elasticity of the crosslinked hydrogel. On the other hand, NMR relaxation studies can be used to show the mobility of water in dependence of gel properties. It should also be emphasized that NMR studies represent a noninvasive method of studying gel properties, which is also quite interesting when embedding living cells.\[38\]

![Figure 1. Relationship between degree of sulfation and the corresponding degree of oxidation of oxidized cellulose sulfates. Sulfite pulp and cotton linters denote the different sources of cellulose used for synthesis of derivatives. Details about the measurement of these quantities can be found in our previous publication.\[39\]](image-url)

In this report, we present an investigation on the properties of hydrogels based on oxidized cellulose sulfates (oxCS) and CMChi, and their potential to be applied as biodegradable scaffolds for the engineering of soft tissue. First studies with such systems have been performed to characterize the cellulose derivatives along with some initial investigations into the physical properties and stability of hydrogels fabricated with them. In particular, we will compare findings of noninvasive studies of intrinsic gel properties by NMR measurements with macroscopic behavior found by rheometry, swelling, and stability studies. In addition, the biocompatibility of these hydrogels toward human-adipose-derived stem cells (hADSCs) under different physiological conditions is investigated to determine the possibility of fabricating hydrogels for tissue engineering applications.

2. Results and Discussion

2.1. Survey on Oxidized Cellulose Sulfates Used in This Study

The synthesis of the oxidized cellulose sulfates has already been discussed in more detail in a previous paper from us.\[39\] Degree of sulfation (DS) values in the range from 0.8 to 2.0 were obtained by sulfation of cellulose and degree of oxidation (DO) values from 0.03 to 0.34 by subsequent oxidation with sodium periodate. The dependence of the degree of oxidation on the degree of sulfation is shown in Figure 1. It can be seen that the higher the degree of sulfation of cellulose is, the lower the degree of oxidation is. Since for the oxidation of the glucose monomers of cellulose vicinal hydroxyl groups are necessary, the reaction can only take place when there is no sulfate group at C2- and C3-positions of the glucose monomeric unit of cellulose. At lower degrees of sulfation, it takes primarily place in the C6-position. However, at higher degrees, hydroxyl groups in the C-2 position are also sulfated. Therefore, cellulose sulfates with higher degrees of sulfation than 2.0 were not synthesized, as this would mean that no significant degree of oxidation could be achieved. A lower limit of 0.8 was chosen as the DS has to be high enough to show any bioactivity.
as observed in previous studies.\textsuperscript{[19]} It is also visible that the source of the cellulose is important, when aiming for a higher degree of oxidation. At higher degrees of sulfation, the cellulose sulfates produced from a sulfite pulp show higher degrees of oxidation in comparison with the samples, made from cotton linters. Cotton linters have a higher $M_w$ than sulfite pulp, which suggests that a lower chain length might possess less of steric hindrance for the oxidizing agent. This is important to note that the gelation time of a hydrogel composed of oxCS and CMChi, and its stability shall be dependent on the amount of aldehyde groups required for the crosslinking reaction.

2.2. Studies on Intrinsic pH Value inside Oxidized Cellulose Sulfate Solutions and Hydrogels

The hydrogels are formed by the click reaction between the aldehyde groups of the oxidized cellulose sulfates and the amine groups of the carboxymethyl chitosan to form imine bonds (Scheme 1). The knowledge of the intrinsic properties of hydrogels is important, as they shall be used for embedding of cells in tissue engineering applications. Therefore, the environment has to be as biocompatible as possible. A pH, which is either too acidic or too basic, will be detrimental to the survival of encapsulated cells. In addition, the bonds formed between the CMChi and oxCS during gelation are imine bonds, which are sensitive to changes of the pH value.\textsuperscript{[40]} A low pH makes the bond unstable and leads to a shift of the equilibrium back to amine and aldehyde groups, and therefore dissolution of the hydrogel. The oxCS samples were dissolved in phosphate-buffered saline (PBS) first, then mixed with the pH-sensitive dye SNARF-4F5-(and-6)-carboxylic acid, and afterward the pH was measured using fluorescence microscopy. Figure 2A shows how the pH values of oxCS solutions are related to sulfation and oxidation degree of derivatives.

At high degrees of sulfation, an increasing amount of aldehyde groups decreases the pH in the sample, because the hydrogen atom in $\alpha$-position to the carbonyl group is strongly acidic. For lower degree of sulfation, the oxidation degree did not affect the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{A) pH value of oxidized cellulose sulfate (oxCS) solutions in dependence on the degree of sulfation and degree of oxidation, dissolved in phosphate-buffered saline. The concentration of the oxCS was 20 mg mL$^{-1}$. B) pH value of hydrogels based on oxCS and carboxymethyl chitosan (CMChi). The concentration of the solutions was 20 mg mL$^{-1}$ for oxCS and 20 mg mL$^{-1}$ for CMChi with a mixing ratio oxCS to CMChi of 1:1.}
\end{figure}

\begin{scheme}
Hydrogel gelation mechanism. Aldehyde and amine groups form the imine bond and crosslink the respective polymer chains.
\end{scheme}
The mobility of water inside the hydrogels was explored with NMR relaxometry by determining the transverse relaxation time $T_2$ in dependence on oxidation and sulfation degree. Smaller $T_2$ means hindered motions of protons.\textsuperscript{[43]} It was measured for the hydrogels and the components CMChi and pure PBS. The $T_2$ values of PBS at $37\,^\circ C$ was $3.2 \pm 0.1\, s$, and of pure CMChi solution was $1.62 \pm 0.06\, s$ due to its higher viscosity. Hence, $T_2$ values are useful to quantify the immobilization of the water inside the hydrogels due to the formation of crosslinks and subsequent gela- tion. The water content of the hydrogels was kept equal for all samples for better comparison. The results of the $T_2$ measurements are shown in Figure 3.

It was found that all $T_2$ values are smaller than in pure CMChi and related to the formation of the gel. Additionally, no free water due to syneresis was found. Particularly the DS was related to keep transverse relaxation time $T_1$ high, which indicates a lower degree of crosslinking due to the lower DO. Samples with a high DS have less free vicinal hydroxyl groups, so that only a low DO can be reached. Hence, the formation of gels appears to have a major effect on the water mobility in these hydrogels, which is visible by smaller $T_2$.

To compare the result of NMR studies on relaxation time $T_2$ with mechanical properties of hydrogels, rheology studies were performed. The gel formation is described and discussed in the Supporting Information. In Figure 4, the E-modulus of the hydrogels in dependence of the DS and DO is shown.

It was found that the E-modulus is related to DO, which represents the amount of aldehyde groups of oxCS, while the E-modulus is inversely related to DS. This corresponds well to the $T_2$ values obtained by NMR studies and shows also the value of the NMR for measurement of intrinsic gel properties in a non-destructive manner without addition of any label as also done in one of our previous studies.\textsuperscript{[38]} It goes along the expectation that higher DO will also lead to a higher E-modulus as one can expect a higher crosslinkingdegree in such systems. The decreasing E-modulus with the increase of DS might also be related to the fact that an increased sulfation degree can cause steric hindrance for the oxCS and the CMC to properly crosslink.\textsuperscript{[42]} However, this becomes only apparent at low degrees of oxidation. In general, results show that the E-modulus of these hydrogels can be tuned by the DO of oxCS. The results were obtained 1 h after gelation, whereas previous studies have shown a much higher E-modulus, if it is measured after 24 h.\textsuperscript{[42]} This suggests that the hydrogels need to incubate for at least 24 h before they are completely crosslinked.

### 2.4. Stability of Hydrogels in Different Environments

Stability studies of the different hydrogel samples were performed in PBS, Dulbecco’s modified Eagle’s medium (DMEM), and Dulbecco’s PBS (DPBS) over a period of 14 days, which was also required for the growth of human-adipose-derived stem cells, used then in cell viability studies. Table 1 shows the survey on the selected oxCS with information about their DO and DS, as well as information about route of synthesis and molecular weight of derivatives. The different synthesis routes were chosen, as they lead to derivatives with different molecular weights.
As illustrated in Table 1, the direct sulfation leads to generally higher molecular weights than aceto- or homogeneous sulfation. The different solvents were selected with regard to the need of cells for isotonic solutions and glucose for a short period of time, medium like DMEM for longer cultures and PBS only as a kind of control.

The stability was related to the wet weight of the hydrogel after certain incubation time in relation to the starting wet weight. The results for the three hydrogels are shown in Figure 5A–C.

After 1 day, the hydrogels swell to more than 100% of their starting weight. Due to osmotic pressure, the gel takes up additional water until the crosslinking density limits its ability to stretch further.[43] After 2 days, the uptake of the water reaches its maximum. It is visible that the highest degree of swelling is found in the case of oxCS1.1/0.24, while the swelling in the other two samples is much lower. As the samples have similar degrees of sulfation and oxidation, their ability to crosslink should be quite similar. Indeed, the relaxation times $T_2$ of the hydrogels was similar for all three DS and DO combinations (around 1.06 s), which indicates a similar density of the polymeric network. In fact, the crosslinking density of gels made of oxCS1.1/0.24 should be higher than of sample oxCS0.8/0.21 due to the higher degree of oxidation, which should lead to a stiffer gel, as shown in Figure 4. Since sample oxCS1.1/0.24 swells more and sample oxCS1.1/0.28 shows a much lower degree of swelling, the decisive factor must be the molecular weight. A higher polymer chain length leads to a stronger entanglement between the different polymer chains, in addition to the covalent imine bonds. Both mechanisms contribute to the stiffness of hydrogels.[44] In almost all samples, the swelling is stronger, if the gel is incubated in DMEM, as it contains amino acids, which compete with CMChi for the aldehyde groups of oxCS. The E-modulus measurements after 1 and 24 h suggest that the hydrogel formation needs more than 1 h of incubation time. Therefore, the amino acids can disrupt the crosslinking process during this time by forming imine bonds with the aldehyde groups. This is especially visible after 7 days like in the sample oxCS1.1/0.24, as it has the lowest overall degree of crosslinking. Therefore, the third experiment was performed, in which the gels were incubated in DPBS for 24 h first, to let them completely crosslinking before switching the surrounding solvent to DMEM.

The change of medium is mandatory, as the hydrogels shall contain cells, which cannot survive longer time without nutrients.

As the selected hydrogel systems had sufficient stability in DMEM and DPBS, which is required when cells are cultured inside the gels, their biocompatibility was investigated by encapsulating hADSCs. DMEM was selected as hADSCs show the best viability in this medium, while hydrogels are stable for a longer time in DPBS. As the gels might be applicable for replacement of cartilage, the incubation medium was changed after 24 h from DMEM to chondrogenic medium (modified Eagle’s medium alpha (αMEM) + chondrogenic differentiation factors). The viability of the cells was studied 24 h and 7 days after the encapsulation. The evaluation of biocompatibility was performed by live/dead staining using fluorescein diacetate (FDA) as vital dye to detect
Figure 5. Swelling and stability of hydrogels in different media. Hydrogel components: oxCS and CMCh. The ratios of oxCS:CMCh were chosen as 1:1 and 1:2. After mixing the precursors, gels were left resting for 1 h before being immersed in phosphate-buffered saline (PBS, black), Dulbecco modified Eagles medium (DMEM, gray), or Dulbecco modified phosphate-buffered saline (DPBS, white). The white samples were incubated in DPBS for 24 h before switching to DMEM. Oxidized cellulose sulfate component: A) oxCS0.8/0.21 (molecular weight: 71.0 kDa). B) oxCS1.1/0.24 (molecular weight: 49.3 kDa). C) oxCS1.1/0.28 (molecular weight: 116.4 kDa).
|               | DMEM low glucose               | DPBS + 1 g/L glucose               |
|---------------|--------------------------------|-----------------------------------|
|               | gelatin | oxCS$_{0.8/0.21}$ | oxCS$_{1.1/0.24}$ | oxCS$_{1.1/0.28}$ | gelatin | oxCS$_{0.8/0.21}$ | oxCS$_{1.1/0.24}$ | oxCS$_{1.1/0.28}$ |
| **Day 1**     | ![Image](image1.png)         | ![Image](image2.png)           | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
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**Figure 6.** Confocal laser scanning microscopy images of encapsulated human-adipose-derived stem cells in hydrogels based on oxidized cellulose sulfates and carboxymethyl chitosan in different media. Cells, which are marked green are living cells, stained with fluorescein diacetate. Blue cells are dead cells, which are stained with the dye TO-PRO-3. Gelatin crosslinked with transglutaminase was used as a positive control. Cell density during seeding was 2000 000 cells mL$^{-1}$. 
of crosslinked hydrogels, a higher degree of aldehyde groups can be beneficial for the cell viability, as the gels become more stable (see Figure 5) and present a better environment for the cells to adhere and grow. Another deciding factor might be the molecular weight, as a decrease in the chain length directly correlates with a decrease of metabolic activity. This could be either because the hydrogel itself is more stable the higher the molecular weight, as seen in the degradation experiments or it could be due to the fact that smaller chains can be more easily taken up by the cells, so that the aldehyde groups are located inside the cells, where they can have a detrimental effect on the viability.[47]

In all cases, the cells adopted a round morphology, which might be favorable for chondrogenic differentiation when compared to gelatin hydrogels where more elongated cell morphology was found.[48] The limited survival of cells in some of these hydrogels might be caused by the soft nature of them and lack of adhesive cues. This would suggest that while the hydrogels are not toxic, but they do not provide sufficient stimulus to cells to grow, which could be related to a lack of integrin ligation. A lack of integrin-mediated survival signals could lead to arrest of cell growth and induce potentially apoptosis.[49] Hence, further studies with these systems are underway to improve survival and promote differentiation of hADSC.

3. Conclusions

The effect of the degrees of sulfation and oxidation on the physical and chemical properties of oxidized cellulose sulfates and hydrogels, prepared from oxCS and CMChi, was analyzed. Higher amounts of sulfation and oxidation lead to lower pH values in the pure oxidized cellulose sulfates and the corresponding hydrogels. The E-modulus is directly influenced by the degree of oxidation, as is the immobilization of water molecules in the hydrogels. A higher aldehyde content leads to stronger gels and a faster gelation time. Degradation experiments have demonstrated that the degradation of the hydrogel is less pronounced with higher degrees of oxidation and higher molecular weights of the oxidized cellulose sulfates. This can also affect the toxicity of hydrogels as was shown that the hydrogels’ cell toxicity is lowered, if the polymer chain length of the oxCS is increased. As the hydrogels can be tuned with regard to their degradability and physical properties, they make a promising material for the field of soft tissue engineering, but potentially also as reservoirs for controlled release of drugs.

4. Experimental Section

Chemicals and Reagents: O-Carboxymethyl chitosan (CMChi (Mw ≈ 321 kDa, degree of deacetylation = 94.2%) was purchased from Heppe Medical Chitosan GmbH (Halle (Saale), Germany). Chlorosulfuric acid, hydroxylationammonium chloride, and PBS were purchased from Merck, and ethanol 99.5% from Frügling, Germany. Sulfur trioxide trimethylamine complex was purchased from Alfa Aesar (Kandel, Germany). Dulbecco’s phosphate-buffered saline, insulin-transferrin-selenite (ITS 100x) liquid media supplement, FDA, Triton X-100, sodium periodate, dimethyl formamide 99% (DMF), and gelatin were purchased from Sigma–Aldrich (München, Germany). N,N-dimethylacetamide (DMA), lithium chloride, sodium hydroxide, sodium acetate trihydrate, glacial acetic acid, ethylene glycol ≥ 99.5%, Roti-Histofix, bovine serum albumin (BSA), and dialysis...
membrane (cutoff weights = 3500 and 100–500 Da) were purchased from Carl Roth (Karlsruhe, Germany). hADSCs, DMEM with a low glucose content of 1 g L\(^{-1}\), glucose, and antibiotic antimycotic solution (AAS) were purchased from Lonza Cologne GmbH (Köln, Germany). mMEM was purchased from Gibco (Life Technologies, Carlsbad, USA). Fetal bovine serum (FBS) was purchased from the company Biochrom (Berlin, Germany). The QBlue viability test solution was purchased from Biozol Diagnostic (Eching, Germany). TO-PRO-3 was purchased from AAT Bioquest (Sunnyvale, USA).

DMF was dried over a molecular sieve with a pore size of 3 Å. The other reagents were used without further purification. All aqueous solutions were prepared using deionized and filtered water.

DPBS was supplemented with 1 g L\(^{-1}\) glucose, 1% ITS liquid media supplement, 1% l-glutamine, 10 ng mL\(^{-1}\) fibroblast growth factor 2 (FGF-2), and 1% AAS. The medium DMEM with a glucose content of 1 g L\(^{-1}\) was supplemented with 1% ITS, 1% l-glutamine, 10 ng mL\(^{-1}\) FGF-2, and 1% AAS. The mMEM was supplemented with 1% FBS, 1% ITS, 1% AAS, 0.1 \(\times\) 10 \(^{-6}\) M dexamethasone, and 50 \(\mu\)g mL\(^{-1}\) ascorbic acid 2-phosphate.

Synthesis and Characterization of Oxidized Cellulose Sulfates: The synthesis of oxCS and the characterization of the degrees of sulfation and oxidation was extensively discussed by Strätz et al.\(^{[39]}\) The oxidized cellulose sulfates were named oxCS\(_{\text{ratiom}}\), where \(x\) stands for the DS (between 0 and 3, as a maximum of three hydroxy groups per cellulose repeating unit can be functionalized) and \(y\) for the DO (between 0 and 2, as a maximum of two hydroxy groups per cellulose repeating unit can be functionalized).

Characterization of Gel Formation, Intrinsic Gel Properties, and Gel Stabilization: For the determination of intrinsic pH value, the pH inside the hydrogels was measured in PBS with a phosphate content of 5.6 \(\times\) 10 \(^{-3}\) m using the pH-sensitive dye SNARF-4F \(5\)-(and-6)-carboxylic acid (Invitrogen, Darmstadt, Germany) and a fluorescence microscope consisting of a light source (PhotoFluor II NIR), a microscope (Leica DM4000B) with Nuance boxymethylchitosan solution (20 mg mL\(^{-1}\)) in PBS was placed in the center of the plate. 170 \(\mu\)L of cellulose solution (20 mg mL\(^{-1}\)) was added by mixing in the center. A 1 mm gap was chosen at 37 °C as incubation temperature. At first a single frequency sweep at 1 Hz and 1% strain was performed for 30 min to monitor the gelation. Afterward the amplitude sweep at 1 Hz from 0.01% to 5% was started. The frequency sweep at 0.1% strain was from 0.01 to 3 Hz. The compression of the sample was 0.3 mm. If a sample was destroyed during this program, a new sample was prepared, and the single frequency sweep was performed and continued with the next experiment.

For gravimetric measurements of gel stability, oxCS (20 mg mL\(^{-1}\)) and CMCh (30 mg mL\(^{-1}\)) were dissolved in PBS. The solutions were mixed in a ratio of 1:1 or 1:2 and then incubated for 1 h at 37 °C for crosslinking. After gelation, the hydrogels were incubated in PBS, DMEM, or DPBS. The DPBS was removed after 24 h and replaced with DMEM. The wet weight of the hydrogels was determined by carefully removing the excess liquid around the hydrogel with a piece of filter paper and weighing the sample.

The degradation of hydrogels was determined as the ratio of the initial wet weight (\(W_{\text{w0}}\)) to the wet weight after specific amounts of time (\(W_{\text{w}}\)) following Equation (1)

\[
\text{Wet remaining weight ratio} (\%) = \frac{W_{\text{w}}}{W_{\text{w0}}} \times 100
\]

Characterization of Biocompatibility of Hydrogels: For fabrication of hydrogels for cell culture studies in all experiments involving hydrogels, oxCS and CMCh were dissolved in phosphate-buffered saline (containing KCl (0.2 G L\(^{-1}\)), NaCl (8.0 G L\(^{-1}\)), KH\(_2\)PO\(_4\) (0.19 G L\(^{-1}\)), and Na\(_2\)HPO\(_4\) \(\cdot\) 2H\(_2\)O (0.76 G L\(^{-1}\)) adjusted to a pH of 7.4) before crosslinking, unless noted otherwise. The specific buffer composition was selected to adjust the osmotic pressure and kind of ions and concentrations as required to be compatible with living cells.

For cell culture, in this study, hADSCs purchased from Lonza were used, which were isolated from the human adipose tissue of a 40 year old female donor. The cell culture work was performed under a laminar-flow clean bench, and all materials and tools were sterilized prior use.

The cells were cultured in a 75 cm\(^2\) tissue culture flask in DMEM low glucose supplemented with 10% FBS and 1% AAS. The media supplements were added to improve cell growth and nutrient supply, and to prevent contamination by various bacteria and fungi. The cells were incubated in a humidified atmosphere of 5% CO\(_2\) and 95% air and 37 °C to provide the growth environment.

For encapsulation of human-adipose-derived stem cells in hydrogels and cell viability experiments, the CMCh solution (20 mg dissolved in 1 mL of DPBS) was placed in a water bath at 37 °C to prevent thermal gelation. oxCS solution (20 mg dissolved in 1 mL of DPBS) was filtered with a 0.2 \(\mu\)m membrane filter and stored at 37 °C prior to mixing with cells. hADSCs were harvested and centrifuged; the supernatant was aspirated, and the cell pellet containing 1.2 \(\times\) 10\(^{6}\) cells was carefully resuspended in CMCh solution. About 33.3 \(\mu\)L of cells per CMCh suspension was transferred into each well of a 12-well cell culture plate and immediately mixed with 16.6 \(\mu\)L of oxCS solution to obtain a final cell density of 1 \(\times\) 10\(^{7}\) cells per 50 \(\mu\)L of hydrogel. To improve the hydrogel stability, the hydrogels were incubated for 3 h at 37 °C before they were transferred into medium, as the medium is detrimental to the network stability. 1 mL of prewarmed growth medium or DPBS was carefully added on the top of hydrogel for 24 h incubation. After 24 h of incubation in the growth medium or DPBS, it was replaced by differentiation \(\alpha\)MEM (supplemented with 0.1 \(\times\) 10 \(^{-6}\) M dexamethasone, 50 \(\mu\)g mL\(^{-1}\) ascorbic acid 2-phosphate, 1% FBS, 1% ITS, and 1% AAS) to promote chondrogenic differentiation. The media were changed every 3 to 4 days to ensure the continued differentiation of the cells. Each experiment was performed in triplicate for each sample.

The preparation of controls was done by embedding hADSCs in gelatin hydrogels. Gelatin was dissolved in PBS at a concentration of 100 mg mL\(^{-1}\) and placed in a water bath at 37 °C to prevent thermal gelation and then filtered through a 0.2 \(\mu\)m membrane filter. The cell suspension of 1 \(\times\) 10\(^6\) cells was carefully mixed with 500 \(\mu\)L of gelatin solution. About 11 \(\mu\)L of microbial transglutaminase (mTG) solution (10 U \(\text{g}^{-1}\) activity) was added with gentle agitation to the gelatin–cell suspension. A volume of 50 \(\mu\)L of cell-encapsulated gelatin solution was transferred into each well of a 12-well tissue culture plate to obtain a final density of 100 000 cells per well. The cell-laden gel construct was incubated for 30 min at 37 °C to crosslink. Once the hydrogel was formed, 1 mL of complete growth medium was carefully added to the top of the hydrogel and incubated for 24 h in a CO\(_2\) incubator.

For visualization of cell viability and cell morphology with FDA and TO-PRO-3, and to qualitatively evaluate cell viability of the hydrogel-encapsulated hADSCs during their differentiation, living cells were visualized with FDA and dead cells were stained with TO-PRO-3 nuclear staining. FDA was taken up and converted by the intracellular esterase to green fluorescent metabolite product by living cells, only. TO-PRO-3 passes through damaged cell membranes, reaches the nucleus, and intercalates with the DNA double helix of the cell. About 5 \(\mu\)L of FDA solution (5 mg mL\(^{-1}\) in acetone) and 1 \(\mu\)L of TO-PRO-3 solution (1 mg mL\(^{-1}\) in acetone) were
added to the wells containing hydrogel constructs to assess the cell viability after 1, 7, 14, and 21 days. Samples were incubated for 5 min at 37 °C. The medium was then aspirated and replaced by fresh DMEM. Viable cells were visualized using a confocal laser scanning microscope. Qualitative image analysis was performed with Zeiss software. FDA positive cells were counted at five different locations within each construct. Only the cells which were completely embedded in the gel, away from any edge, were analyzed.

For the measurement of metabolic activity of cells, the metabolic activity of cells cultured inside the gels was analyzed using QBlue kit (Biochain, Heidelberg, Germany), which is based on the conversion of nonfluorescent redox dye resazurin to a highly fluorescent product (resorufin) by living cells. Embedding and culturing of cells were performed as described above. QBlue reagent solution was prepared at a ratio of 1:10 with colorless DMEM, added to each gel, and incubated at 37 °C for 3 h. After incubation, duplicates of 100 µL of the supernatant were transferred to a black 96-well plate. The converted product was fluorometrically quantified at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a plate reader (FLUOstar, BMG LabTech, Offenburg, Germany).

Statistical Analysis: The statistical analysis of cell data was performed using Origin Pro 2019 software with a one-way analysis of variance, evaluated by a posthoc Tukey test. The number of samples is described in the caption of figure 7. The statistical significance was determined for an error probability of $p \leq 0.05$ and marked by asterisks in the figure.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
Research data are not shared.

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