Porous cellulose-collagen scaffolds for soft tissue regeneration: influence of cellulose derivatives on mechanical properties and compatibility with adipose-derived stem cells

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Abstract This study compares two types of biore sorptive cellulose, i.e., calcium-sodium salt of oxidized cellulose (OC) and sodium salt of carboxymethylcellulose (CMC). It investigates which type would be preferable as an implant material in terms of biocompatibility, biomechanical and biological properties, and also in terms of its behavior in combination with collagen fibrils (Col) in composite Col/OC or Col/CMC scaffolds. OC significantly supported the stiffness and elasticity of Col fibrils, whereas CMC significantly reduced these properties. OC also enabled a strong interaction with Col fibrils even in a moist environment, accompanied by a significant drop in elastic modulus. The addition of cellulose did not significantly influence scaffold porosity; however, changes in surface morphology and the lower swelling capacity of OC, with a degree of oxidation of its chains between 16 and 24%, supported the idea of improved cell-material interaction. The elasticity and the stiffness of Col/OC guided human adipose-derived stem cells (hADSCs) to significantly higher adhesion, proliferation, and metabolic activity. On the contrary, the Col/CMC provided only limited mechanical support for the cells and inhibited their attachment and proliferation, although without any signs of cytotoxicity. This phenomenon could be used for future control of the differentiation of hADSCs towards a desired phenotype to generate advanced tissue replacements using modern methods of tissue engineering. The oxidation of cellulose resulted in a firmer scaffolding material, as required in vascular or skin tissue engineering. CMC is suitable for moist wound healing, e.g. as a mucoadhesive gel, where cell adhesion is not desirable.
Introduction

Today, soft tissue engineering places emphasis on finding optimal sources of three-dimensional (3D) scaffolds for the construction of tissue replacements. These scaffolds should provide a suitable microenvironment for subsequent ingrowth and differentiation of cells, particularly stem cells, which are an important cell component of practically all tissues of the human organism and play a key role in tissue regeneration (Bacakova et al. 2018). Stem cells have emerged as an important novel cell component of advanced tissue replacements created by methods of modern tissue engineering. Similarly, as differentiated cells, these cells can be used as autologous, but they are usually available in greater quantities, by less invasive methods (e.g., liposuction in the case of mesenchymal stem cells derived from subcutaneous adipose tissue), they have a higher proliferative capacity, a lower tendency towards senescence, and can be differentiated towards various desired cell phenotypes for the purposes of soft and hard tissue engineering (Bacakova et al. 2018). The interaction between stem cells and the microenvironment of an implanted material is fundamental for cell processes, followed by tissue morphogenesis. Scaffolding materials with specific mechanical and chemical properties (i.e., mechanical strength, elasticity, porosity, biodegradability, non-immunogenicity, antibacterial properties, etc.) can direct all these multiple processes of stem cell behaviour. For this purpose, porous scaffolds are usually used with the aim of mimicking the extracellular matrix (ECM) of various tissues, e.g. in a skin wound site or in the cardiovascular tissue. Some of the processing techniques used for making porous structures including both soft and hard scaffolds involve, for instance, freeze-drying, fibre bonding, foaming, salt leaching, or 3D (bio)printing. (Ghasemi-Mobarakeh et al. 2015; Stojic et al. 2019; Guo and Ma 2014; Svatík et al. 2021; Lepcio et al. 2022).

Collagen (Col) is the most abundant protein in ECM, consisting of three α-domains (polypeptide chains) (Shoulders and Raines 2009). In vivo, collagen molecules are naturally crosslinked by covalent intramolecular and intermolecular interactions to form fibres. Collagen contributes to maintaining the biological and structural integrity of ECM and provides physical support to tissues. With its biocompatibility, biodegradability, and permeability, it has important functions in regulating the morphology, adhesion, migration, growth, and differentiation of cells. Specific amino acid sequences of collagen, such as the Arg-Gly-Asp (RGD) motif and the Asp-Gly-Glu-Ala (DGEA) motif, induce cell adhesion by binding to integrin adhesion receptors on cells (Parenteau-Bareil et al. 2010). However, the most important
limitation on the use of a collagen-based material is its low mechanical strength, considering the high pressures and stresses encountered, e.g., by vascular tissues. This limitation is then accompanied by the rapid biodegradation rate of collagen. Therefore, it is recommended to crosslink collagen to form a highly organized 3D structure that will provide appropriate mechanical and biochemical signals for cells (Dong and Lv 2016; Ruszczak 2003; Copes et al. 2019). The mixing of collagen with other materials, both natural and synthetic, is also frequently used to improve the mechanical strength (Dong and Lv 2016) or the antibacterial properties of collagen scaffolds (Dorazilová et al. 2020).

Carboxymethylcellulose (CMC) is an anionic and water-soluble cellulose derivative, prepared by swelling cellulose in an aqueous NaOH solution and organic solvents, followed by carboxymethylation with monochloroacetic acid or its sodium salt, with the aim of converting the hydroxyl groups into carboxymethyl groups (Heinze and Pfeiffer 1999). The number of hydroxyl groups replaced by carboxymethyl groups per repeating unit referred to as the degree of substitution (DS), is usually within the range of 0.4 to 1.5 (An et al. 2014). CMC chains consist of D-glucopyranose ring units in a 4 C1 configuration. Each unit is linked by β-1,4-glycosidic linkage which results in an alternate turning of the cellulose chain axis by 180° (Thein-Han et al. 2009). Due to a hydrophobic polysaccharide backbone and many hydrophilic carboxyl groups, amphiphilic characteristics (Dutta et al. 2019; Hollabaugh et al. 1945). CMCs possess non-toxicity, biocompatibility, and biodegradability, CMC has a wide range of applications in biomedicine, since it is added to pharmaceutical formulations and cosmetics, as an emulsifier, a viscosity modifier, a lubricant, and a stabilizer. It has also been widely studied as a skin tissue implant in the form of films, hydrogels, foams, or nanocomposites (Basu et al. 2018). CMC is used to absorb wound exudates and autolytic debridement in wound healing. CMC has already proven its efficacy against infection, by attaching to microbes and removing them from the wound, and by inhibiting microbial growth (Jantrawut et al. 2019; Wong and Ramli 2014). Various types of CMC are used as delivery systems for active compounds (Abdollahi et al. 2019; de Lima et al. 2018; Oliveira et al. 2017) mainly to promote the antibacterial effect or to improve angiogenesis (Li et al. 2020). CMC creates a 3D gel network through physical crosslinking of hydrophilic sites or by chemical crosslinking (Ke et al. 2014). In combination with collagen, CMC forms a porous 3D structure that increases fluid absorption, providing an excellent water-retaining structure, suitable for drug delivery or as a hemostatic material (Paprskářová et al. 2021). Many studies have incorporated CMC in bone tissue engineering, but always in combination with mechanically stable structures, such as ceramic-based composites (Clarke et al. 2007; Teti et al. 2015). There is a lack of studies on the use of CMC in vascular tissue engineering. One study showed that oxidized CMC blends with gelatine in an electrospun tubular scaffold can mimic a blood vessel (Joy et al. 2018). Among cellulose derivatives, only bacterial cellulose has been used in vascular tissue engineering, because it has the strongest mechanical properties (Bäckdahl et al. 2006; Fink et al. 2011; Malm et al. 2012; Wippermann et al. 2009).

Oxidized cellulose (OC) is a cellulose derivative with potential applications in the field of soft tissue engineering. The structure of cellulose presents anhydroglucose (AGU) units linked by β-1,4 glycosidic bonds. Aldehyde and hydroxyl groups of the OC can react with other functional materials and improve the final biological and mechanical properties (Zimnitsky et al. 2005). New functions to cellulose can be added by oxidization by nitrogen tetroxide (N₂O₄) or nitroxyl radicals, such as 2,2,6,6-tetramethylpiperidine-1oxyl (TEMPO). Oxidation makes cellulose bioresorbable and biocompatible (Silvestre et al. 2021; Tang et al. 2017). TEMPO-oxidized cellulose possesses a negative surface charge introduced to the fibrils, and the consequent electrostatic repulsion forces keep the fibrils in a stable colloidal dispersion (Levanič et al. 2020). Bioresorption is enabled via chemical depolarization and enzymatic hydrolysis mediated by glycosidases, which leads to nontoxic final products of glucuronic acid and glucose. OC is characterized by nonimmunogenicity, antibacterial and anti-tumor activity, high absorbability, and anti-adhesive effects. It also has great hemostatic activity and is promising as a carrier for controlled drug delivery (Zimnitsky et al. 2004; Novotna et al. 2013). OC can also act effectively against antibiotic resistant microorganisms (Spangler et al. 2003). OC can
likely eliminate the inflammatory phase in chronic wounds (Martina et al. 2009). The combination with gelatine has already been studied in the form of a nanofibrous scaffold, which is suitable for medical applications, especially for modeling lung disease (Švachová et al. 2016). OC-based scaffolds have been further functionalized with arginine and chitosan to improve their suitability for the adhesion and growth of vascular smooth muscle cells (VSMC) (Novotna et al. 2013). A combination of TEMPO-mediated oxidation and carboxymethylation of cellulose created a mechanically suitable bioink for wound healing (Rees et al. 2015). OC was also used as a support for a cross linked alginate sponge in soft tissue engineering applications (Lin et al. 2012). OC-based biomaterials for vascular or skin tissue engineering and wound healing are still being explored, and so far, only limited research has been carried out (Wagenhäuser et al. 2016; Wei et al. 2020). Moreover, to the best of our knowledge, CMC and OC have not yet been compared in terms of cell adhesion and growth for purposes of tissue engineering. Such a comparison has usually been made from the point of view of the antibacterial, antitumor, and drug delivery properties of both cellulose types (Alavi and Nokhodchi 2020; Basta et al. 2020), or in terms of their ability to prevent postoperative tissue adhesions (Park et al. 2011).

Previous studies have evaluated collagen-based scaffolds, namely Col/OC-based scaffolds, and their modifications for specific skin tissue engineering applications (Babrnáková et al. 2019). In this work, we focus on how the addition of cellulose derivatives can influence either the tensile strength or the elongation of collagen fibrils and thus how it can create a new cell environment for soft tissue engineering applications. We have evaluated the in vitro biocompatibility of collagen scaffolds modified with two types of bioreversible cellulose, a fully soluble sodium salt of CMC and a semi soluble calcium sodium salt of OC, with an oxidation degree of 16–24%. We found that these two types of cellulose show a diverse biomechanical effect when combined with collagen. The scaffolds were studied to prove their nontoxic effect on adipose-derived stem cells (ADSCs) cultivated in a growth medium under conventional static culture conditions. Cell responses to both Col/CMC and Col/OC were related to the chemical and physical properties of these scaffolds (particularly swelling capacity and biomechanical properties).

Materials and methods

Materials

This study was carried out using the following materials, chemicals and other ingredients: bovine collagen type I, in the form of an 8 wt% aqueous solution (Collado s.r.o., Brno, Czech Republic), carboxymethylcellulose sodium salt having number average molecular weight $M_n = 250$ kg/mol and the degree of substitution $DS = 0.7$ (Holzbecher, Zlíc, Czech Republic), oxidized cellulose calcium-sodium salt (degree of oxidation 16–24%) and $M_n = 350$ kg/mol (Synthesia, Pardubice, Czech Republic), $N$-(3-dimethylaminopropyl)-$N'$-ethylcarbodiimide hydrochloride (EDC), $N$-hydroxy succinimide (NHS), ethanol p.a. 99.8%, sodium phosphate dibasic, sodium chloride, calcium chloride, sodium phosphate dibasic dodecahydrate, potassium dihydrogen phosphate, potassium chloride (KCl), collagenase from *Clostridium histolyticum* (≥ 125 CDU/mg) (all from Sigma-Aldrich, Darmstadt, Germany), isopropanol p.a. (Penta, Prague, Czech Republic), phosphate-buffered saline (PBS; Sigma Aldrich, St. Louis, MO, USA) Dulbecco’s modified Eagle medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), gentamicin (LEK, Ljubljana, Slovenia), fibroblast growth factor (FGF2; Z03116, GenScript, Piscataway, NJ, USA), ethidium homodimer-1 (E1169, ThermoFisher Scientific, Waltham, MA, USA), calcein (C1430, ThermoFisher Scientific, Waltham, MA, USA), resazurin (R7017, Sigma-Aldrich, St. Louis, MO, USA), bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), phalloidin-Atto 488 (49409, Sigma-Aldrich, St. Louis, MO, USA), 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI; 32670, Sigma-Aldrich, St. Louis, MO, USA).
Sample preparation

Col/cellulose scaffolds were prepared by the freeze-drying process from bovine type I collagen, as described previously (Slovíková et al. 2008). The collagen was modified with cellulose derivatives either with OC or CMC (Babrnáková et al. 2019). Briefly, the calculated amount of collagen (0.5 wt%) and cellulose derivative (0.5 wt%) in a ratio of 1:1 (wt/wt) was homogenized at 4 °C. Col/cellulose suspensions were poured either in a volume of 500 μl into 24-well plates (for in vitro evaluation) or in a volume of 80 ml into square 12 × 12 cm plastic plates (for an evaluation of the physical properties), and were freeze-dried in an Epsilon 2-10D lyophilizer (Martin Christ, Osterode am Hartz, Germany). Primary freeze-drying at −35 °C under 1 mBar for 15 h was followed by a secondary drying process at 25 °C under 0.01 mBar until the decreasing Δp (change in pressure) was up to 10%. The carbodiimide crosslinking system (EDC/NHS in a molar ratio of 2/1) was used for lyophilized scaffolds. After a 2 h crosslinking process, the scaffolds were washed twice with 0.1 M Na₂HPO₄ followed by a pure water wash to remove by-products. The final products were freeze-dried again, as described above.

Swelling behaviour and porosity

The crosslinked Col/cellulose scaffolds were cut into 1 × 2 cm strips and were immersed in PBS to test their hydrolytic stability at 37 °C. Each piece of the scaffold was weighed in its dry state before immersion, and the weight after gently removing the surface water with the use of a filter paper was noted in the following intervals: 1, 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150 and 180 min. The swelling ratio was calculated to define the exact amount of swelling caused by water absorption, and the swelling curve was obtained. The swelling ratio was calculated as follows:

\[
Swelling\ Ratio = \frac{W_s}{W_i}
\]

where \(W_s\) is the weight of the scaffold in the swollen state and \(W_i\) is the initial weight of the dry scaffold sample.

To determine the pore size and porosity of the cellulose scaffolds, images with the ×100 magnification and a scale of 500 μm of each sample were used to establish uniform conditions. The measurements were undertaken in the ImageJ software, using the thresholding function for porosity and line measuring in accordance with the resolution for pore size. The minimum of 30 pore diameters were measured in either horizontal or vertical axis.

Enzymatic stability

The enzymatic stability was observed in simulated in vitro conditions with a solution of collagenase from C. histolyticum in PBS at physiological pH 7.4 at 37 °C. A corresponding amount of collagenase was dissolved in PBS to obtain a solution with a collagenase concentration of 2.2 mg·l⁻¹. The solution was then tempered at 37 °C for 1 h. Scaffold samples were immersed in PBS for 10 min (according to the swelling behaviour results) at 37 °C, were deprived of excess water, and were weighed in a wet state.

Afterward, the PBS solution with collagenase was placed into glass vials with samples inserted into the vials. For the degradation experiment, samples were put into an incubator (37 °C) to simulate physiological conditions. Each sample was removed from the solution, dried on filtration paper, and weighed in 1, 2, 4, 8, 24, 48, and 72 h intervals from the first immersion. To calculate the mass loss of each sample, the obtained data were put into the following equation:

\[
WL = \frac{w_i - w_t}{w_i}.100
\]

where \(W_L\) indicates the weight loss, \(w_i\) is the initial weight of the wet scaffold after PBS swelling, and \(w_t\) is the weight of that scaffold after the corresponding elapsed period.

Charge density and stability

To measure the charge density of cellulose, an acid–base titration was performed with a pH meter (Milwaukee Instruments, Inc. Rocky Mount, United States) under atmospheric conditions. To simultaneously measure the pH of the prepared dispersion,
the pH meter was carefully fixed to the beaker containing the continuously mixing dispersion with the magnetic stirrer. The samples at a concentration of 0.5 wt% were titrated from acidic to alkaline between 2 < pH < 12, using 0.1 M NaOH as a titrant. The temperature was kept constant during the experiment, and the titrant was dispensed with an automatic micropipette. In order to completely neutralize the negative charge distributed along the cellulose chains, an excess of 2 ml of 0.1 M HCl was added. Titration was carried out by continuously adding NaOH under stirring (250 rpm). All measurements were repeated four times. The midpoint of the titration curve (the half-equivalence point) was quantitatively determined using the first derivation. The spike in the graph (plotted pH as x and the first derivative as y) points to the midpoint. The buffer range was estimated according to the Henderson-Hasselbalch principle, which states that, halfway to the equivalence point, the concentrations of the acid and base form are equal. At that point, the pH is numerically equal to the pKa at half-equivalence (midpoint). One unit around the pKa defines the buffer region. Before the midpoint, there is acid form predominance and pKa<1 and, after the midpoint, there is basic form predominance and pKa>1. The width of this buffer region was used to estimate the average charge density on the cellulose spheres. The charge density on an average cellulose sphere is calculated from the titration using the following process, which has already been described elsewhere (Li 2021). First, the number of cellulose spheres N in 100 ml solution is computed with the following equation, where m is the weight of the cellulose, A is the Avogadro number, and \( M_n \) is the molecular weight of the cellulose.

\[
N = \frac{m \cdot A}{M_n}
\]

The following equation is used to calculate the charge density, \( \delta \), of a cellulose sphere:

\[
\delta = \frac{A \cdot e \cdot c \cdot V}{N \cdot S}
\]

where A is the Avogadro number, e is the charge of the electron, c is the concentration of HCl (mol·l⁻¹), V is the volume of the base used to neutralize the charge on the cellulose spheres (µl) obtained from the buffer region, N is the number of particles, and S is the surface area of an average particle determined from its diameter, D, and assuming a spherical shape.

The diameter of the cellulose spheres was measured using the dynamic light scattering (DLS) detector (Wyatt Technology, Santa Barbara, USA) operating with a 658 nm laser at 25 °C while detecting a scattering angle at 90°. The diluted solution of cellulose was poured into a DLS cuvette and placed in the instrument cell. Each sample was measured five times, and the average diameter of the sphere was calculated, as well as a surface area, assuming a spherical shape. The average charge density was calculated along with the standard propagation of the error yields from the titration and diameter measurements.

To measure the stability of cellulose in solution, the zeta potential was determined using the Zetasizer Nano ZS (Malvern, UK) at 25 °C using the 633 nm laser. The measurements were performed using glass cuvettes and a dip electrode by Malvern, UK. Each sample was always measured at least 5 times.

Biomechanical analysis

Samples prepared on 12×12 cm plastic plates were cut into strips 4 cm in length, 10 mm in width (and approx. 3 mm in thickness). TA Instruments RSA-G2 (Dynamic Mechanical Analyzer DMA, New Castle, USA) was used to measure the tensile properties of the sample. A film tension clamp was used for testing. Samples were separately mounted into the grip, in a central position. The first biomechanical testing was carried out at room temperature (23 °C), while the second testing involved constant hydrating conditions, where the samples were immersed in PBS at 37 °C in the chamber surrounding the clamps. Before each measurement, 10 min of swelling was provided for each sample. The elastic modulus \( E \) was determined by the stress-to-strain ratio on the initial linear portion of the tensile curve, using the TRIOSDMA Data Analysis program (TA Instruments Inc., New Castle, USA).

In vitro testing with cells

For in vitro testing of the scaffolds, human adipose-derived stem cells (hADSCs) were used. The scaffolds were tested in terms of their potential indirect
cytotoxicity (e.g., mediated by substances released from the material), and later for direct cell growth within the scaffolds.

Previously, isolation of the hADSCs had been performed in compliance with the Declaration of Helsinki and had been approved by the Ethics Committee of Na Bulovce Hospital in Prague, according to a previously described procedure (Travnicková et al. 2020). The isolated hADSCs (passage 2) were characterized by flow cytometry for the presence of specific CD markers typical for mesenchymal stem cells and for the absence of hematopoietic and endothelial cell markers. The percentage of cells positive for the markers mentioned above was as follows: CD105 (99.9%), CD90 (99.5%), CD73 (100%), CD29 (100%), CD146 (4.7%), CD45 (3.8%), CD34 (0.2%), CD31 (0.5%).

Testing of scaffold cytotoxicity

The potential cytotoxicity of Col/cellulose scaffolds was tested by evaluating the visual morphology, the viability, and the metabolic activity of hADSCs cultured in extracts from the scaffolds, using Live/Dead staining and a resazurin conversion assay. First, the scaffolds were inserted into a 24-well polystyrene (PS) cell culture plate (TPP, Trasadingen, Switzerland), and 1.5 ml of DMEM without FBS was added into each well. The tissue culture PS of the 24-well plate was used as a control. Subsequently, the extracts were prepared by incubating the scaffolds in DMEM without FBS for 7 days (at a temperature of 37 °C, 5% of CO₂, 90% of humidity). Meanwhile, the hADSCs (passage 2) were seeded at a density of 5 000 cells/well in 150 µl of DMEM supplemented with 10% (vol·vol⁻¹) FBS, gentamicin (40 µg·ml⁻¹) and FGF2 (10 ng·ml⁻¹) into 96-well plates (TPP, Trasadingen, Switzerland). After 24 h of initial cell adhesion and spreading, the growth medium was exchanged for 150 µl of the extract supplemented by 10% (vol·vol⁻¹) FBS and FGF2 (10 ng·ml⁻¹), referred to as the “extract medium”. The extract medium was left unchanged for 7 days of cell culture.

Live/dead staining The visual morphology and viability of the hADSCs were evaluated by Live/Dead staining on days 1, 3, and 7. In brief, the cells were washed with PBS and were incubated with ethidium homodimer-1 (3 µg·ml⁻¹ in PBS) and calcein (2 µg·ml⁻¹ in PBS) for 10 min at a temperature of 37 °C. Then, the cells were washed with PBS and microphotographs were taken under an Olympus epifluorescence microscope IX51 (DP74 digital camera, objective magnification of ×10; Olympus, Tokyo, Japan). The living cells were visualized by calcein (green colour) and the dead cells were visualized by ethidium homodimer-1 (red colour).

Metabolic activity of the cells Conversion of resazurin was used to evaluate the metabolic activity (i.e., the activity of the mitochondrial respiratory chain enzymes) of the hADSCs on days 1, 3, and 7. In brief, the stock resazurin solution of 1 mg·ml⁻¹ in PBS was added to DMEM without phenol red to a final concentration of 10 µg·ml⁻¹. The cells were pre-washed with PBS, and 150 µl of the final solution was added to the cells in each well. The tissue culture PS of the 96-well plate without cells was used as a background control.

The plate with cells and the PS control was incubated at 37 °C for 3 h and 30 min on all measurement days. Subsequently, 100 µl of resazurin solution from each well was transferred to a new 96-well plate and the fluorescence (Ex/Em = 530/590 nm) was measured by a Synergy™ HT Multi-Mode Microplate reader (BioTek, Winooski, VT, USA).

Direct cell seeding and subsequent cell growth in static culture conditions

The hADSCs were used to study the biocompatibility of the Col/cellulose scaffolds in direct contact with the cells under static culture conditions. The sterile scaffolds were inserted into 24-well plates, were fixed with glass circles to prevent them floating in the culture medium and were prewashed with DMEM without FBS. The hADSCs (passage 2) were seeded at a density of 150,000 cells/well (i.e., 83,000 cells cm⁻²) in 1.5 ml of DMEM supplemented with 10% (vol·vol⁻¹) FBS and 10 ng·ml⁻¹ of FGF2.

Live/dead staining The visual morphology, cell number, and viability of the hADSCs was evaluated by Live/Dead staining on day 7. This procedure is described in detail in Sect. “Live/dead staining” (above). The viability and the cell number were then
counted from 10 microphotographs for each sample type of Live/Dead staining.

Fluorescence staining Fluorescence staining was used to visualize the cell morphology within the scaffolds on day 14 after seeding. First, the cells were fixed with 4% paraformaldehyde (for 10 min). Then the cells were pre-treated in PBS with 1% (vol·vol⁻¹) BSA and 0.1% (vol·vol⁻¹) TritonX-100 (for 20 min), and they were then incubated in PBS with 1% (vol·vol⁻¹) Tween 20 (for 20 min). Between each step, washing in a pure PBS solution was applied. To visualize the cell morphology, filamentous (F) actin was stained with phalloidin-Atto 488 (0.04 nmol·ml⁻¹ in PBS), and the cell nuclei were counterstained with DAPI (1 μg·ml⁻¹ in PBS) for 1 h at room temperature (RT). Photographs were taken under a Dragonfly 503 scanning disk confocal microscope with a Zyla 4.2 PLUS sCMOS camera, objective HC PL APO 20x/0.75 IMM CORR CS2 (Andor Technology Ltd., Belfast, UK).

Statistical analysis

Microsoft Excel data analysis was used for a statistical evaluation of the biomechanical and structural analysis, using five samples of each type. The data were evaluated using a 2-sample t-test, which assumes unequal variances and unequal sample sizes. The level of significance was set at 0.01 and 0.001. Data concerning in vitro testing are represented as mean ± SD; three parallels from each sample type were used unless otherwise specified. A statistical comparison of the samples was performed by the One-way ANOVA, Student–Newman–Keuls test, p < 0.05 or p < 0.001, using SigmaStat 3.5 software.

Results

Influence of cellulose on swelling, porosity and degradation

The ability of a scaffold to retain water is an important aspect in designing materials for soft tissue regeneration. The swelling ratio of all tested scaffolds increased with time and reached constant values, as illustrated in Fig. 1. The Col/CMC scaffold curve revealed that CMC contributed to the increased swelling capacity because of strong water adsorption. The internal architecture of each scaffold, revealed on the cut-formed cross-section, was assessed by a closer examination of the SEM micrographs. Images of all scaffolds with a magnification of ×100 are shown in Fig. 2. A large portion of the purely collagenous scaffold had a fibrous structure; fibres were also frequently present in the Col/OC scaffold. The Col/CMC scaffold suppressed the fibrous structure in its formation and formed plate-like units instead. These plates contain circular-shaped cavities that connect the neighbouring pores. The porosity of all scaffolds was between 60 and 70%, which was not significantly influenced by the addition of cellulose, and the material showed approximately the same porosity Fig. 3a. The differences in pore sizes among the

Fig. 1 The swelling behaviour of the Col/cellulose scaffolds. The tested materials were as follows: collagen (Col), collagen/oxycellulose (Col/OC) and collagen/carboxymethyl cellulose (Col/CMC)
tested materials did not show statistical significance with the average pore size of about 170 μm Fig. 3b. Cellulose scaffolds lost most of their mass over the course of their degradation. A graphical representation of the mass loss of the scaffolds in time is shown in Fig. 4. The integrity of the 3D network of the scaffolds was severed as the collagen degraded in the collagenase environment and small pieces of cellulose in the network were separated from the scaffolds. Small pieces of separated cellulose could be seen in the solutions of the composite scaffolds. No pieces were observed in the solution with pure collagen and the cellulose derivatives were also part of the mass loss. The hydrophilicity of the material also influenced the degradation process. Finally, the scaffold containing CMC was slightly more degraded than the scaffold with less hydrophilic OC.

Charge density of cellulose and stability

The acid–base titration curves of the solution containing cellulose are shown in Fig. 5. The midpoint of the titration curve was estimated at 3.42 ± 0.27 for OC with a buffer region that existed with pH between 2.42 and 4.42 with a total added volume of 14,800 μl. The midpoint for CMC was estimated at 3.72 ± 0.14, and the buffer region for CMC existed at pH between 2.72 and 4.72, with a total added volume of 14,100 μl. The measured values of the average diameter of cellulose spheres, charge density, and zeta potential are summarized in Table 1. Comparing OC to CMC, CMC spheres showed larger diameter and the average zeta potential indicated their higher stability in solution. The higher average charge density was attributed to the OC with lower diameters of spheres and lower stability. The number of spheres in 100 ml solution depended on the molecular weight of the cellulose and reached 1.20·10^{18} for CMC and 8.60·10^{17} for OC.

Influence of the cellulose derivatives on the biomechanical properties of the collagen scaffold

The resistance of the material against an induced force was examined with a measurement that yields the elastic modulus of a specimen in a uniaxial tensile test, in which the sample is grasped at the two ends and pulled, while the strain and the stress are measured simultaneously. All samples were subjected to a deformation test. The following stress–strain curves in Fig. 6a and b represent, under certain in vitro conditions, three regions and one point: the region of elastic deformation, the yielding region, one point of maximum stress, called the ultimate tensile strength (UTS), and the region of material tearing.

The region of elastic deformation represents the continually increasing stress applied to the material, accompanied by the material tension. In this area, the
The tensile curves, which differ in dry and hydrated conditions, are displayed in Fig. 6a and b. In the hydrated state, both Col/cellulose scaffolds showed the greatest plastic deformation with the lowest UTS compared to pure Col Fig. 6c. However, these Col/cellulose scaffolds displayed the most significant elongation Fig. 6d. Beyond the UTS point, the Col/cellulose materials gradually started to tear. In hydrated conditions, the stress is low (in the tearing phase), and the material continues to elongate more than in dry conditions until the ultimate fracture of the material Fig. 6b. Representative values of the maximum elongation and UTS of the materials are shown in Fig. 6c, d.

In the dry measurement condition, the most significant material resistance was attributed to the Col/OC scaffold with UTS of 188 ± 38 kPa, followed by...
**Fig. 4** The mass loss of the Col/cellulose scaffolds during the process of enzymatic degradation in a collagenase solution in PBS. The tested materials were as follows: collagen (Col) black coloured, collagen/oxycellulose (Col/OC) white coloured and collagen/carboxymethyl cellulose (Col/CMC) grey coloured.

![Graph showing mass loss of Col/cellulose scaffolds](image)

**Fig. 5** pH titration of cellulose solution to calculate charge density, where oxidized cellulose OC (a) and carboxymethyl cellulose CMC (b). The displayed borders point to a buffering regions.

![Graph showing pH titration of cellulose solutions](image)
pure Col with UTS of 86 ± 19 kPa and Col/CMC with UTS of 32 ± 4 kPa Fig. 6c. The addition of CMC contributed to a significant decrease in stress but resulted in the greatest elongation in both environments Fig. 6d. Col/OC is stiffer, but is inflexible according to the tensile curves, as it exhibits less elongation. In the hydrated state, only a small amount of stress (UTS = 9.4 ± 2.0 kPa for Col/OC and UTS = 2.5 ± 0.3 kPa for Col/CMC) was required to cause permanent deformation after 10 min of swelling, as depicted on Fig. 6c.

Pure Col in a hydrated environment showed the highest resistivity (28 ± 5 kPa) of all materials tested. In general, the addition of both types of cellulose could place the collagen material under significant stress in both dry and wet environments. In general, the presence of CMC stretched the collagen fibres even more in aqueous experimental conditions than in a dry environment. The breaking point occurred at 19.5 ± 0.2% of the initial length. When the material was swollen, water molecules spread through the collagen fibrils, where they also interacted with polar groups of cellulose, thereby mediated conformational and segmental motion and increased the tension of the material (Coenen et al., 2018). However, much less stress had to be applied in order to stretch the material to a greater length.

In dry conditions, Col/OC significantly increased the Elastic modulus (E) from 3.0 ± 0.7 MPa (for pure collagen) to 5.4 ± 1.0 MPa (Fig. 7a) and reduced the E

### Table 1

| Cellulose type | Spheres diameter [nm] | Charge density [C/m²] | Zeta potential [mV] |
|---------------|-----------------------|-----------------------|---------------------|
| OC           | 59 ± 9                | 1.52·10⁻² ± 0.11      | -15.9 ± 2.58        |
| CMC          | 162 ± 41              | 1.41·10⁻³ ± 0.21      | -71.8 ± 7.22        |

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**Fig. 6** Influence of the addition of cellulose types on the stress and strain of collagen fibrils: **a**—tensile curves of scaffolds in a dry state, **b**—tensile curves of scaffolds in a hydrated state, **c**—ultimate tensile strength points in a dry state and in a hydrated state, **d**—maximum elongation at the ultimate tensile strength points in a dry state and in a hydrated state. The tested materials were as follows: collagen (Col), collagen/oxycellulose (Col/OC) and collagen/carboxymethyl cellulose (Col/CMC). A significant difference was noted between Col and Col/cellulose; Student’s t-test for two independent populations with unequal variances (**p ≤ 0.001), (**p ≤ 0.01)**
from 352 ± 38 kPa (Col) to 104 ± 19 kPa in a hydrated state (Fig. 7b). Col/CMC showed a significant drop in E from 3.0 ± 0.7 MPa (Col) to 0.36 ± 0.04 MPa in a dry state (Fig. 7a). The same happened in a hydrated state (Fig. 7b), where the E of Col/CMC showed a significant decrease from 352 ± 38 kPa (Col) to 17.2 ± 1.8 kPa. The swelling of each material before the initiation of the test highlighted the importance of hydration in the mechanical response. Collagen and cellulose fibrils swelled as water penetrated the structure; the hydrogen bonds became hydrated and weak, which led to a drop in tensile strength, and in the E of all scaffolding materials. In addition, the formation of water bridges between peptide chains could be responsible for a decrease in the modulus (Grant et al., 2008).

Testing in cell cultures

Testing of scaffold cytotoxicity

Live/Dead staining was used to visualise the morphology and the viability of hADSCs cultured in extracts from the scaffolds. On day 1, the hADSCs were well spread in the cultures in all extract samples.

On day 7, the density of the hADSCs had increased in all extracts, and the cells were still well spread. High viability of the hADSCs (days 1, 3, and 7) was proved in all extracts. Interestingly, some of the scaffold fragments (shown in red colour in the microphotographs) occurred during Live/Dead staining, mainly in Col/OC extracts (Fig. 8).
Measurements of the cell metabolic activity were used to estimate the growth of cells cultured in the scaffold extracts. On days 1, 3, and 7, the metabolic activity of the hADSCs in the Col/CMC and in the Col/OC extracts was significantly higher than in the Col and PS control extracts (Fig. 9).

On day 7, Live/Dead staining revealed a high number of well-spread cells on the Col scaffolds. The Col/OC scaffolds also supported cell adhesion and cell spreading, although the cell number and the cell spreading were slightly worse than on the Col scaffolds (Fig. 10). In contrast, the Col/CMC
scaffolds showed a small number of round cells, i.e., the cells were not properly attached and were not well spread (Fig. 10). A quantitative cell count on day 7 showed significant differences among all tested samples ($p < 0.001$) (Fig. 11a).

Specifically, the highest cell numbers were found on the Col scaffolds (i.e., 31,383 cells cm$^{-2}$). Col/OC reached a mean value of 20,290 cells cm$^{-2}$ and the lowest mean value was obtained for Col/CMC, i.e., 7330 cells cm$^{-2}$ (Fig. 11a). Col and Col/OC reached mean cell viability values of over 95%, whereas the cell viability on the Col/CMC scaffolds was slightly lower, i.e., 89.9% (Fig. 11b).

Fluorescence staining The morphology of the hADSCs on the scaffolds was visualized by staining of F-actin on day 14. The hADSCs reached almost confluent layers on the Col/OC and Col scaffolds (Fig. 12), depending on the specific shape of the scaffold. In particular, the 3D microscopy projections showed clearly that the cells followed the shape of the scaffold fibres. The cells on the Col/CMC scaffolds formed only sporadic spheroidal structures (Fig. 12).

Discussion A set of experiments was performed to verify the suitability of Col/cellulose scaffolds for soft tissue engineering applications, with an emphasis on vascular and skin tissue engineering. The addition of both OC and CMC does not have a radical influence on the enzymatic stability, and only a higher mass loss is recorded as a result of cellulose, which is gradually released from the network as collagen is degraded. The CMC supported water absorption into
the scaffold, compared to the OC or to the pure Col, confirmed as well by the highest swelling ratio. CMC is fully soluble in water with a higher negative zeta potential of its anionic chains than OC, that leads to a better stability of CMC in solution. The swelling can be also modulated by a higher charge density of Na⁺ ions or different CMC concentrations (Chang et al. 2011). Good solubility and bioabsorption of CMC are also assigned to DS > 0.7, while DS < 0.4 leads to low or no bioabsorption. However, high water absorption of CMC can cause undesirable effects when creating mechanically strong scaffold. Otherwise, a lower negative zeta potential of OC shows incipient instability, which can be explained by a lower percentage of oxidation (16–24%), and OC therefore has a lower capacity to absorb water. The oxidation of cellulose is an important parameter, which also influences solubility and bioabsorption. It resulted in a lower content of water-stabilizing hydroxyl groups, and thus in a lower swelling ratio. However, the swelling of the Col/OC scaffold was comparable to that of the Col scaffold. The porosity and the pore sizes of freeze-dried foams are already well explored in soft tissue engineering. The results of our study are similar to those of already modified cellulose-based scaffolds. Pore sizes in the range of 50 μm to millimeters support cell infiltration, migration and signalling (Chen and Fan 2007; Mirtaghavi et al. 2020; Wiwatwongwana and Promma 2019). As shown in our study, a similar morphology was also obtained on S-sulfo keratin sponges with controlled pore size and porosity (Katoh et al. 2004). In this research, the change in porosity influenced by both types of cellulose showed a slight reduction (from 70 to 60%). This reduced the rate of cell infiltration and ingrowth, since the number of hADSCs seeded on the Col scaffold was significantly

![Fig. 12](image-url)
higher than on the Col/cellulose scaffolds. A change in pore size was not significant when comparing both types of cellulose, the average pore size in the Col/CMC and Col/OC scaffolds was around 170 μm. However, OC and CMC acted differently in terms of the attachment and proliferation of hADSCs. Thus, the different cell responses in this study can be attributed mainly to the differences in the stiffness, elasticity, and swelling ability of the two types of scaffolds, rather than to their porosity, which apparently played no significant role. Different charge densities can also influence further properties of materials, e.g., water absorption, viscosity, transparency, rheology, stiffness, and elasticity (Moradian et al. 2021). Detailed studies of the charge density of cellulose have mainly focused on its nanocrystals (Wu et al. 2017, 2021). It seems that the charge density of polyelectrolytes is also one of the main parameters in complexation with other charged molecules. Moreover, a higher charge density resulted in more stretched chains, because of the stronger electrostatic and steric repulsions (Li and Zhong 2021). In this article, OC had a higher charge density than CMC, and the carboxylate groups of cellulose were highly negatively charged above a pKa of 3.42. Therefore, they were able to provide stronger binding with positively charged basic amino acid residues of collagen, and this binding could also result in more stable complexes. It seems that the smaller diameter of OC spheres, in comparison with CMC, together with a lower ability to form a gel, can also be influenced by a higher charge density, as was once observed (Abitbol et al. 2018). On the other hand, it has been shown that the higher DS of the CMC correlates with a higher charge density, which leads to increased electrostatic attraction with positively charged molecules. No precipitation of cellulose with collagen occurred during mixing, suggesting an adequate amount of charge densities in the cellulose chains (Xiong et al. 2017).

According to the mechanical analysis, the significant drop in UTS and in the elastic modulus (E) of material in a moist environment maintained the mechanical resistivity mainly of the Col and Col/OC scaffolds compared to the Col/CMC. This leads to a positive response of hADSCs in the stiffness and elasticity of Col and Col/OC scaffolds after direct cell seeding. These scaffolds supported the initial adhesion, spreading and growth, and the hADSCs reached almost confluent layers by day 7 or by day 14. Therefore, these scaffolds are expected to induce or to support an early stage of cell differentiation. The culture of hADSCs in the scaffold extracts revealed that there was no negative effect on the viability or metabolic activity of hADSCs within all Col/cellulose scaffolds. On the contrary, the hADSCs seeded in Col/CMC did not spread and formed only sporadic clusters. Similarly, in several studies carried out on CMC alone or in combination with various other biomaterials, Kilic Bektas et al. (2018), Ke et al. (2018), and Wiegand et al. (2019) showed no fibroblast attachment and little to no support for proliferation on CMC, and they advised blending it with another material such as collagen. As revealed by the present study, the combination of CMC with collagen did not overcome the limitations of CMC for further cell performance. It seems that CMC can provide only slight support for cell viability, as has also been confirmed by Basu et al. (2018). The proliferation of stem cells was confirmed only on a CMC scaffold loaded with platelet-rich plasma, while CMC alone showed only cell migration (Diaz-Gomez et al. 2022). The structure of OC in this research presents a rougher surface of more fibrillar morphology, which is considered to be an advantageous factor for tissue engineering, as wider pores and greater porosity provide suitable support for cell processes. On the contrary, the relatively flat surfaces present in CMC prevented cells from adhering and proliferating. Further measurements of the roughness thresholds of cellulose surfaces would need to be performed to modulate cellular responses. Courtenay et al. (2018) have shown that modulating the stiffness and the charge of cellulose from negative to positive resulted in differential cell responses, and enabled cell attachment, also cell spreading without the need for additional cell adhesive proteins or ligands. Likewise, Courtenay et al. (2017), evaluated that positively charged cellulose increased cell attachment by 70% in comparison with unmodified cellulose, or in comparison with negatively charged cellulose. Charge plays an important role in establishing an appropriate environment for various types of cells (Dadsetan et al. 2011). In our earlier study by Pajorova et al. (2020), a negatively charged nanocellulose was preferred for adhesion and growth by human skin fibroblasts, while the behaviour of hADSCs was less dependent on the charge of the nanocellulose substrate. In this article, both types of cellulose are negatively charged, which gave them the potential
to be ionically attracted to positively charged collagen. However, these ionic interactions are still weak and must therefore be supported by a chemical crosslinker. This neutralizes the charges, but strongly increases the biomechanical properties and the stability of the material.

The addition of cellulose among collagen fibrils has already shown some good mechanical prospects for applications in soft tissue engineering. A similar collagen-cellulose material was used for engineered arteries (Steele et al. 2013) with the tensile strength of 10–15 MPa, and strain of 20–25%. Special bacterial cellulose has remarkable biomechanical properties (tensile strength of 250 MPa) and extremely high modulus (greater than 15 GPa) (Nishi et al. 1990), which is attributed to the super-molecular nanostructure (Feng et al. 2015). The E of cellulose can be also dependent on the electrical charge, which modulates the cell adhesion and proliferation, as mentioned above (Pajorova et al. 2020). Synthetic polymers, such as polyester elastomers often showed growth support of cells for vascular tissue engineering (Pêgo et al. 2003; Bat et al. 2010; Song et al. 2010). Tuning the composition of the synthetic polymers, the elastic modulus can be modulated between 12 and 1343 MPa (Fernández et al. 2012). They also possess perfect elongation at break of 250% (Kwon et al. 2001). Fibrous keratin/poly(vinyl alcohol) (Ker/PVA) and poly(ε-caprolactone) (PCL) with elongation at break about 44% and the E=17 MPa was considered to be optimal for application in skin regeneration (Ranjbar-Mohammadi et al. 2021). Generally, synthetic polymers offer greater control over the structural and mechanical properties of the scaffolds, and greatly support cell interactions. However, they may lack the stimuli necessary for differentiating stem cells towards a specific cell lineage (Zhu and Marchant 2011). Cellulose-based biomaterials can offer important advantages over conventional synthetic materials and may show greater promise for advancing the differentiation.

In this paper, mechanical properties of Col/Cellulose scaffolds were much lower than of synthetic polymers or bacterial cellulose. The addition of CMC to Col significantly decreased the E approximately tenfold in dry measurements. Even if CMC improves the tension of collagen fibrils, the moisture in the cell culture environment disabled the mechanical support, followed by an approximately 20-fold drop in the E. This led to poor cell adhesion, and highly probable it will lead to a deficient later growth, as the cells were enabled only to penetrate the Col/CMC scaffold or attach incompletely on its surface. Highly soft and deformable substrates cannot resist the traction forces generated by the cells during cell adhesion and collapse (Engler et al. 2004). Here, the OC increased the stiffness of collagen by approximately 45%. In a moist environment, as more physiological conditions were applied, the stiffness radically decreased and approached the values of native tissues. E of native soft tissues and organs ranges from 0.1 kPa to 1 MPa, depending on the function and the location of the tissue (Liu et al. 2015; Akhtar et al. 2011). There is large variability among the examined values, due to the different realization protocols, and variability of the subjects (Griffin et al. 2016). Qiu et al. (2010) used atomic force microscopy (AFM) to measure the E in force mode using silicon nitride cantilevers in tissue using VSMCs from the aorta of young (13 ± 0.3 kPa) and old (42 ± 0.5 kPa) male monkeys. The E of Col/OC scaffold presented here was more adaptable to the old aorta under physical conditions. Snowhill and Silver (2005) obtained E = 9.6 MPa and UTS = 2.5 MPa, as the highest value from various blood vessels (aorta, carotid artery, iliac artery, and vena cava in a porcine model). Under dry measurements, Col and Col/OC scaffolds are more alike these values. Crichton et al. (2011) showed by performing AFM that the E of the dermis component is 7.33–13.48 MPa. The Col/OC scaffold (with the E of 5.4 ± 0.95 MPa) approached the stiffness of the native skin most closely. The E of the epidermal components (0.75–1.62 MPa) reported in the same study (Crichton et al. 2011) shows that Col/CMC scaffold (which lacks the ability to support cell adhesion) can also reach the values of the native skin.

**Conclusion and further perspectives**

Cellulose plays an important role in modifying the biomechanical and biological properties of porous cellulose/collagen material and can modulate cellular responses. We compared two specific types of bioabsorptive cellulose, one prepared by oxidation (OC) and second one by carboxymethylation (CMC). They differ mainly in solubility and charge density, which was influenced by different preparation processes
(oxidization and carboxymethylation). Cellulose types were combined separately with collagen to enhance the mechanical and structural properties. The scaffolds differ mainly in swelling capacity, biomechanical behaviour, cell attachment, and proliferation. OC supported the rigidity, stability, and cell-material interactions, even after a significant reduction in biomechanical properties caused by swelling of the material. The elasticity and stiffness of the Col/OC scaffold led the cells to adhere and spread, and also to be metabolically active, without any signs of cytotoxicity of the material. Col/OC is denoting as a “scaffolding” material which can further facilitate air permeation and support vascularization. CMC was most compromised by the addition of water and by both low stiffness and elasticity. In addition, the flat structure with decreased porosity creates inhospitable conditions for cell attachment. The study introduces the idea of incorporating CMC into already mechanically stable structures, ideally synthetic polymers, where it can support physiological properties (oxygen flow, nutrient delivery, or exudate removal during wound healing), or it may enhance cell differentiation in later stages. However, the most appropriate role that CMC can play is as a bioinert superficial secondary cover, e.g., as a wound dressing with a high absorption capacity for exudate removal. Finally, tuning the cellulose concentration, tuning the degree of oxidation, and modulating the charge can also have a positive influence on the mechanical properties and thus on the cell-material interactions.

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**Declarations**

**Conflict of interest** The authors declare that they have no competing interests.

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