



Ionically-crosslinked carboxymethyl chitosan scaffolds by additive manufacturing for antimicrobial wound dressing applications

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ABSTRACT

Chitosan chemical functionalization is a powerful tool to provide novel materials for additive manufacturing strategies. The main aim of this study was the employment of computer-aided wet spinning (CAWS) for the first time to design and fabricate carboxymethyl chitosan (CMCS) scaffolds. For this purpose, the synthesis of a chitosan derivative with a high degree of O-substitution (1.07) and water soluble in a large pH range allowed the fabrication of scaffolds with a 3D interconnected porous structure. In particular, the developed scaffolds were composed of CMCS fibers with a small diameter ($< 60 \mu\text{m}$) and a hollow structure due to a fast non solvent-induced coagulation. Zn^{2+} ionotropic crosslinking endowed the CMCS scaffolds with stability in aqueous solutions, pH-sensitive water uptake capability, and antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. In addition, post-printing functionalization through collagen grafting resulted in a decreased stiffness ($1.6 \pm 0.3 \text{ kPa}$) and a higher elongation at break ($101 \pm 9 \%$) of CMCS scaffolds, as well as in their improved ability to support *in vitro* fibroblast viability and wound healing process. The obtained results encourage therefore further investigation of the developed scaffolds as antimicrobial wound dressing hydrogels for skin regeneration.

1. Introduction

Chitosan (CS) is a naturally derived cationic polysaccharide with physical-chemical and biological properties suitable for tissue engineering and other biomedical applications (Kou et al., 2022). Its main disadvantage from a processing point of view is a limited solubility in aqueous media at neutral pH (Szulc & Lewandowska, 2023). CS chemical modification to obtain the water soluble derivative carboxymethyl chitosan (CMCS) has attracted increasing interest for the development of various products depending on the reaction conditions, i.e., N-CMCS, O-CMCS or N,O-CMCS, if the carboxymethylation is achieved at the amino, hydroxyl, or both groups, respectively. CMCS advantages in comparison with CS have motivated impactful studies for drug/enzyme delivery, wound healing, bioimaging, and tissue engineering applications (Geng et al., 2023). Indeed, CMCS has an excellent *in vitro* and *in vivo* biocompatibility (Douette et al., 2020) with a positive effect on fibroblasts proliferation (Geng et al., 2023) and collagen mineralization (Li et al., 2023). Furthermore, CMCS has hemostatic properties (Qian et al.,

2021) and stands out for its antioxidant capacity (Chaiwong et al., 2020). The presence of both carboxyl and amino groups in O-CMCS endows the polysaccharide structure with peculiar biological properties (Poon et al., 2007) thanks to its increased similarity with the anionic structure of glycosaminoglycans (GAGs), including improved interaction with the cell membrane (Zhu et al., 2007). In addition, O-CMCS has shown *in vivo* anti-inflammatory effect (Adnan et al., 2020) and increased antibacterial properties compared to CS (Geng et al., 2023). O-substitution is favored by a lower reaction temperature, while N-substitution increases by raising the reaction temperature (Upadhyaya et al., 2014). In any case, at room temperature it is unlikely that no substitution occurs on $-\text{NH}_2$ groups, at least as a minor reaction (de Abreu & Campana-Filho, 2009). Other chemical modification parameters, including NaOH concentration and CS/monochloroacetic acid ratio, also play a key role in the preferential substitution site (Poon et al., 2007; Zhu et al., 2007).

Additive manufacturing (AM) is a class of computer-controlled techniques for 3D structure design and fabrication through a layer-by-

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layer process. Several AM approaches have been developed to process CS for wound healing and tissue engineering applications (Agarwal et al., 2023). However, only few works have been focused on AM of CMCS, always in combination with other polymers to achieve suitable processing properties (Taghizadeh et al., 2022). For instance, CMCS was blended with gelatin methacryloyl (GelMA) and then processed by extrusion/photopolymerization for vascular tissue engineering (Wang et al., 2023). Ionic crosslinking with a Ca^{2+} solution was instead investigated to stabilize 3D printed CMCS/alginate porous constructs for bone tissue regeneration (Mohabatpour et al., 2022; Müller et al., 2015). Other 3D (bio)printing studies have used CMCS blended with starch (Naseri et al., 2021), agarose (Butler et al., 2021), or gelatin (Huang et al., 2016), usually also submitted to Ca^{2+} ionotropic crosslinking. However, these approaches have not allowed the production of scaffolds in the form of a 3D layered fibrous structure providing an interconnected porosity along their thickness, suitable for optimized wound healing and skin tissue engineering applications (Kai et al., 2022; Naseri et al., 2021). In this context, computer-aided wet-spinning (CAWS) has emerged in the past decade as a hydrogel processing method that couples the advantages of AM with those of wet-spinning to fabricate scaffolds with a tunable macroporosity along their thickness, according to the designed lay-down pattern and the applied processing parameters. This AM technology involves extruding a polymeric solution directly into a non solvent bath to induce polymer coagulation. The solidifying polymeric fiber resulting from solvent/non-solvent counterdiffusion across the transversal cross-section of the extruded filament is deposited with a predefined pattern to form layer-by-layer a 3D scaffold (Puppi & Chiellini, 2020). CAWS has been effectively employed to process both synthetic and natural polymers, including 3D hydrogels made of CS-based polyelectrolyte complexes (Braccini et al., 2024; Puppi et al., 2016). However, to the best of the author's knowledge CAWS approach has not been applied yet to CMCS processing, nor to wound dressing development.

The aim of this study was the design and CAWS manufacturing for the first time of CMCS scaffolds with a predefined porous structure. The main hypothesis to be verified was therefore that a binary CMCS/water mixture with an optimized composition could be extruded and deposited into a polymer non-solvent bath by following a predefined layer-by-layer pattern. Indeed, as highlighted earlier, previous literature reported on AM of CMCS only when blended with other polymers, in order to achieve suitable rheological properties and apply effective crosslinking strategies. The ionotropic crosslinking stabilization in aqueous media of CMCS layered structures fabricated by means of this novel method, as well as collagen I (COL) immobilization on their polymeric matrix through reaction with carboxylated groups, were other relevant hypotheses to be proved in order to assess scaffolds suitability for cell culture and wound healing applications. As a consequence, a key objective of the research was also to synthesize a CMCS with a high substitution degree resulting in suitable water solubility, as well as a high number of functional groups for collagen grafting and complexation with divalent cations. Among other cations investigated in the past years for polysaccharide crosslinking (e.g., Cu^{2+} , Co^{2+} , Ni^{2+} , and Sr^{2+}) (Yan et al., 2023), Zn^{2+} was selected in this study due to its remarkable antibacterial activity, as well as the high mechanical strength of polysaccharide hydrogels obtained by means of its complexation, suitable as wound healing matrices (Cui et al., 2023; P.-H. Lin et al., 2018; Wu et al., 2022; Zhou et al., 2018). In particular, Zn-crosslinking resulted in increased CS hydrogel biocompatibility in comparison to what was achieved with Cu-crosslinking (Rogina et al., 2019), as well as in a higher CMCS hydrogel antibacterial activity than in the case of Ag- or Cu-crosslinking (Wahid et al., 2017). Furthermore, Zn ions can endow the material with anti-inflammatory and scavenging properties (Mutlu et al., 2022; Yan et al., 2023). However, Zn hydrogel content and its concentration upon release in physiological media should be accurately considered since they can affect material biocompatibility depending on the investigated cell line (Cui et al., 2023; Salama & Abdel Aziz, 2020).

The CMCS investigated in this study was synthesized through CS reaction with monochloroacetic acid and characterized by FTIR and ^1H NMR spectroscopy, solubility analysis, and viscometry. CAWS parameters for CMCS processing were optimized and ionic crosslinking through ZnSO_4 treatment was explored as an effective means to stabilize the fabricated hydrophilic scaffolds in aqueous medium. In addition, scaffold functionalization through surface grafting of COL, the main component of the extracellular matrix, was explored as an effective means to increase cell adhesion to the polymeric matrix (Kuo & Yeh, 2011). The developed CMCS scaffolds were characterized in comparison to analogous CS scaffolds used as a reference, by means of FTIR spectroscopy, scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM-EDS) analyses, water contact angle, water uptake, differential scanning calorimetry (DSC), mechanical tensile test, and antibacterial activity against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). The biocompatibility and ability to support wound healing of the developed hydrogels were investigated through Balb/3T3 clone A31 cell culture and *in vitro* scratch assay.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan (CS, L-448869), collagen type I from rat tail (COL, L-093 K37781) monochloroacetic acid, sodium hydroxide (NaOH), glacial acetic acid, sodium acetate, ethanol (EtOH), isopropanol, deuterium oxide (D_2O), trifluoroacetic acid-*d* (CF_3COOD), 2-(N-morpholino)-ethanesulfonic acid (MES, $\geq 99\%$), N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), and compounds for the preparation of phosphate-buffered saline (PBS: Na_2HPO_4 ; NaH_2PO_4 ; NaCl; KCl) were purchased from Sigma-Aldrich (Italy) and used as received. Deionized water (dH_2O) was used for the preparation of polymeric and reactive solutions.

2.2. Carboxymethyl chitosan (CMCS) synthesis

5 g of CS were added to 30 mL of an aqueous solution of NaOH (50 % wt) and the obtained mixture was kept under constant magnetic stirring for 2 h at 30°C . Subsequently, 14.2 g of monochloroacetic acid were dissolved in 120 mL of isopropanol and the obtained solution was slowly added dropwise to the alkalized CS at 30°C . After stirring for 24 h, the solid was filtered and suspended in 50 mL of EtOH. The pH was adjusted to 7 using acetic acid. The product was dissolved overnight in dH_2O for further purification. The solution was then filtered through a porous glass membrane and precipitated upon the addition of EtOH. The precipitate (CMCS-Na) was collected and thoroughly washed with EtOH/ dH_2O solutions with increasing EtOH content (80–99 % v/v). The acid form (CMCS-H) was prepared based on a previously described procedure (Upadhyaya et al., 2014). Briefly, 0.5 g of CMCS-Na were suspended in a EtOH/HCl solution (50 mL/5 mL, 80/37 % v/v) for 30 min, filtered, and rinsed with 80–90 % EtOH for neutralization. CMCS-Na and CMCS-H were frozen at -20°C for 24 h and then lyophilized for 24 h at -50°C and 0.04 Torr.

2.3. Scaffolds fabrication

CS was dissolved at a given concentration (3.3 % w/v) in a 1 % (v/v) acetic acid aqueous mixture under magnetic stirring at room temperature overnight. Purified CMCS was added to dH_2O (4 % w/v) and the mixture was kept under stirring overnight at room temperature until a homogeneous solution was obtained. Before printing, all the solutions were filtered using a cellulose acetate filter (cut-off 5 μm). The wanted solution was loaded into a 10 mL plastic syringe equipped with a blunt-tip stainless-steel needle (Gauge 22) and extruded into an EtOH coagulation bath using a programmable syringe pump (NE-1000, New Era

Pump Systems Inc., Wantagh, NY) to control the extrusion flow rate ($F = 1$ mL/h). Scaffolds were manufactured by CAWS using a home-modified subtractive rapid prototyping system (MDX-40 A, ROLAND DG Mid Europe Srl, Italy) to produce layer-by-layer 3D scaffolds composed of wet-spun polymeric fibers (Mota et al., 2013). Samples with a base size of (20×20) mm² and composed of 32 layers were fabricated by employing a 0–90° lay-down staggered pattern. The initial distance between the needle tip and the bottom of the beaker (z_0) was 4 mm. The deposition velocity ($V_{dep} = 500$ mm/min), theoretical distance between the axes of two consecutive fibers ($d_{xy} = 1$ mm), and interlayer needle translation ($d_z = 0.2$ mm) were optimized. Dog-bone shape samples for tensile mechanical test with customized base dimensions (75.0×12.5) mm², $d_{xy} = 1.1$ mm, $d_z = 0.1$ mm and 12 layers were also printed. After printing, the scaffolds were removed from the coagulation bath and ionically crosslinked in a ZnSO₄·7H₂O (2 % w/v) solution in dH₂O/EtOH (70/30 v/v) for 24 h. The samples were then immersed in dH₂O three times for 5 min each, frozen at -20 °C for 24 h, and then lyophilized for 48 h at -50 °C and 0.04 Torr.

2.4. Films preparation

CS and CMCS films were prepared by solvent casting using polymeric solutions with the same composition employed for the CAWS technique. Polymeric solutions (100 µL) were cast on square coverslips (3.2 cm²), left under a fume hood for 24 h, and stored in a desiccator. Films were then ionically crosslinked following the same procedure used for 3D-printed scaffolds.

2.5. Collagen (COL) grafting

COL was dissolved for 2 h at room temperature in an acetic acid 0.1 M aqueous solution. The scaffold was immersed in 600 µL of a 50 mM MES buffer at pH 6 with 60 mM EDC, 30 mM NHS, and 1 mg/mL COL for 3 h under orbital shaking at room temperature to graft the protein to CMCS carboxyl groups. The sample was washed three times with dH₂O to remove weakly bound COL and unreacted EDC/NHS residues, frozen at -20 °C for 24 h and lyophilized for 48 h.

2.6. Polymers molecular weight

Polymers viscosity average molecular weight (M_v) was calculated according to the Mark-Houwink-Sakurada (MHS) equation from intrinsic viscosity ($[\eta]$) by one-point method. The flux times were measured within an Ubbelohde capillary viscometer at 25 °C or 30 °C for CS and CMCS (2 mg/mL), respectively (Costa et al., 2015). The MHS constants employed in the case of CS were $K = 7.0 \times 10^{-2}$ mL/g and $\alpha = 0.81$, determined in a previous study (Brugnerotto, Desbrières, et al., 2001) through size exclusion chromatography (SEC) analysis of a CS with a 75 % degree of deacetylation (DD) dissolved in acetic acid 0.3 M/sodium acetate 0.2 M. The MHS constants for CMCS were $K = 7.92 \times 10^{-5}$ mL/g and $\alpha = 1.0$, according to a previous study reporting on M_v calculation of a polymer solution in NaCl 0.1 M with a deviation lower than 5 % (Mourya et al., 2010). The following equations were used to calculate $[\eta]$ of CS (eq. 1) and CMCS (eq. 2).

$$[\eta] = \frac{[2(\eta_{sp} - \ln \eta_r)]^{\frac{1}{2}}}{c} \quad (1)$$

$$[\eta] = \frac{4\eta_{sp}^{1.02} \times \ln \eta_r}{c^{1.01}(3\eta_{sp} + \ln \eta_r)} \quad (2)$$

where c (g/mL) is the concentration, η_r and η_{sp} are the relative and specific viscosity, respectively.

2.7. Solubility and isoelectric point of CMCS

CMCS solubility in dH₂O was estimated by analyzing mixture turbidity as a function of pH, following a previous method with modifications (de Abreu & Campana-Filho, 2009). The samples were prepared at 1.0 g/L concentration and the pH was adjusted between 1 and 14 by adding HCl or NaOH aqueous solutions. CMCS was considered insoluble at a given pH when the transmittance at 450 nm, measured by using a UV–Vis spectrometer (JASCO Co. UV-530, Italy), was lower than 80 %, and partially soluble when between 80 and 95 %.

The isoelectric point (pI) was determined based on a method described in the literature (Kong, 2012). A previously prepared aqueous solution (CMCS 10 g/L; NaCl 0.1 M) was divided into equal volumes and the pH of the obtained samples was adjusted with HCl to values in the range 2–6. The solid was separated by centrifugation and the pH of the supernatant was adjusted to 8 using NaOH. The pI corresponded to the initial pH of the sample with the lowest absorbance at 220 nm.

2.8. Polymers structural analysis

The ATR-FTIR spectra of the starting and processed polymers were recorded using a Spectrum 100 FTIR Spectrometer (Perkin Elmer, Waltham, MA) in the wavenumber range of 4000 – 650 cm⁻¹, at a resolution of 2 cm⁻¹ and 64 scans. The average degree of deacetylation (DD) was calculated based on an absorbance band ratio (A_{1320}/A_{1420}) previously reported and calibrated by NMR ($r = 0.990$), $DD = 1 - [(0.3192 \times A_{1320}/A_{1420}) - 0.122]$ (Brugnerotto, Lizardi, et al., 2001). The average degree of substitution (DS) was determined with an absorbance band ratio equation, $DS = A_{1410}/A_{1320}$, previously monitored by conductivity and pH measurements in agreement with the carboxylic content (de Abreu & Campana-Filho, 2009).

For micro ATR-FTIR analysis of COL, a Nicolet iN10 MX spectrometer (Thermo Scientific, Italy) was used. The scan line was recorded using an ATR 'slide-on' accessory equipped with a germanium crystal (0.66 µm IR penetration at 1000 cm⁻¹), a liquid nitrogen-cooled MCT-A detector and a (200 × 200) µm² aperture. The resolution was 4 cm⁻¹ between 4000 and 650 cm⁻¹ with 64 scans. A total of 12 spectra were acquired at a distance of 100 µm.

Polymers DD and DS were also determined by potentiometric titration and confirmed by ¹H NMR spectroscopy. Briefly, 0.5 g of CS or CMCS were dissolved in 60 mL of an aqueous solution of HCl (0.1 M) and titrated with a calibrated NaOH (0.1 M) solution (Xu et al., 2021). The pH was recorded with a calibrated pH-meter. The two parameters were calculated according to the following equations (eq. 3 and 4.):

$$DD = \frac{(M_x \times C_{NaOH} \times V_{NaOH})}{m_x} \quad (3)$$

$$DS = \frac{(M_{CS} \times C_{NaOH} \times V_{NaOH})}{(m - 80 \times C_{NaOH} \times V_{NaOH})} \quad (4)$$

where M (g/mol) is the average molar mass of basic units of CS or CMCS, C (mol/L) is the concentration, V (L) is the added volume, and m (g) the sample weight.

The polymers' structure was analyzed by ¹H NMR spectroscopy using a JEOL ECZR 500 MHz spectrometer (Jeol Ltd., Japan). CMCS samples were dissolved for 24 h in D₂O (10–20 mg/mL) and placed in a 5 mm diameter tube. CF₃COOD (1 % v/v) was added to CS water-insoluble sample. The parameters were set as follows: 500 MHz spectral window, 32 k data points, 45° pulse angle, acquisition time of 3.49 s, 16 scans with a 4 s delay, and solvent pre-saturation at 80 °C. Polymer DD was calculated according to eq. 5 (Sivashankari & Prabakaran, 2017). Moreover, the carboxylic substitution sites (f_6 , f_3 , f_2) were identified according to eqs. 6–8, and DS was calculated as $DS = f_2 + f_3 + f_6$ (Bukzem et al., 2016). The intensities as integrals (I_x) were normalized to those of the *N*-acetyl peaks used as references.

$$DD = 1 - \left(\frac{1/3 \times I_h}{I_g} \right) \quad (5)$$

$$f_2 = \frac{1/2 \times I_f}{(I_a + I_b)} \quad (6)$$

$$f_3 = \frac{I_c}{(I_a + I_b)} \quad (7)$$

$$f_6 = \frac{1/2 \times (I_d - I_c)}{(I_a + I_b)} \quad (8)$$

2.9. Morphological characterization

Dry samples top-view and cross-section were analyzed by means of scanning electron microscopy combined with energy-dispersive X-ray (SEM-EDS) using a LSM 5600LV JEOL instrument (Jeol, Japan) at different magnifications (100–4000×) and 5 kV of accelerating voltage. The samples were previously subjected to fracture in liquid nitrogen and platinum sputter coating. The average fiber diameter and pore size, defined as the distance between two adjacent fibers, were measured by means of ImageJ 1.54d software on top-view micrographs with at least 20 randomly selected measurements per scaffold.

2.10. Contact angle measurements

Printed scaffolds and solvent cast films wettability at room temperature were measured using an FTA 200 Camtel goniometer (First Ten Ångströms, Portsmouth, VA) through the sessile drop method. Briefly, 10 µL of dH₂O were freely dropped onto the sample surface, and the contact angle (θ) was recorded for up to 140 s after dropping. Four samples for each kind of scaffold or film were analyzed.

2.11. Water uptake (WU) measurements

Scaffolds water uptake (WU) was assessed by measuring the weight of the samples as a function of immersion time at 37 °C in 6 mL of PBS 0.01 M at pH 7.4 or 5.8. WU was determined according to eq. 9.

$$WU (\%) = \frac{(W_t - W_0)}{W_0} \times 100 \quad (9)$$

Where W_0 is the initial weight and W_t is the weight of the swollen sample at a given time. Three samples for each kind of scaffold were analyzed.

2.12. Thermal analysis

Thermal properties of the unprocessed polymers and scaffolds were investigated by means of differential scanning calorimetry (DSC) under a nitrogen flow of 50 mL/min, using a Discovery Instrument DSC250 (TA Instrument, Milan, Italy). The samples were accurately weighed (5 mg), sealed in a hermetic aluminum cup, and an empty cup was used as reference. Two heating cycles at a heating rate of 10 °C/min were performed: the first one from 20 to 150 °C and the second one from 20 to 400 °C. The heating cycles were separated by a cooling cycle (−10 °C/min) and two isothermal steps (1 min). The glass transition temperature (T_g) was determined by analyzing the inflection point in the second cycle. The onset temperature (T_{onset}) and the peak temperature (T_{peak}) were taken at the intersection of base tangents and maximum of exothermic peak, respectively, and the enthalpy of decomposition (ΔH) as the exothermic area. Three samples for each kind of material were analyzed.

2.13. Mechanical testing

The tensile mechanical properties of dog-bone shape scaffolds were

evaluated using an Instron 5564 instrument (Norwood, MA, USA) according to ASTM standard D882–18 (ASTM, 1, 2018). The test was carried out by applying a 4 mm/min rate of grip separation. The specimens were left in PBS 0.01 M at 37 °C for 4 h before testing and then fixed to pneumatic tensile clamps (1.8 atm) at room temperature and humidity. The elastic modulus (E), tensile strength (σ) and elongation at break (ε) were calculated from the tensile stress-strain curves by using the software recording data Merlin Series IX. Six samples for each kind of scaffold were analyzed.

2.14. Antimicrobial activity assessment

E. coli ATCC 8739 and *S. aureus* ATCC 6538 strains were used as target microorganisms. Bacterial strains were cultivated in Nutrient Broth (NB, Oxoid, Milan, Italy) for 16 h at 37 °C under aerobic conditions and then centrifuged at 7000 rpm for 10 min. Subsequently, the bacterial cells were washed in sterile saline (0.9 % w/v NaCl) and resuspended in the same solution to obtain a concentration of 10⁵ Colony Forming Unit (CFU)/mL. The halo inhibition zone test method was conducted according to a previously published protocol (Cheah et al., 2019) with some modifications. 15 mL of PCA medium (VWR, Milano, Italy) were poured in a Sterile Petri dish (60 mm). After solidification, 50 µL of bacterial suspension (10⁵ CFU/mL) were spread on the plates. The material was placed on the surface, incubated at 37 °C for 24 h and the antimicrobial activity was evaluated following the bacterial growth inhibition halo. Moreover, a cells-scaffold contact test was conducted by transferring the scaffolds (20 mm²) into 2 mL tubes containing 1.5 mL of bacterial cell suspension (10⁵ CFU/mL). The tubes were placed in a shaker (80 rpm) for 24 h at 23 ± 1 °C. At the end of the incubation period, each cell suspension was serially diluted (1:10) and each dilution was plated on PCA medium. After incubation at 37 °C for 24 h under aerobic conditions, the colonies corresponding to viable cells were counted and the results expressed as CFU/mL (Totaro et al., 2020). The decrease in the number of cells upon contact with the specimen was quantified using a modified version of the equation proposed in a previously published article (Lala et al., 2007):

$$R (\%) = \frac{(B - A)}{B} \times 100 \quad (10)$$

where $R\%$ is the cell mortality percentage, A the average number of viable bacterial cells after 24 h of exposure to the specimen, and B the average number of viable bacterial cells after 24 h of incubation without any specimen. Each analysis was performed in triplicate.

2.15. Biocompatibility assessment

The Balb/3T3 clone A31 mouse embryo fibroblast cell line (ATCC CCL-163) was selected for materials and scaffolds characterization. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Milan, Italy) supplemented with 4 mM L-glutamine, 1 % penicillin:streptomycin solution (10,000 U/mL:10 mg/mL), 10 % Calf serum, and antimycotic (37 °C, 5 % CO₂-enriched environment). The assays were performed in triplicate, and the average values were expressed as a percentage of the control.

2.15.1. Extract assay

The cytocompatibility of the polysaccharides was evaluated after sterilization under UV light for 20 min. CS and CMCS powders were placed in a centrifuge tube containing complete growth medium (1 % w/v) and incubated at 37 °C and 5 % CO₂ for 24 h to allow the formation of the extracts. In parallel, Balb/3T3 clone A31 cells were seeded in 96-well culture plates at a concentration of 1 × 10⁴ per well and incubated at 37 °C in a 5 % CO₂-enriched atmosphere and allowed to proliferate for 24 h. Cells were then incubated with diluted sample extracts (dilution ratios up to 1:16 in cell culture medium) for 24 h. Cells incubated with

the complete growth medium were used as a control. Finally, cells were incubated for 4 h at 37 °C and 5 % CO₂ with the WST-1 reagent at 1:10 dilution in cell culture medium in order to evaluate their viability. Formazan dye absorbance was measured by means of a microplate reader (Biorad, Milan, Italy) at 450 nm (655 nm reference wavelength).

2.15.2. Direct contact assay

Viability of cells cultured on CS/Zn, CMCS/Zn, and CMCS/Zn/COL scaffolds was evaluated up to 7 days. Samples were placed in a 96-well plate, sterilized with UV radiation for 20 min on each side, and then thoroughly washed with Dulbecco's phosphate buffer saline (DPBS), containing a 1 % penicillin/streptomycin solution. The samples were then incubated in cell culture medium for 24 h at 37 °C, 5 % CO₂. Subsequently, 1×10^3 cells were seeded onto each scaffold and, after 30 min of incubation at 37 °C and 5 % CO₂, complete medium volume was adjusted to 100 μ L, followed by incubation in a humidified atmosphere at 37 °C and 5 % CO₂. Cells cultured on tissue culture polystyrene (TC-PS) for 24 h were used as a control. At prefixed time points, cell-seeded samples were analyzed by means of the WST-1 assay, as described in the previous section, or Live/Dead assay. In the second case, cell/scaffold constructs were thoroughly rinsed three times with DPBS before being treated with Live/Dead stain (Thermo-Fisher Scientific, Waltham, MA) at a concentration of 2 μ M calcein-AM and 4 μ M ethidium homodimer-1 for 40 min at room temperature. The incidence of living cells (stained green) and non-viable cells (stained red) was evaluated using a Nikon Eclipse TE2000 inverted microscope with an EZ-C1 confocal laser (Nikon, Tokyo, Japan).

2.15.3. Wound healing assay

Balb/3T3 clone A31 cells were seeded onto a 24-well plate at a concentration of 1.5×10^5 and incubated for 24 h at 37 °C and 5 % CO₂. Once the cells reached confluence, a 10 μ L pipette tip was used to create a linear scratch on the cell monolayer. The wells were rinsed with DPBS to remove any non-adherent cells. Subsequently, cells were incubated with scaffold extracts, obtained by incubating one scaffold in 6 mL of complete growth medium for 24 h at 37 °C (2.5 mg/mL). Cells treated with growth medium were used as a control. The scratches were imaged at 0, 12, 24, and 48 h after wound creation using a Nikon Eclipse TE2000 inverted microscope (Nikon, Tokyo, Japan), and the wound closure was analyzed by means of ImageJ 1.54d software (National Institutes of Health, New York, NY). The wound closure percentage was calculated using the following equation:

$$\text{Wound closure (\%)} = \frac{(WA_{t0} - WA_{tX})}{WA_{t0}} * 100 \quad (11)$$

where WA_{t0} is the initial wound area and WA_{tX} is the wound area at a given time. Three samples for each kind of scaffold were analyzed.

2.16. Statistical analysis

The experimental data are presented as mean $\pm$ standard deviation. Statistical differences were analyzed using one-way analysis of variance (ANOVA), and Tukey's test was used for *post hoc* analysis. Statistical significance was set at $p < 0.05$.

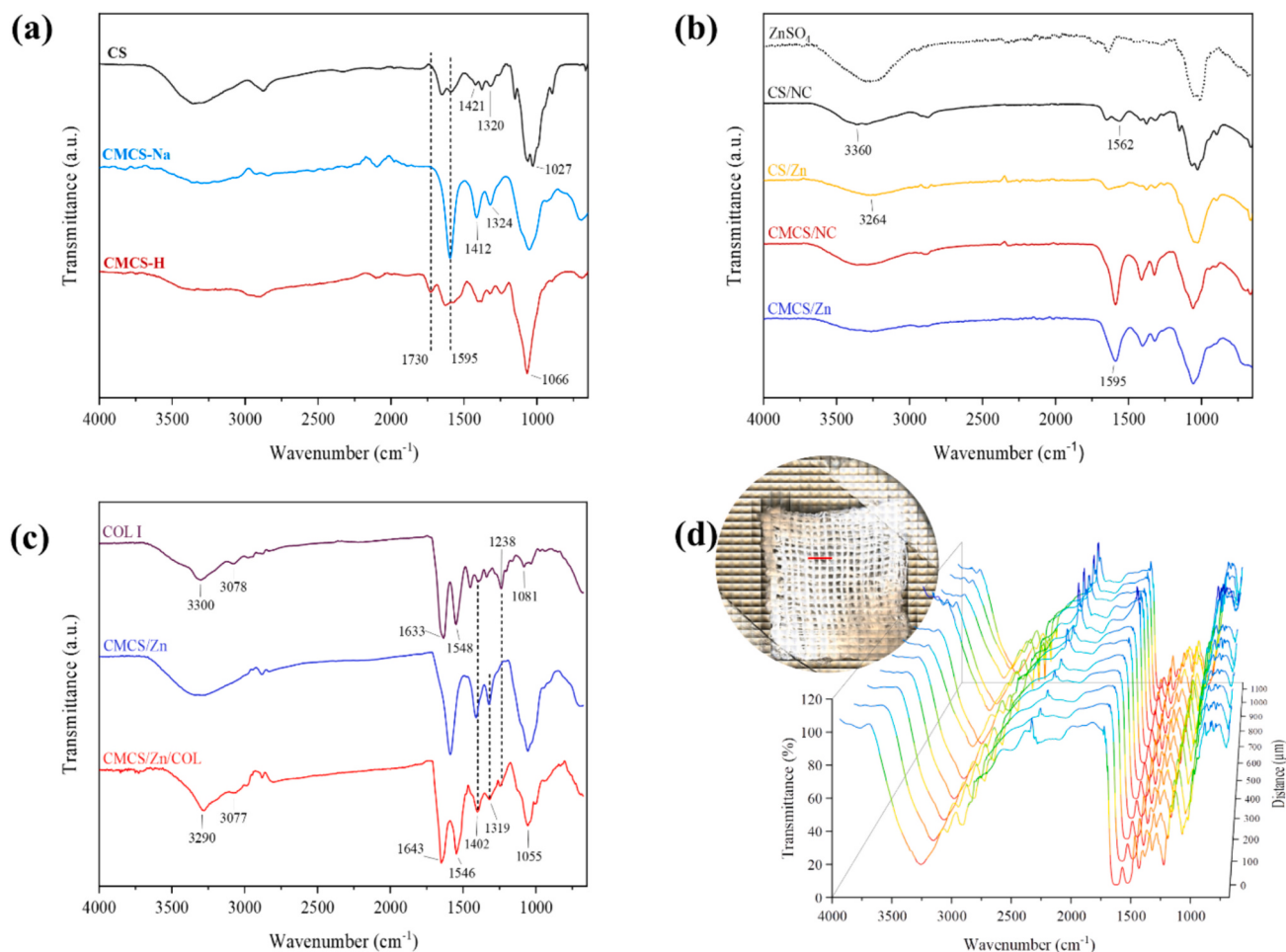


Fig. 1. FTIR-ATR Spectra of a) CS, CMCS-Na and CMCS-H polymers; b) scaffolds before crosslinking (non-crosslinked: CS/NC and CMCS/NC) and after ZnSO₄ crosslinking (CS/Zn and CMCS/Zn); c) collagen (COL) and collagen-grafted CMCS scaffolds (CMCS/Zn/COL); d) micro-ATR analysis of CMCS/Zn/COL scaffolds.

3. Results and discussion

3.1. Characterization of CS and CMCS

3.1.1. Polymers structural analysis

Polysaccharide carboxymethylation was explored by analyzing the FTIR spectra of CS and CMCS (Fig. 1a) through a comparison of their characteristic bands (Table S1) (Kong, 2012; Medeiros Borsagli et al., 2015).

In contrast to the spectrum of the starting CS, two new intense absorption bands characteristic of CMCS-Na, *i.e.*, the antisymmetric ($1600\text{--}1580\text{ cm}^{-1}$) and symmetric ($1415\text{--}1410\text{ cm}^{-1}$) stretching of the COO^- group, were observed. The vibration of amides I and II in sodium salt overlapped with the asymmetric stretching of COO^- . COONa conversion to COOH caused a shifting of the relevant absorption band to 1730 cm^{-1} and the NH_2 group changed to NH_3^+ with a resulting broad band ($1500\text{--}1640\text{ cm}^{-1}$) overlapping with the COOH band. This is consistent with the acidic form of O-CMCS characterized by limited substitution at amino groups (Upadhyaya et al., 2014). The method based on FTIR spectroscopy analysis provided a deacetylation degree (DD) for CS of 0.81. In the case of CMCS, the selected internal reference absorption band (A_{1420}) and the COO^- symmetrical stretching band in CMCS-H were not resolved. The effect of CH_3 on the COO^- band was not expected to be significant for DS analysis by FTIR, since a DS >1 was obtained (Table 1) (Kong, 2012).

Effective carboxymethylation was confirmed by comparing ^1H NMR spectra of the two polymers (Fig. 2). CS and CMCS resonances (Table S2) were those previously reported in the literature (Bukzem et al., 2016). The strong resonances between 4.47 and 4.05 ppm (d) proved that the substitution reaction mostly occurred on hydroxyl groups. The amino groups were slightly carboxymethylated as suggested by the signal centered at 3.2 ppm (f). Despite the overlap of signals (f) and (g), the proposed method allowed DS determination, with $f_6 = 0.65$, $f_3 = 0.42$, and $f_2 = 0.17$ as relative DS at the possible positions OH-6, OH-3, and NH_2 , respectively. The order of reactivity $\text{OH-6} > \text{OH-3} > \text{NH}_2$ was that expected for the employed reaction conditions.

Overall, high DS values (≥ 1) were obtained by means of the three used analytical techniques, *i.e.*, ATR-FTIR, pH measurements, and ^1H NMR, the latter employed as an absolute method (Table 1). In addition, the CS derivative had a high degree of O-substitution and the carboxymethylation ratio between O- and N- positions was 44:7. So far, no criteria have been established to consider a derivative as O-CMCS or N-CMCS based on the relative substitution fraction. However, other authors have proposed considering O-CMCS a derivative with 15–20 % of N-substitution if DS >1 (Adnan et al., 2020; Poon et al., 2007).

Excess NaOH could cause deacetylation of the polysaccharide, with an obvious low effect in the case of a high DD. However, in the case of a NaOH 50 % wt solution and an initial DD of 75 %, the DD could increase by about 10 % (Chen et al., 2003). In addition, under strong alkali conditions, other substantial degradation of the macromolecule during carboxymethylation was reported (Bochek et al., 2022). In our study, the DD did not increase significantly and the viscosity average molecular weight (M_v) of CMCS was higher than that of CS (Table 1), indicating that substitution predominated over depolymerization. This was probably due to a shorter alkalization time and a lower reaction temperature than those typically reported in the literature. The relatively low intrinsic viscosity ($[\eta]$) of CMCS in comparison to that of CS was

considered during the optimization of the scaffold fabrication process. Indeed, solution viscosity is among the most influential parameters affecting polymer coagulation and deposition during CAWS (Puppi & Chiellini, 2020). In summary, the carboxymethylation product described in this study was a complex derivative with residual acetylated units ($\sim 70\%$ of free NH_2 groups) and at least one average COONa group per monomeric unit.

3.1.2. CMCS solubility

CMCS solubility is a key aspect to be considered for optimizing the AM approach investigated in this study involving the extrusion of a polymer aqueous solution into a non-solvent bath. O-CMCS is a poly-ampholyte soluble in water in a wide range of concentrations, depending on its DD, DS, molecular weight, and pH. According to the test carried out, based on turbidimetric analysis and isoelectric point (pI) determination, the developed CS derivative was soluble at basic pH values and under acidic conditions down to pH 4 at which it appeared partially soluble, and non-soluble at pH 3, this latter value identified as the pI (Fig. S1). The solubility is the result of the prevalence of positive or negative charges at a given pH. Between pH 3–4 the COO^- concentration is enough to compensate the NH_3^+ charges (Medeiros Borsagli et al., 2015). A similar derivative with a DS = 1.26 but a higher DD (90 %) showed a non-solubility range near neutral pH (Poon et al., 2007), demonstrating that the COOH/NH_2 ratio plays a key role on this aspect. The narrow insolubility range at a low pI observed in the current study suggests that the obtained derivative had a higher COONa/NH_2 ratio.

3.2. Scaffolds fabrication and post-printing treatment

CS and CMCS scaffolds were fabricated using CAWS technique (Fig. 3a). In particular, the developed AM protocol involved extruding into an EtOH bath an aqueous polysaccharide solution at a given concentration and the layer-by-layer deposition of the resulting coagulating filament by following a predefined $0\text{--}90^\circ$ lay-down pattern. Tuning design parameters, such as inter-layer needle translation (d_z) and inter-fiber axis distance (d_{xy}), and processing variables, such as solution extrusion rate (F) and deposition velocity (V_{dep}), allowed the optimization of the control and reproducibility of the resulting hydrogel shape and porosity (see Section 2.3) (Fig. 3b and c). After fabrication, the scaffolds were ionically crosslinked by means of divalent cation-polysaccharide complexation through Zn^{2+} interactions with COO^- or NH_2 in a $\text{dH}_2\text{O}/\text{EtOH}$ solution (Fig. 3d and e). Non-crosslinked (NC) scaffolds and films were referred to as CS/NC and CMCS/NC. On the other hand, crosslinked scaffolds and films were labeled as CS/Zn or CMCS/Zn.

The coagulation and crosslinking process caused a macroscopic shrinkage of the scaffold with resulting base dimensions smaller than the theoretical ones ($20 \times 20\text{ mm}^2$). However, the optimized experimental protocol allowed the fabrication of scaffolds with reproducible shape and dimensions of around $(13 \times 13 \times 2)\text{ mm}^3$ and $(16 \times 16 \times 2)\text{ mm}^3$, in the case of CS/Zn and CMCS/Zn, respectively (Fig. 3d).

Collagen I (COL) grafting procedure optimized for CMCS scaffolds and films involved the reaction between the carboxyl groups of CMCS and the amino groups of COL with the formation of amide bonds using EDC/NHS as the catalyst (Fig. 3f). After functionalization, the CMCS scaffolds did not show macroscopic changes in terms of dimensions and color. COL-functionalized scaffolds and films were referred to as CMCS/

Table 1

Results of deacetylation degree (DD), substitution degree (DS), intrinsic viscosity ($[\eta]$) and viscosity average molecular weight (M_v) calculations for CS and CMCS.

Sample	$[\eta]$ (mL/g)	M_v (Da)	DD			DS		
			IR	Titration	^1H NMR	IR	Titration	^1H NMR
CS	537	6.26×10^4	0.81	0.75	0.757	–	–	–
CMCS	333	4.21×10^6	–	0.74	0.764	1.18	1.10	1.24

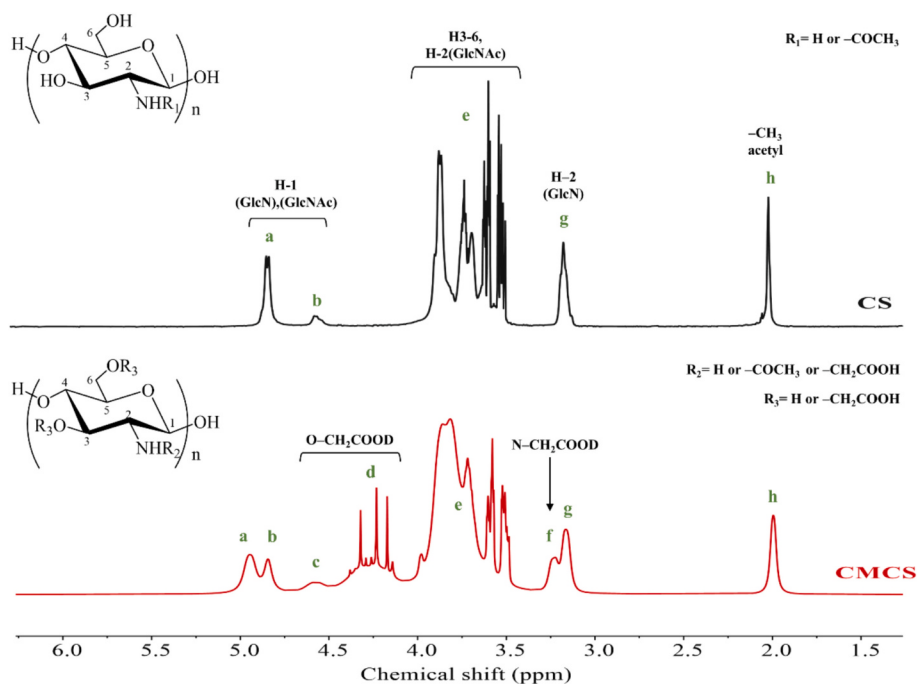


Fig. 2. ^1H NMR spectra of CS and CMCS at 80 °C in CF_3COOD or D_2O , respectively.

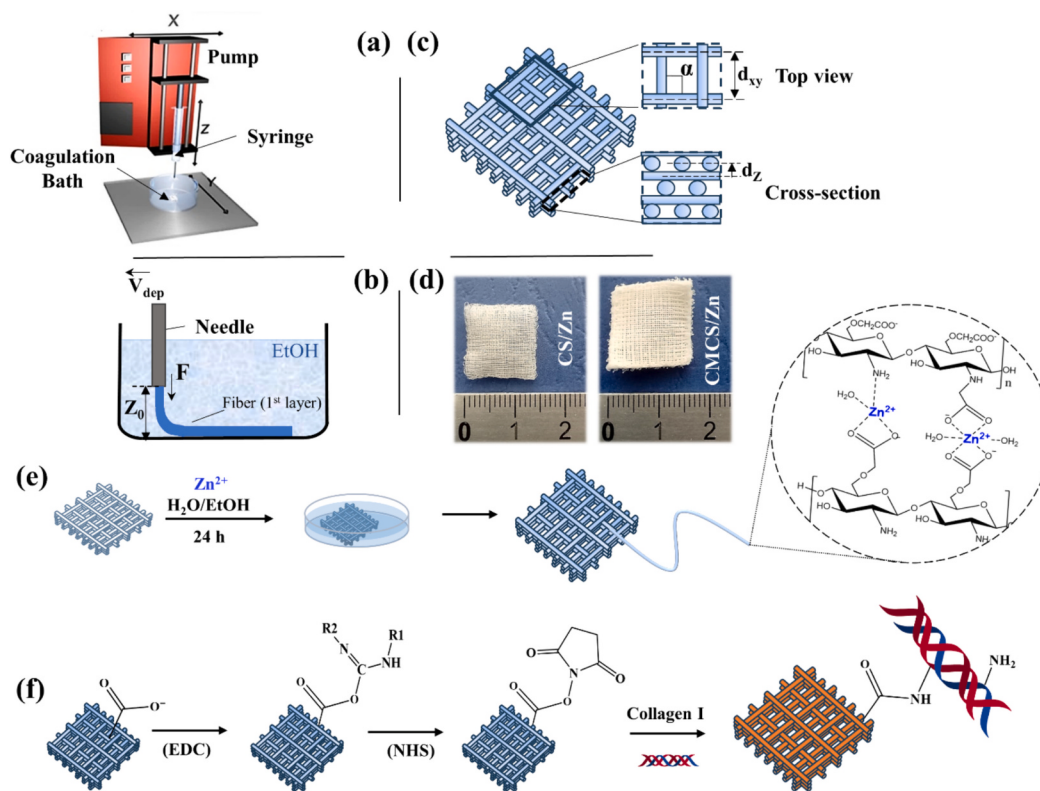


Fig. 3. Schematic representation of a) CAWS equipment, b) fiber deposition into the coagulation bath, c) structural design and dimensional parameters. d) Representative pictures of crosslinked CS and CMCS scaffolds. Schematic representation of e) CMCS scaffold ionic crosslinking procedure, f) CMCS scaffold functionalization by collagen grafting procedure. (V_{dep} , deposition velocity; F , solution feed rate; z_0 , initial distance between needle tip and deposition plane; d_{xy} , distance between deposition lines; d_z , interlayer needle translation along Z-axis; α , lay-down pattern angle; $R_1 = -\text{CH}_2\text{CH}_3$; $R_2 = -(\text{CH}_2)_3-\text{NH}^+(\text{CH}_3)_2\text{Cl}^-$).

Zn/COL.

Extruding a CMCS/water solution into an ethanol bath is here described for the first time to fabricate 3D scaffolds by AM. Indeed, previous studies used CMCS blended with other natural polymers in

order to achieve a uniform filament deposition and apply an effective crosslinking method, *i.e.*, photo-crosslinking of a gelatin derivative (Wang et al., 2023) or ionotropic gelation of alginate (Mohabatpour et al., 2022). Coagulating in a non-solvent bath allowed a fast CMCS

solidification as a key requirement to obtain a scaffold with a well-defined 3D layered structure, characterized by a highly interconnected macroporosity. In addition, Zn^{2+} crosslinking of a 3D printed CMCS scaffold has never been demonstrated before. Indeed, this ionic bonding approach was previously employed only to stabilize CMCS-based vacuum-dried films (Yan et al., 2023) or single fibers (Y.-L. Wang et al., 2020). A recent article achieved effective Ca-crosslinking to stabilize CMCS scaffolds in aqueous media, but only upon ethylenediaminetetraacetic acid (EDTA) modification to increase the polysaccharide degree of carboxylation (He et al., 2020). Indeed, Ca^{2+} solution treatment was not effective to stabilize the CMCS hydrogels developed in this study, resulting in their fast water erosion. On the other side, the achieved degree of functionalization was adequate to exploit Zn-crosslinking and obtained hydrogels stable in aqueous medium for weeks (see Section 3.3.3). The successfully applied COL grafting method is another noteworthy novelty aspect of this research since it has been previously demonstrated only in the case of freeze-dried CMCS hydrogels (Cheng et al., 2019; Cheng et al., 2020).

3.3. Scaffolds characterization

3.3.1. FTIR spectroscopy characterization

Non-crosslinked CS and CMCS scaffolds (CS/NC; CMCS/NC) showed the characteristic bands observed in the case of starting polymers (Fig. 1b). However, in comparison to raw polymers, the amide II band of CS scaffold was shifted to a lower wavenumber (1562 cm^{-1}) indicating a partial protonation of amino groups ($-\text{NH}_3^+$) (He et al., 2011), whereas this effect was negligible for $-\text{COO}^-$ in CMCS since these groups remained ionized after coagulation in EtOH. After crosslinking, the O—H and N—H symmetric stretching bands of CS shifted to lower frequencies. The C—O symmetric stretching merged into a broad band, indicating that —OH electron pairs interacted with Zn^{2+} . The intensity of amide I and II decreased due to the coordination bonds between $-\text{NH}_2/\text{Zn}^{2+}$ and the ionic interaction between $-\text{NH}_3^+/\text{SO}_4^{2-}$ (Mutlu et al., 2022). CMCS scaffolds crosslinking caused a widening and a slight decrease in intensity of the band at 1595 cm^{-1} , showing that crosslinking was mainly the result of the coordination bonds between $-\text{COO}^-/\text{Zn}^{2+}$. The S—O antisymmetric stretching band of sulfate ions at 1020 cm^{-1} overlapped with the symmetric stretching band of C—O (Rahmani et al., 2018).

The characteristic spectrum signals of COL, such as the symmetric stretching of N—H amide A (3300 cm^{-1}) and B (3078 cm^{-1}), amide I and II ($1633\text{--}1548\text{ cm}^{-1}$), and amide III ($1280\text{--}1200\text{ cm}^{-1}$), are highlighted in Fig. 1c (Y. Lin et al., 2023). In the spectrum of CMCS/Zn/COL scaffolds, the N—H stretching and the typical amide I/II bands of COL partially overlapped with the CMCS bands of O—H/N—H symmetric stretching and $-\text{COO}^-$ antisymmetric stretching, clearly demonstrating the successful functionalization of the scaffold. The symmetric stretching band of $-\text{COO}^-$ at 1402 cm^{-1} weakened due to a decrease of carboxylate groups in the macromolecules as a consequence of their reaction during the crosslinking. Two amide III bands ($1319\text{--}1238\text{ cm}^{-1}$) from the CMCS and COL structures as well as from the newly formed C—N bonds, were evident (Y. Lin et al., 2023). The band with a maximum at 1055 cm^{-1} due to C—O and C—O—C bonds in the CMCS was also observed in functionalized scaffolds.

Micro-ATR analysis of single fibers composing a functionalized scaffold allowed the obtainment of $1100\text{ }\mu\text{m}$ length scan lines to verify the linear distribution of COL grafting in the polymeric matrix by monitoring protein amide I and II bands. The 12 spectra (one every $100\text{ }\mu\text{m}$) obtained in the case of one of the three scan-lines performed are shown in the 3D graph reported in Fig. 1d. The two amide group bands were detected in all the 12 spectra in the case of two scan-lines (100 % functionalization degree) while in the other case they were detected in 7 out of 12 spectra (58 % functionalization degree).

3.3.2. SEM and EDS characterization

Top-view and transversal cross-section micrographs analysis corroborated the macroscopic observation of the CS and CMCS fibrous structure, showing a good degree of fiber alignment for both kinds of scaffold, as well as a defined layered architecture characterized by an interconnected network of macropores (Fig. 4a). This is a remarkable result considering that in the case of polysaccharide hydrogels, fibers distortion and misalignment, as well as a limited porosity along the scaffold cross-section are often observed by SEM analysis, due to different factors, such as a limited control over filament deposition and the effect of the drying and sputtering procedure for sample preparation (Puppi & Chiellini, 2020). Both systems presented fusion at fiber-fiber contact points between different layers. In the case of CMCS scaffolds this phenomenon was evident also between non-adjacent layers due to fibers bending. Polymer chains interdiffusion and entanglement at the fiber-fiber contact points resulting in effective layers welding, are affected by polymer coagulation kinetics and local polymer redissolution due to solvent diffusion from a newly deposited filament. Optimal fibers welding at the contact points results in a cohesive 3D structure with suitable mechanical properties (Puppi et al., 2023). The average fiber diameter was smaller than $60\text{ }\mu\text{m}$, with CMCS scaffolds having a larger mean value but a narrower distribution (Fig. 4b). In addition, the average X-Y pores size of CS and CMCS scaffolds in the dry state were in the range $600\text{--}800\text{ }\mu\text{m}$ and significantly different, as consequence of different fiber shrinkage during sample coagulation and crosslinking.

High-magnification micrographs analysis revealed marked differences in fiber morphology between CS and CMCS scaffolds. While single CS fibers had a dense cross-sectional morphology, CMCS scaffolds presented a hollow fiber morphology characterized by a dense wall with a rough outer surface/smooth inner surface and a longitudinal channel. Hollow fibers seem to be interconnected at contact points forming a microchannel network mimicking to some extent a blood capillary structure.

A smaller difference in the solubility parameter (δ) between the polymer and the non-solvent typically leads to a slower coagulation rate and thus to a less porous and more uniform structure (Peng et al., 2012; Rissanen et al., 2008). CMCS ($\delta = 79.2\text{ MPa}^{1/2}$) was likely characterized by a higher coagulation rate than CS ($\delta = 39.9\text{ MPa}^{1/2}$) due to a larger difference with the δ value of EtOH ($\delta = 26.5\text{ MPa}^{1/2}$) (Pillai et al., 2009). As a consequence, EtOH mixing could have favored instantaneous CMCS/ H_2O demixing and fiber surface solidification with a resulting dense external skin. This outer surface layer could have entrapped the solvent within the filament, resulting in a hollow structure (Lavin et al., 2012; Rissanen et al., 2008). Similar fiber structures due to particular solidification and phase inversion rate conditions have been observed in the case of polysulfone fibers (Kim et al., 2016; Puppi et al., 2023). However, to the best of author's knowledge, the self-formation of hollow CMCS fibers without the use of coaxial needles has not been previously described. Indeed, hollow polymeric fibers, employed for instance in blood purification, are typically obtained by flowing a polymeric solution (dope solution) through an external nozzle, and a non-solvent or air/nitrogen (bore solution) through an inner nozzle (Rohani Shirvan et al., 2022). The grafted COL was evident on CMCS fibers, forming fibrillar structures and partially filling the pores of CMCS/Zn/COL scaffolds. In addition, the COL functionalization process affected the scaffold macroporous structure leading to a decreased porosity along the Z-axis as result of partial fusion between different layers.

The obtained X-Y pores size ($> 100\text{ }\mu\text{m}$), interconnected macroporosity, and vascular-like fiber morphology of CMCS/Zn and CMCS/Zn/COL scaffolds are suitable to support the required diffusion of oxygen, cell nutrients, and other biomolecules, as well as for effective cell seeding, distribution, migration, and subsequent tissue neo-vascularization, all critical aspects in skin tissue engineering (Neves et al., 2016). In addition, the remarkable rougher surface of CMCS fibers

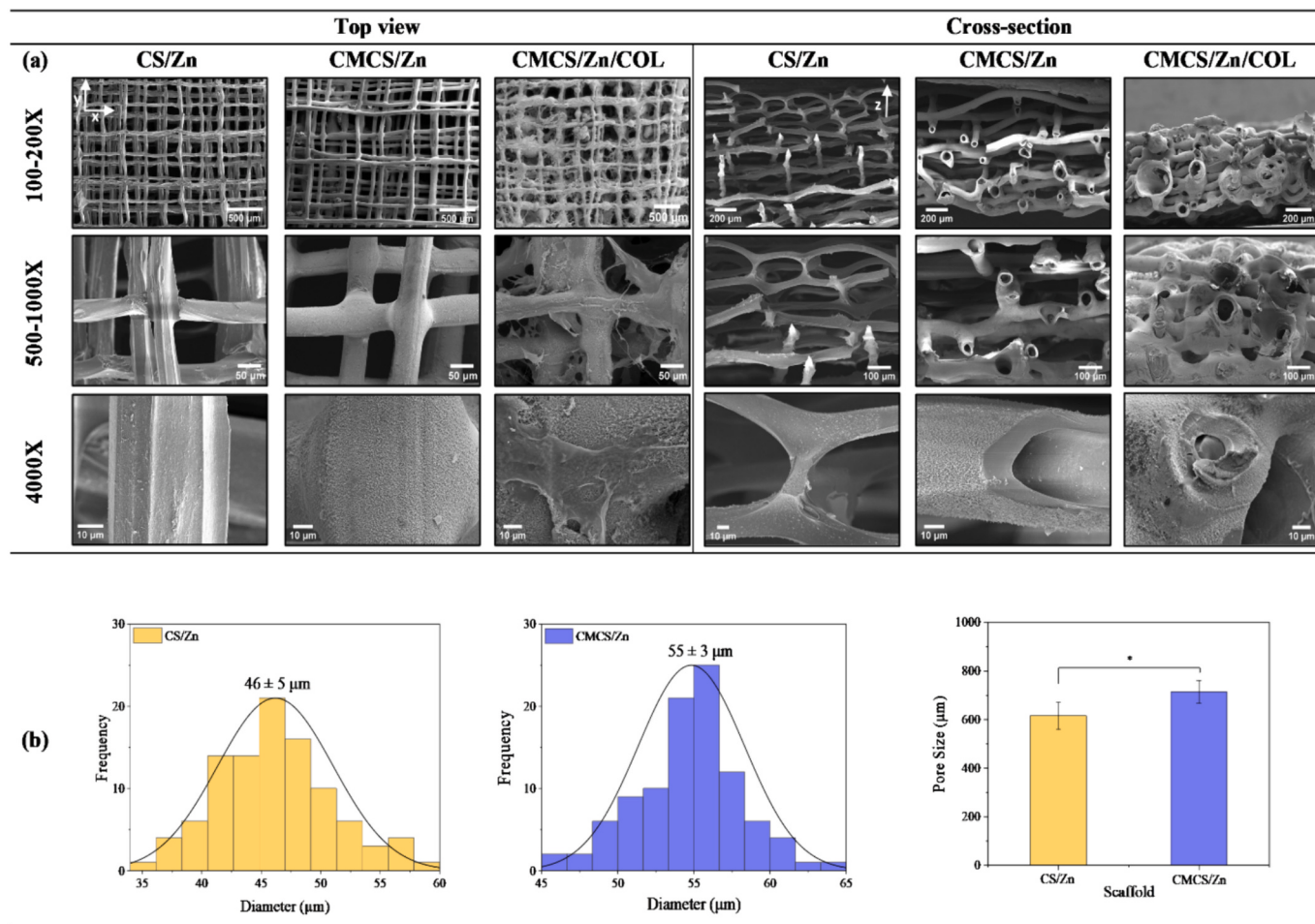


Fig. 4. a) SEM micrographs taken at different magnifications of scaffolds top-view and cross-section; b) CS/Zn and CMCS/Zn scaffolds fiber diameter distribution and average value $\pm$ standard deviation ($n = 100$), as well as mean pore size (error bars representing standard deviation, $n = 100$).

in comparison to that of CS fibers, can provide a larger specific surface area for cell adhesion and scaffold colonization (Cai et al., 2020). Particularly referring to wound dressings designed for tissue healing, a hydrophilic macroporous structure is also required to maintain the wound hydrated and protect it from infection, as well as to absorb exudates and blood, which can cause separation in the wound tissue layers (Dabiri et al., 2014).

The presence of Zn in the developed hydrogels was demonstrated through elemental composition assessed by EDS (Fig. 5). The peaks at around 1.01 and 8.6 keV confirmed that Zn^{2+} was successfully coordinated to functional groups of CS or CMCS (Mutlu et al., 2022). The relative Zn/O ratio was 0.31, 0.53, and 0.40 wt. for CS/Zn, CMCS/Zn and CMCS/Zn/COL, respectively (Fig. S2). As expected, CMCS-based scaffolds had a higher Zn concentration than CS scaffold due to a higher concentration of chelating groups. The reduced Zn/O ratio after COL functionalization can be due to partial Zn ions loss during treatment in MES buffer.

3.3.3. Wettability and water uptake (WU) analysis

Water contact angle (θ) was measured 30 s after dropping on CS- and CMCS-based films and scaffolds (Fig. 6a). As expected, the uncrosslinked CMCS films had a significantly lower θ ($31 \pm 3^\circ$) than CS films ($81 \pm 3^\circ$) due to the higher hydrophilic nature of the COOH groups. Crosslinking did not alter the CS films wettability, while CMCS/Zn films had a lower wettability than CMCS ones, probably due to a lower availability of free $-\text{COO}^-$ and $-\text{NH}_2$ groups, as a consequence of their coordination with Zn^{2+} . A similar result was previously described

for alginate/CMCS films crosslinked with Ca^{2+} and Cu^{2+} (Yan et al., 2023). Film functionalization with COL resulted in a θ of $44 \pm 3^\circ$, between those of CMCS and CMCS/Zn films. Even though in this kind of reaction part of $-\text{COO}^-$ groups are involved in grafting, immobilization of a protein with hydrophilic functional groups can lead to an increase in material wettability, as well as to increased adsorption of cell adhesion proteins (Jiang et al., 2017; Klimek & Ginalska, 2020). While θ was significantly increased in the case of CS scaffold ($117 \pm 4^\circ$) due to the inability of the droplet to penetrate through the porous substrate because of the air trapped beneath it (Drelich et al., 2020), in the case of CMCS/Zn and CMCS/Zn/COL scaffolds the high polymers hydrophilicity allowed the droplet to penetrate the pores instantly ($\theta \approx 0$).

Water uptake (WU) experiments were carried out in PBS at 37°C and two different pH, i.e., 7.4 and 5.8, to simulate the average pH of different physiological environments (e.g., blood or injured skin, and healthy skin, respectively) (Fig. 6b). The test demonstrated that in general CMCS scaffolds had a higher WU than CS scaffolds during the whole experiment. This result can be explained by the different hydrophilicity of the two polymers, as demonstrated by means of θ measurements. The pH-dependent balance between ionic attraction/repulsion and hydrogen bonding interactions is responsible for the expansion/contraction and WU of the polymeric structure. Indeed, CMCS based scaffolds were characterized by lower WU values at pH 5.8 than at pH 7.4. At pH 5.8, above the pI of CMCS and below the $\text{pK}_a = 6.4\text{--}7.6$ of its NH_3^+ groups (Wang et al., 2008) there was more probability of $\text{NH}_3^+/\text{COO}^-$ ionic attraction and hydrogen bonding between the relevant non-ionized pairs. These interactions increased the crosslinking density, leading to

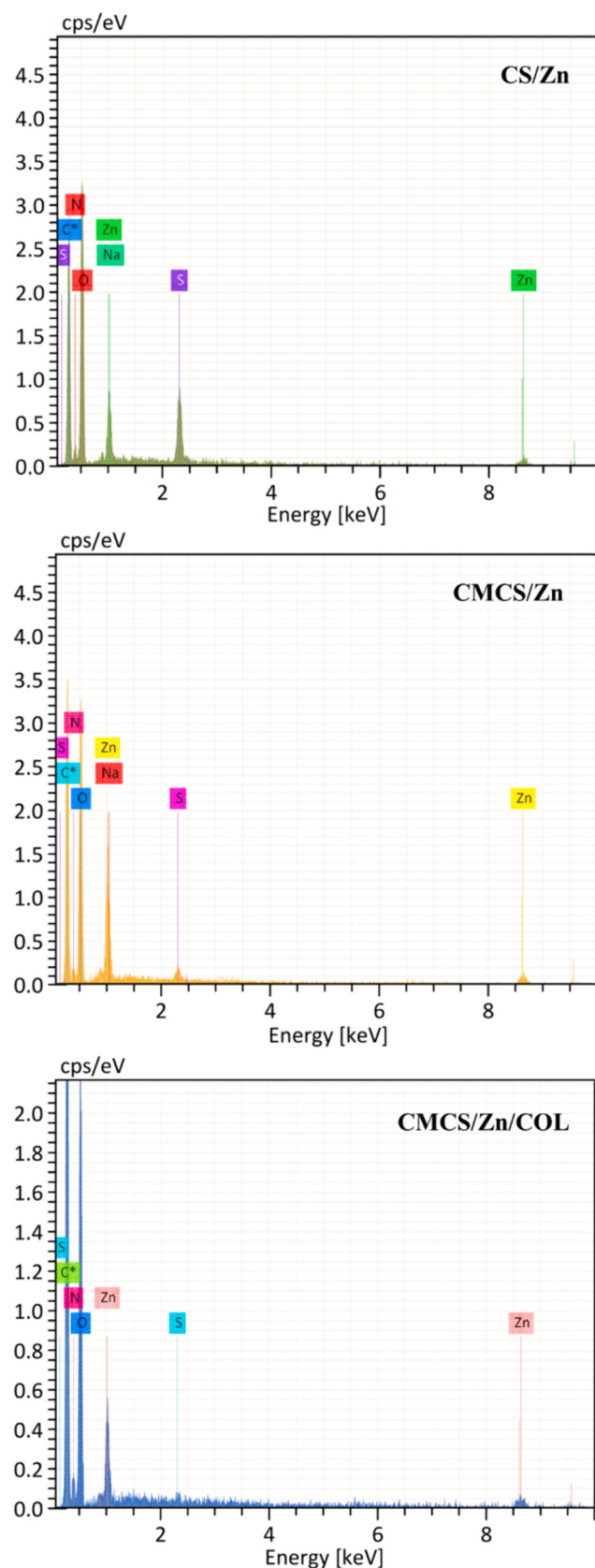


Fig. 5. EDS spectra of the developed CS/Zn, CMCS/Zn, and CMCS/Zn/COL hydrogels.

a lower absorption. Furthermore, the slope of CMCS scaffolds curve at pH 7.4 increased rapidly after 21 days. At this pH, anionic repulsion (COO^-) favored diffusion within the scaffolds of water and phosphate ions, competing with carboxylate groups for Zn^{2+} complexation. Furthermore, insoluble phosphate compounds could be formed ($\text{Zn}_3(\text{PO}_4)_2$, $K_{sp} = 9.0 \times 10^{-33}$) (Mutlu et al., 2022). A continuous release of Zn^{2+} decreased the crosslinking density progressively, leading to a lower stability at longer times (Mishra & Mishra, 2020; Mutlu et al., 2022). WU increase with time was smaller in the case of CS scaffolds at pH 5.8 since under these conditions $\text{NH}_3^+/\text{SO}_4^{2-}$ interaction was less compromised.

While the pH of healthy skin is typically between 4.1 and 5.8 (Proksch, 2018), chronic wounds and other skin diseases exhibit a pH increase to values also higher than 8.5. As tissue healing progresses and exudate levels decrease, the pH also decreases (Jones et al., 2014). The sensitivity of CMCS/Zn hydrogels to pH changes can be exploited to maximize the volume of absorbed biological fluids in case of skin defects, thus favoring the wound healing process. This behavior is also attractive for other biomedical applications based on physiological pH changes, e.g., in the case of biosensors or stimuli-controlled drug release (Kalliola et al., 2017).

3.3.4. Thermal properties

During the second DSC heating cycle (Fig. 7a) all samples showed a main exothermic event at $T > 200^\circ\text{C}$ that was associated with polymer decomposition.

As described in the literature, the temperature and area of this peak is influenced by CS/CMCS degree of polymerization (dp), DD, and DS. The decomposition enthalpy (ΔH) and onset temperature ($T_{\text{onset}} \sim 290^\circ\text{C}$) recorded for CS in this study (Table 2) were consistent with what previously reported for low molecular weight CS and DD of 70–90 % (Kittur et al., 2002). CMCS ΔH was higher than that of CS as a consequence of a high DS value. In addition, the T_{onset} and peak temperature (T_{peak}) of CMCS shifted to lower values in comparison to those of CS, due to a stability decrease associated with an increase of the polymer amorphous fraction during the carboxymethylation reaction (Kittur et al., 2002; Sheikholeslami et al., 2017). The influence of COOH groups on macromolecule thermal degradation mechanism, in terms of main chain scission and side groups cleavage, should also be considered. However, the main CMCS decomposition peak overlapped with a lower intensity peak close to that of decomposition of the starting CS, similarly to what described in previous studies (Kittur et al., 2002). Another broad band of low intensity ($\sim 374^\circ\text{C}$) suggested the possibility of a second process of decomposition of higher molecular weight macromolecules, which may be produced through the formation of amide bonds during the first step of decomposition (Sheikholeslami et al., 2017). No glass transition temperature (T_g) was observed for CS or its carboxymethylated derivative, although the amorphous phase fraction of the latter should have been higher (Peniche et al., 2019).

After cross-linking (CS/Zn), T_{onset} , T_{peak} and ΔH decreased significantly in the case of CS. The decomposition temperatures shift was related to a decrease in stability due to a lower crystalline fraction, while the ΔH value decrease was attributed to the lower amount of free amino groups after ionic interaction with SO_4^{2-} , as suggested by a previous study on TPP-crosslinked CS (Bhumkar & Pokharkar, 2006). In the case of CMCS/Zn scaffolds, ΔH decreased likely because of the same reason (i.e., free NH_2 and COOH groups decrease), while the T_{peak} increased as presumably the result of a higher macromolecular stability due to the coordination between $\text{COO}^-/\text{Zn}^{2+}$. The observation of a glass transition with a T_g of around 150°C for both crosslinked scaffolds corroborated the hypothesis of an increase of amorphous fraction upon crosslinking.

No additional thermal events associated with COL were identified after functionalization (CMCS/Zn/COL), probably due to its low concentration in the sample. It has been reported that COL undergoes glass transition and thermal denaturation in a wide range of temperatures ($80\text{--}220^\circ\text{C}$), in relationship to various aspects, such as its source,

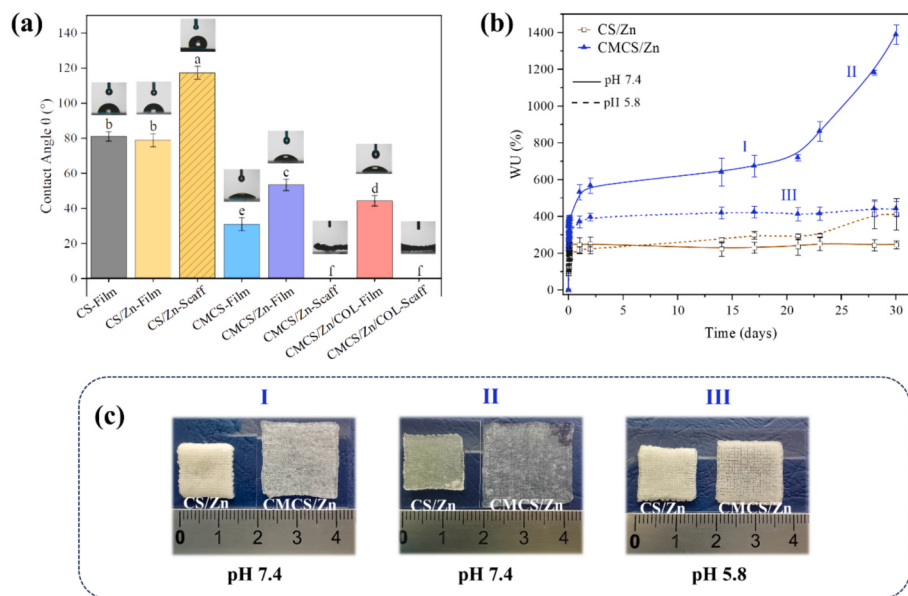


Fig. 6. a) Water contact angle (θ) of non-crosslinked films (CS-Film and CMCS-Film), crosslinked films (CS/Zn-Film, CMCS/Zn-Film and CMCS/Zn/COL-Film) and scaffolds (CS/Zn-Scaff, CMCS/Zn-Scaff and CMCS/Zn/COL-Scaff) (data reported as mean, error bars representing standard deviation, $n = 3$, ^a, ^b, ^c, ^d, ^e, ^f The values that do not share the same letter are significantly different); b) Water uptake (WU) in PBS at pH 5.8 and 7.4 of crosslinked scaffolds (CS/Zn and CMCS/Zn) (data reported as mean, error bars representing standard deviation, $n = 3$); c) representative pictures of CS/Zn and CMCS/Zn scaffolds at 16 days (I) and after 20 days of swelling at pH 7.4 (II) or after 20 days at pH 5.8 (III).

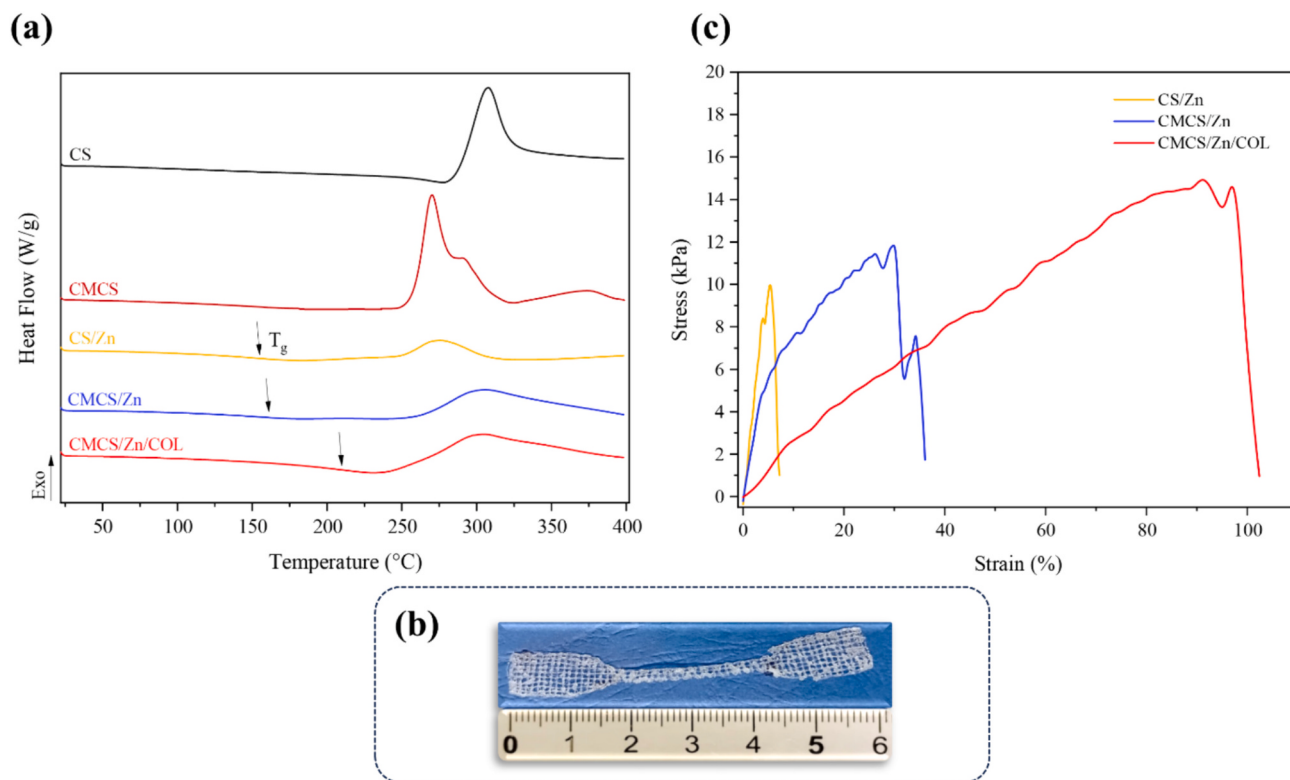


Fig. 7. a) DSC analysis of raw materials (CS and CMCS) and scaffolds (CS/Zn, CMCS/Zn, and CMCS/Zn/COL) (second heating scan, arrows indicating glass transition); b) representative picture of dog bone shape CMCS/Zn specimen; c) stress-strain curves of swollen scaffolds (CS/Zn, CMCS/Zn, and CMCS/Zn/COL) after incubation in PBS at pH = 7.4 and $T = 37^\circ\text{C}$.

denaturation degree (Perez-Puyana et al., 2019), and structure (Bozec & Odlyha, 2011). However, COL-functionalized scaffolds were characterized by a significantly higher T_g (210 °C) likely because of an increase in crosslinking density due to amide bonds between COL and CMCS, CMCS-

CMCS, and COL-COL. Moreover, COL undergoes degradation in two stages approximately in the range 300–340 °C (Bozec & Odlyha, 2011). The relevant exothermic peaks may have overlapped with the decomposition peak found for the crosslinked CMCS scaffold, contributing to

Table 2

Thermal parameters of the samples analyzed by DSC.

Sample	T _g (°C)	T _{onset} (°C)	T _{peak} (°C)	ΔH* (J/g)
CS	–	286.5 ± 1.4 ^a	306.8 ± 0.6 ^a	–156.6 ± 0.9
CS/Zn	151.9 ± 0.9 ^a	253.9 ± 5.2 ^c	278.9 ± 3.4 ^b	–50.0 ± 10.0
CMCS	–	257.9 ± 0.8 ^{b,c}	270.2 ± 0.2 ^c	–283.4 ± 7.3
CMCS/Zn	153.1 ± 2.7 ^a	261.9 ± 1.0 ^b	303.6 ± 1.2 ^a	–189.8 ± 3.3
CMCS/Zn/ COL	210.2 ± 1.4 ^b	254.9 ± 1.4 ^c	303.4 ± 0.6 ^a	–263.7 ± 5.0

Values reported as mean ± standard deviation (n = 3).

a,b,c The values that do not share the same letter are significantly different (p < 0.05).

* All values relevant to ΔH are significantly different (p < 0.05).

the higher ΔH of the latter.

3.3.5. Mechanical characterization

Stress-strain curves of crosslinked (CS/Zn, CMCS/Zn) and functionalized scaffolds (CMCS/Zn/COL) (Fig. 7b) after incubation in PBS at 37 °C for 4 h are reported in Fig. 7c. CS/Zn and CMCS/Zn/COL curves exhibited a roughly linear profile up to the breaking point, which indicated their elastic properties. However, the CMCS/Zn curve was characterized by a decreasing slope before breaking, suggesting a detectable sample plastic deformation. All samples showed stress fluctuations near the end of the test due to failure of single fibers.

The presence of polar groups along the macromolecular chains generally leads to an increase in the stiffness of the polymer due to the strength of intermolecular interactions, such as hydrogen bonding and ionic complexation (Pecorini et al., 2022). In particular, the CMCS ability to form a polymer-metallic cation crosslinking complex should result in an increased stiffness (Yan et al., 2023). However, the elastic modulus (E) of CMCS/Zn scaffold was lower than that of CS/Zn one (Table 3), likely because of the different WU values at pH 7.4, as previously described. Indeed, in the case of hydrophilic polymers in a swollen state, interactions with water molecules can compete with the aforementioned inter/intra-macromolecular interactions. Under these conditions dynamic/reversible macromolecular interactions can occur (Yan et al., 2023) and anionic repulsion between carboxylate groups can also contribute to more flexible polymer chains.

Functionalization significantly increased the scaffold tensile strength (σ_{max}) probably because of the higher density of covalent crosslinks among CMCS chains, as described in the DSC analysis section. This is consistent with previous results about crosslinking with EDS and its effect over σ_{max} (Chen et al., 2006). Moreover, COL can function as a long-range crosslinker (Lin et al., 2023) interconnecting CMCS chains over long distances, further contributing to mechanical strength enhancement, as well as to the observed significant increase of elongation at break (ε_{break}). In addition, the reversible nature of supramolecular interactions, fibrillar structure, and flexibility of COL could have contributed to decrease scaffold stiffness (Soliman et al., 2024; Varma

Table 3

Tensile mechanical parameters of the developed scaffolds.

Sample	E (kPa)*	σ _{max} (kPa)	ε _{break} (%)*
CS/Zn	25.6 ± 3.9	103.3 ± 23.0 ^a	5.5 ± 1.6
CMCS/Zn	13.0 ± 1.3	111.3 ± 17.9 ^a	38.7 ± 6.0
CMCS/Zn/COL	1.6 ± 0.3	153.8 ± 35.5 ^b	101.0 ± 8.9

Values reported as mean ± standard deviation (n = 6).

a,b The values that do not share the same letter are significantly different (p < 0.05).

* All values relevant to E or ε_{break} are significantly different (p < 0.05).

et al., 2016). Indeed, CMCS/Zn/COL scaffolds exhibited the smallest E value and highest ε_{break} among the tested samples. A significant effect of the change in scaffold macroporosity upon COL functionalization treatment, as demonstrated through SEM analysis, should be also considered to better understand these changes in material mechanical behavior.

Surface stiffness, expressed via Young's or elastic modulus, plays a key role in cell adhesion, migration, and differentiation (Cai et al., 2020). The stiffness of the tested scaffolds is suitable for mimicking the *in vitro/in vivo* mechanical properties of soft tissues, such as adipose connective tissue (1–6 kPa). Particularly referring to wound healing, the developed CS- and CMCS-based scaffolds have a stiffness close to those of the skin dermal layer (E = 35 kPa) and the substrates considered optimal for *in vitro* fibroblasts growth modelling (0.5 kPa < E < 20 kPa) (Guimarães et al., 2020).

3.3.6. Antimicrobial activity assessment

The *in vitro* antibacterial activity of CS/Zn, CMCS/Zn, and CMCS/Zn/COL scaffolds was evaluated against a Gram-negative *E. coli* strain (Fig. 8a-d), and a Gram-positive *S. aureus* strain (Fig. 8e-h) using the halo inhibition zone test. A clear inhibition zone was evident against both target microorganisms, in particular in the case of CMCS/Zn (Fig. 8c and g) and CMCS/Zn/COL (Fig. 8d and h) samples. Moreover, a slight inhibition was observed in the case of CS/Zn samples (Fig. 8b and f), whereas inhibition was completely absent in non-crosslinked CS samples (Fig. 8a and e) for both bacterial strains. The antimicrobial activity of CS functionalized with Zn²⁺ has been described in the literature against these pathogen strains (Mutlu et al., 2022). Although its antimicrobial activity in the form of fibers (Y.-L. Wang et al., 2020) and injectable hydrogels (Wahid et al., 2018) has been already described, this is the first report on Zn-crosslinked CMCS in the form of additively manufactured scaffold.

The obtained qualitative results were corroborated using a more precise quantification of the antimicrobial activity based on the contact test, as shown in Table 4. A significantly higher antibacterial activity towards *E. coli* and *S. aureus* (> 90 %) was obtained for CMCS/Zn and CMCS/Zn/COL compared to CS/Zn samples, likely because of the lower Zn content in the latter, as demonstrated by EDS analysis. The lower inhibition percentages for both strains displayed by CS/Zn scaffolds are, however, significantly higher than that of CS samples, confirming the antimicrobial action of Zn²⁺, as previously reported in the literature (Krishnaveni & Thambidurai, 2013; Mutlu et al., 2022). Indeed, Zn ions can bind to proteins leading to their denaturation, which can cause a structural and permeability change in the cell membrane (Wang et al., 2012). In this way, they interact with nucleic acids, preventing microbial replication. The accumulation of Zn²⁺ can therefore cause membrane disorganization and consequent internalization in the microbial cell (Wu et al., 2022). Although the anti-microbial activity against the two microbial targets was similar, CMCS scaffolds were most effective against *E. coli* than *S. aureus*. This difference can be ascribed to the easier access of antimicrobial components within the Gram-negative cells due to the thinner peptidoglycan layer (Pagliarini et al., 2024).

3.3.7. Cell culture

The biocompatibility of the polysaccharides employed for scaffolds fabrication was assessed through extracts assay. The mouse embryo fibroblast cell line Balb/3T3 clone A31 was used for this purpose, and the sample extracts were tested at different dilutions (up to 1:16) with an extraction time of 24 h. Results showed the cytocompatibility of all extracts and dilutions, since cell viability was >75 % (Fig. 9a) (ISO, 2009). These findings provided evidence that the investigated CS and CMCS are compatible and therefore suitable for the development of scaffolds for wound healing and skin tissue engineering applications.

Furthermore, cells seeded directly on the surface of CS/Zn, CMCS/Zn, and CMCS/Zn/COL scaffolds exhibited viability values comparable to each other on day 1 of culture. Cell viability was roughly constant

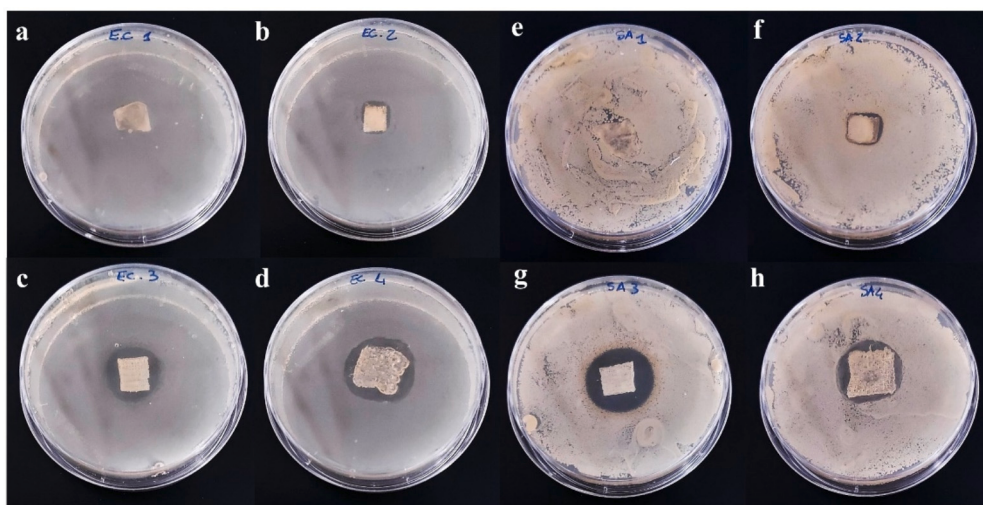


Fig. 8. Evaluation of the antimicrobial activity by halo inhibition method of (a) CS, (b) CS/Zn, (c) CMCS/Zn, (d) CMCS/Zn/COL, against *E. coli*; (e) CS, (f) CS/Zn, (g) CMCS/Zn, (h) CMCS/Zn/COL against *S. aureus*.

Table 4

Cell mortality (R%) of Gram-negative and Gram-positive strain in contact with scaffolds.

Sample	<i>E. coli</i>	<i>S. aureus</i>
CS	0 ^d	0 ^d
CS/Zn	54.07 ± 0.96 ^c	50.68 ± 0.97 ^c
CMCS/Zn	99.49 ± 0.06 ^a	99.53 ± 0.04 ^a
CMCS/Zn/COL	95.15 ± 0.09 ^b	91.96 ± 0.36 ^b

Data reported as average ± standard deviation (n = 3).

a,b,c For a given microorganism, different letters indicate significant differences ($p < 0.01$).

throughout the experiment period in the case of CS/Zn and CMCS/Zn scaffolds, while cells grown on CMCS/Zn/COL scaffold significantly increased their viability on day 7 of culture up to a value statistically higher than that relevant to CMCS/Zn scaffold at the same time point (Fig. 9b). This could be due to the presence of COL grafted onto the surface of the CMCS/Zn scaffold fibers. Indeed, grafted COL presents the tripeptide arginine–glycine–aspartic acid sequence (RGD), a cell adhesion motif that can improve hydrogel biocompatibility by favoring cell adhesion, proliferation, migration, and differentiation (Dai et al., 2021; Guzmán-Soria et al., 2023; Kuo & Yeh, 2011; Y. Lin et al., 2023).

Live/Dead assay showed the intrinsic fluorescence of all analyzed hydrogel fibers (Fig. 9c), corroborating the findings of previous articles investigating CMCS-Zn hydrogels as optical indicator (Y.-L. Wang et al.,

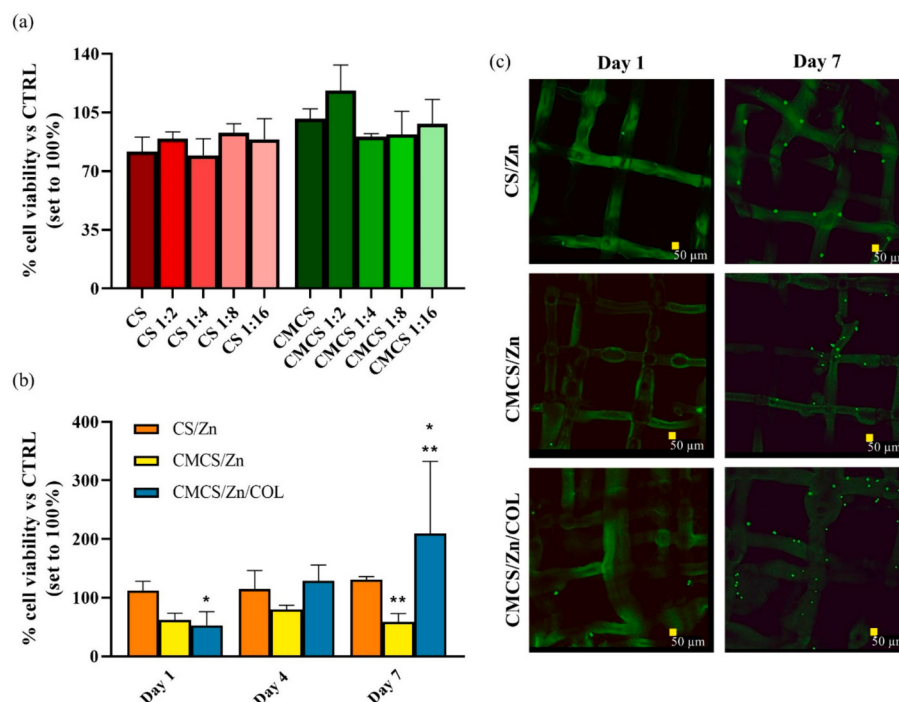


Fig. 9. Balb/3T3 clone A31 cell viability values from (a) extract assay on raw polymers (CS and CMCS) and (b) direct contact assay on CS/Zn, CMCS/Zn, and CMCS/Zn/COL scaffolds up to 7 days of cell culture; (c) Live/Dead micrographs of cells grown on CS/Zn, CMCS/Zn, and CMCS/Zn/COL scaffolds at day 1 and 7 of cell culture (scale bar: 50 μ m). Data reported as mean ± standard deviation (n = 3). *** Values with the same symbol are statistically different ($p < 0.05$).

2020). In addition, the obtained results confirmed the comparative analysis made by means of WST-1 assay, since after 7 days of cell culture a higher number of cells was detected on CMCS/Zn/COL scaffolds than on CMCS/Zn ones. Although a relatively small number of adhered cells were visible at the different experimental time points (1 and 7 days) on all samples, only viable cells (in green) were detected confirming the cytocompatibility of the developed scaffolds.

An *in vitro* scratch test was also carried out to assess the capability of the developed scaffolds to support wound healing (Nanditha & Vinod Kumar, 2022; Kalirajan et al., 2022). As shown in Fig. 10, the wound closure percentage after 12 h was significantly lower in the case of cells treated with CS/Zn and CMCS/Zn scaffold extracts than in the case of the control. This result could be due to the presence of Zn^{2+} since previous studies have demonstrated that high concentrations of this ion can have a cytotoxic effect (Salama & Abdel Aziz, 2020). In any case, CMCS/Zn/COL scaffolds showed a wound healing value comparable to that of the control already after 12 h, confirming the well-known beneficial effect of collagen on skin regeneration (Liu et al., 2019). Moreover, wound healing percentages of all the tested polysaccharidic samples were comparable to that of the control after 24 h with a complete wound closure after 48 h, confirming the suitability of the developed scaffolds for the investigated application.

4. Conclusion

The main result of this research activity was the development of a novel protocol for the fabrication of 3D CMCS scaffolds by AM. Indeed, the hypothesis that a CMCS water solution could be extruded and deposited into an ethanol bath with a layer-by-layer process was verified. This represents a noteworthy innovative aspect, considering that previous relevant studies described blending with other polymers as a key requirement for AM of CMCS scaffolds with a 3D layered structure.

The employed CS carboxymethylation process carried out at room temperature with a short alkalization time, allowed minimizing the possibility of polysaccharide deacetylation and depolymerization, which could compromise its processing properties. Although the obtained CMCS was readily soluble in aqueous media, after Zn^{2+} cross-linking the resulting additively manufactured scaffolds were stable in PBS with a pH-sensitive swelling behavior. These CMCS scaffolds showed improved thermal stability due to the strong metal-polymer interactions, compared to analogous crosslinked CS scaffolds, as well as a highly-interconnected macroporous structure with a hollow fiber morphology. The high DS of the synthesized CMCS made it possible to graft COL onto the scaffold fibers and relevant solvent cast films, with a resulting increase in bulk material surface hydrophilicity. COL functionalization also had a significant effect on scaffold porous structure, as well as on CMCS glass transition and mechanical properties. The antimicrobial activity of the developed scaffolds as a consequence of Zn loading was demonstrated employing a Gram-negative and a Gram-positive bacterial strain, highlighting that CMCS hydrogels had a significantly higher activity than CS ones. *In vitro* cell culture experiments employing murine fibroblasts demonstrated the biocompatibility of the investigated raw polysaccharides, i.e., CS and CMCS, and Zn-crosslinked scaffolds by AM. In addition, COL functionalization led to a significant increase in the ability of the developed CMCS scaffolds to support *in vitro* fibroblast viability and wound healing process.

The obtained results represent therefore the basis for future research focused on the investigation of additively manufactured CMCS scaffolds for wound healing and *in vitro* tailored skin tissue engineering strategies. The versatility of CMCS in terms of chemical functionalization, thanks to reactive functional groups in its repeating unit, will be further exploited to immobilize drugs and other functional molecules for advanced bioactive material strategies.

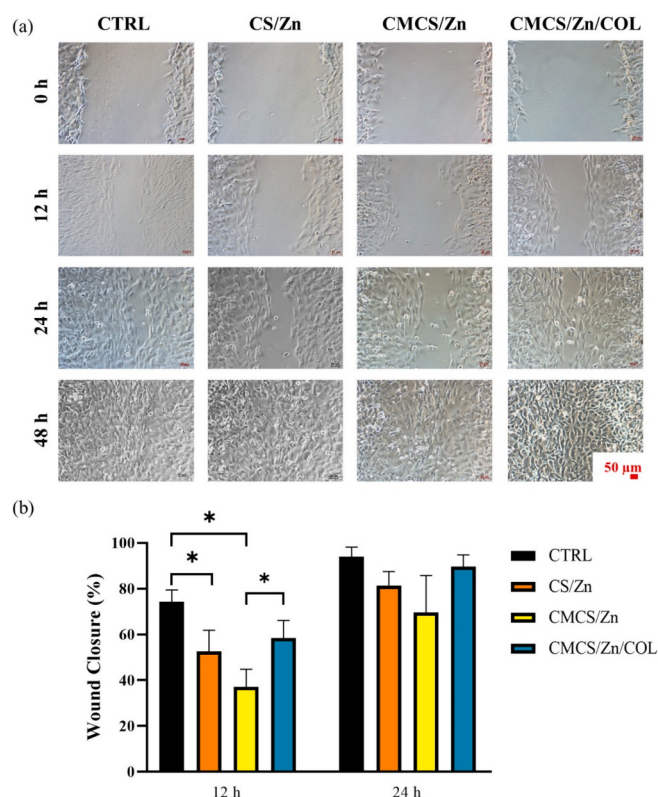


Fig. 10. Wound healing assay on Balb/3T3 clone A31 cells treated or non-treated (CTRL) with scaffold extracts: (a) optical microscopy micrographs of cells at 0, 12, 24 and 48 h after wound creation (scale bar: 50 µm); (b) percentage wound closure as a function of time. Data reported as mean $\pm$ standard deviation (n = 3). * Values statistically different (p < 0.05).

CRediT authorship contribution statement

Lorenzo García: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Simona Braccini:** Writing – original draft, Methodology, Investigation, Conceptualization. **Elia Pagliarini:** Investigation, Methodology, Writing – review & editing. **Viola Del Gronchio:** Investigation. **Diana Di Gioia:** Funding acquisition, Methodology, Supervision. **Hazel Peniche:** Methodology, Conceptualization. **Carlos Peniche:** Writing – review & editing, Validation, Methodology, Conceptualization. **Dario Puppi:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.carbpol.2024.122640>.

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