

Development and assessment of a novel silk fibroin methacrylate and keratin hydrogel for human hip acetabular labrum regeneration

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ABSTRACT

The acetabular labrum is a fibrocartilaginous ring that surrounds the acetabular rim, anchoring to bone and ensuring hip joint stability and sealing. It is mainly composed of fibrochondrocytes and type I collagen fibers, providing resistance to mechanical stress during daily activities. Femoroacetabular impingement can compromise these properties, leading to labral degeneration, hip pain, reduced quality of life, and early osteoarthritis. To address this, biomaterials that mimic the native labral and support regeneration are under investigation.

This study developed composite hydrogels combining, for the first time, *Bombyx mori*-derived silk fibroin methacrylate (SilMA) and wool-derived keratin microfibers (KI) to create a bioartificial labrum prototype. Two KI concentrations (12.5% and 25% w/w), mixed with SilMA, were tested and compared to SilMA alone. KI powder was dispersed into SilMA, and ultraviolet (UV) exposure time was optimized for hydrogel crosslinking. Morphological and mechanical characterization of the hydrogels was performed, along with the evaluation of cell biocompatibility and cell behavior with human chondrocytes (C28/I2) using a lactate dehydrogenase assay, transmission electron microscopy, and histology.

Increasing KI concentration enhanced viscosity, shear stiffness, and elastic modulus under dry and wet conditions. Biocompatibility assays confirmed cell viability up to 14 days.

Overall, SilMA-KI hydrogels demonstrated favorable mechanical performance and cytocompatibility, highlighting their potential as scaffolds for acetabular labrum regeneration.

1. Introduction

The acetabular labrum is a fibrocartilaginous ring of tissue attached to the external edge of the acetabulum of the hip joint, merging with the acetabular hyaline cartilage on the articular side, and with the bony acetabulum on the capsular side [1]. The labrum is composed mainly of type I collagen fibers and the fibrochondrocytes that maintain it. Its main functions are to increase the contact surface area with the femoral head, deepening the acetabular socket, providing a fluid seal, and contributing to the load transfer across the hip joint during activities of daily living, thus conferring overall more stability to the joint and improving joint lubrication [2–4].

However, labral function and integrity can be impaired due to

deterioration caused by hip pathologies, including acute trauma, dysplasia, capsular laxity, extreme ranges of motion, and femoroacetabular impingement (FAI) [1,5,6]. Degeneration of the labrum often leads to the formation of labral tears, which impair the joint's ability to maintain intra-articular fluid pressure, thus resulting in abnormal loading conditions. This scenario causes hip pain, leading to a decreased quality of life, and lastly, even to early osteoarthritis (OA) [2,7].

Currently, tendon allografts or autografts are the main sources for labrum reconstruction [8,9], but several limitations are reported, such as: (i) the shape of the tendon graft, which is circular in cross-section and does not ensure an efficient load-bearing and sealing effect of the joint, resulting in altered function [10]; (ii) the handling and preparation of

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the graft (both autologous and homologous) may lead to further lengthening of the surgical time [11]; (iii) the need for an additional skin incision, in case of autograft (from gracilis/hamstring, quadriceps tendon, hamstring, iliotibial band, ligamentum teres, or tensor fascia lata [9,12–15], (iv) extended traction times during the surgery, (v) the difficulties with the introduction and fixation of the graft, as well as (vi) the risk of iatrogenic injury during the procedure.

Therefore, the development of new techniques to regenerate the native joint function more effectively is highly needed [16]. Tissue engineering has emerged as a promising approach offering alternative solutions for cartilage regeneration by developing biomimetic tissue substitutes, as a new strategy to reduce the incidence of end-stage hip OA and total hip replacement (THR), especially in adolescents and young adults [17,18].

In the case of acetabular labrum regeneration, an ideal biomaterial-based graft to substitute the native labrum should have the following characteristics: i) sufficient mechanical properties to partially withstand the mechanical stresses in the joint until the natural tissue has been restored; ii) ability to support fibro-chondrocyte viability as well as support their proper phenotype and matrix production; iii) anatomical profile to best restore the load bearing and “seal” effect; iv) ready to use in surgical room for primary arthroscopies, avoiding a second surgical intervention for a more effective and rapid surgical technique.

Hydrogels have emerged as promising scaffolds for fibrocartilage repair because their highly hydrated three-dimensional networks can mimic the native extracellular matrix (ECM) and support cell survival, migration, and matrix deposition [19]. In recent years, increasing attention has been directed toward hydrogels derived from natural biomaterials because they more closely resemble the biochemical composition and biological signaling of native tissues. In particular, these biomaterials provide both structural support and biological cues similar to native ECM. As a result, the use of collagen, chitosan, alginate, gelatin, silk fibroin, and extracellular matrix-derived components in material fabrication has proven successful for supporting fibrocartilage regeneration due to their inherent bioactivity, biodegradability, and cell-adhesion characteristics [20]. Nevertheless, as far as we are aware, no study has specifically investigated natural biomaterials for the regeneration of the acetabular labrum.

Among naturally derived biopolymers, Silk Fibroin, a structural fibrous protein derived from the *Bombyx mori* silkworm, has attracted significant attention in biomaterials research [21]. Its outstanding mechanical strength, biodegradability, and biocompatibility, made SF a promising candidate for labral reconstruction [22–24]. To broaden its applicability, SF can be chemically modified by introducing a methacryloyl group into the amino acids side chains, enabling its photocrosslinking under ultraviolet (UV) light to form stable hydrogels [25]. Including other materials in a SilMA matrix further represents an appealing strategy to overcome some of the inherent limitations of SilMA hydrogels [23,26].

Keratin (KI) is a fibrous structural protein with remarkable mechanical properties, including hardness and tensile strength, which together provide effective reinforcing mechanisms [27]. Beyond its mechanical role, KI exhibits notable resistance to enzymatic degradation and thermal stability [28,29], while also promoting cell adhesion, proliferation, and regeneration, closely mimicking the behavior of native fibrous networks [30]. Moreover, KI confers to SF additional strength and rigidity by permanent, thermally stable crosslinking, thus improving the strength of the final composite biomaterial.

This study reports the first development of a SilMA/KI composite hydrogel and proposes it as a biomaterial prototype for labral regeneration. Unlike conventional approaches that rely on soluble extracted forms of keratin requiring multiple chemical processing steps, here a fibrous form of keratin was employed, preserving its native architecture and simplifying preparation by eliminating unnecessary solubilization steps. Rather than claiming immediate clinical translation, the material is presented as a proof-of-concept platform to explore the feasibility of

combining photocrosslinkable silk fibroin with bioactive keratin to generate a tunable hydrogel system with potential applicability in fibrocartilaginous repair. A preliminary mechanical characterization was conducted using unconfined compression testing and rheological analysis. Changes in the chemical structure of the hydrogels were investigated using Fourier Transform Infrared Spectroscopy (FTIR). Finally, chondrocytes were encapsulated within the hydrogel to perform a biological evaluation.

2. Materials and methods

2.1. Silk fibroin regeneration and methacrylation

To extract SF from *Bombyx mori* cocoons (social cooperative Socio-lario ONLUS, Como, Italy), a degumming process was performed to remove sericin proteins, which can interfere with the downstream applications of fibroin. Briefly, silk cocoons were cut into small pieces and boiled sequentially in two aqueous sodium carbonate (Na_2CO_3) baths at 98 °C (first in a 1.1 g/l solution for 45 min, then in a 0.4 g/l solution for an additional 45 min). This dual-step treatment ensured efficient sericin removal while preserving fibroin integrity. The degummed fibers were then thoroughly rinsed with deionized water several times to eliminate residual salts and sericin fragments and dried for three days under the hood. This process yielded to degummed SF fibers suitable for subsequent chemical modification. SilMA was obtained by methacrylation with glycidyl methacrylate (GMA), as previously published by the authors [31–33]. Briefly, the degummed SF was dissolved in a 9 M lithium bromide (LiBr; Sigma-Aldrich, Zwijndrecht, The Netherlands) solution at 60 °C for at least three hours, to obtain a 20% SF-LiBr solution. Then 1 ml of glycidyl methacrylate (GMA, Sigma-Aldrich) per every 4 g of dissolved SF was added to the solution. GMA was let to react under mixing for four hours at 500 rpm and 65 °C. The final product was dialyzed against deionized water (DDW) at room temperature for three days using a 3.5 kDa Spectra/Por™ (ThermoFisher Scientific, Waltham, MA, USA) dialysis tube. Water was changed at least two times per day to ensure the complete release of unreacted GMA, LiBr and shorter fragment of SF. Lastly, the resulting SilMA water solution was left in the chemical hood to evaporate inside the dialysis membrane measuring its concentration two times per day until a final concentration of 150 mg/ml of SilMA was reached.

The concentration was measured by letting water evaporate from the small volume of SilMA solution and then weighing the residue of protein.

By knowing the initial volume, the concentration was easily determined by Eq. 1.

$$C \left[\frac{\text{mg}}{\text{ml}} \right] = m[\text{mg}] / V[\text{ml}] \quad (1)$$

The solution was then filtered using a 100 µm and, subsequently, a 70 µm cell strainer (BD Biosciences, Erembodegem, Belgium) to ensure no debris was present. It was then stored at 4 °C until further use.

2.2. Development of SilMA-KI hydrogels

KI (Tokyo Chemical Industry CO., LTD., Japan) was mixed with SilMA in two different proportions, in particular 12.5%_{w/w} (SilMA-KI12.5) or 25%_{w/w} of KI (SilMA-KI25), using the material without KI (SilMA alone) as control.

In all the uncrosslinked solutions, 1%_{w/w} of lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP) was added as photoinitiator.

Due to the small amount of LAP to be added, highly concentrated water solution of LAP (20 mg/ml) was prepared. It should be noticed that the percentages must be considered in respect to the weight of SilMA. As example, to prepare 1 ml of SilMA-KI12.5 to 1 ml of SilMA solution (with a concentration of 150 mg/ml) 18.75 mg of KI and 1.5 mg of LAP (75 µl of LAP solution) were added; while to prepare 1 ml of

SilMA-KI, from the same starting solution, 37.5 mg of KI and 1.5 mg of LAP (75 μ l of LAP solution) were added. The materials were homogenized by gentle pipetting using a positive displacement pipette (Gilson Microman® E, Middleton, WI, USA) to avoid bubble formation. Successively, to allow the photocrosslinking, 200 μ l of solution was poured in cylindrical (8 mm diameter, 5 mm height) silicone mold and placed under a UV light lamp (VL-6.LC; Vilber Deutschland GmbH, Eberhardzell, Germany), with a 365 nm wavelength for 10 min, keeping the distance of the samples from the source to 15 cm (resulting in an intensity of 4.58 mW/cm²). The cylindrical hydrogels were then gently extracted from the mold.

2.3. Fourier transform infrared (ftir) analysis

Changes in the chemical structure were evaluated using Fourier-transform infrared spectroscopy with attenuated total reflection (ATR-FTIR; Perkin Elmer Spectrum Two).

For each sample, 32 spectra were acquired at a resolution of 1 cm⁻¹ and averaged to improve the signal-to-noise ratio. To evaluate changes in the protein secondary structure, the primary amide region (1580–1720 cm⁻¹) was isolated and subjected to an initial smoothing using a 5-point moving average, followed by Fourier self-deconvolution (smoothing factor 0.3, gamma function 30) to enhance resolution and highlight overlapping components. The second derivative of the deconvoluted spectrum was then calculated to precisely identify peak positions. These peak centers were used as initial parameters for the iterative fitting of Gaussian functions, repeated until the χ^2 between the experimental curve and the model was minimized. The area of each Gaussian was then compared to the total area of the Amide I band to quantify the relative percentage of each secondary structure. Peak assignment was carried out using Table I.

2.4. Morphological and mechanical characterization

To evaluate the morphological reconstitution of biomaterials, Transmission Electron Microscopy (TEM) was performed on biomaterial samples (SilMA, SilMA-KI12.5, and SilMA-KI25) maintained 1 day in cell culture medium, i.e. Dulbecco's Modified Eagle Medium (DMEM), 1% penicillin-streptomycin (11330–032 and 15,140–122 respectively, Gibco - Thermo Fisher Scientific, MA, USA) and 10% fetal bovine serum (F7524, Sigma-Aldrich, MO, USA) (hereafter referred to as complete DMEM). Successively, biomaterial samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 3 h, incubated with 1% osmium tetroxide in the same buffer for one hour, alcohol dehydrated, and embedded in araldite, as previously reported [34]. Thin sections (0.5 μ m) were collected on 400 mesh nickel grids and stained with uranyl acetate and lead citrate. Analysis was carried out with a Philips CM 10 electron microscope at 80 kV.

Rheological characterization was performed on a Discovery HR-2 hybrid rheometer (TA Instruments, New Castle, Delaware). The instrument was equipped with a stainless-steel cone/plate geometry (50 mm diameter, 2° cone angle, 100 μ m truncation gap) and a Peltier plate for temperature control. The uncrosslinked SilMA-KI solutions were tested in flow mode, recording shear stress and viscosity over a shear rate range

from 0.1 to 100 s⁻¹ at room temperature to characterize shear-thinning behaviors.

The mechanical characterization was performed on cylindrical hydrogels (6 mm diameter and 5 mm of height) obtained by pouring the SilMA/KI/LAP solutions into silicon mold and crosslinking them for 10 min under UV light (365 nm). Three cylinders for each KI concentration (0%_{w/w}, 12.5%_{w/w}, 25%_{w/w}) were tested both in dry and pseudo physiological conditions (indicated as Wet, after 24 h of swelling at 37 °C in PBS). The test was performed using a single cycle of unconfined compression loading and unloading, between two flat impermeable stainless-steel platters, with a constant compression rate of 1 mm/min using a universal testing machine (Instron 5969, Norwood (MA), USA) with a load cell of 2.5 N. A preload of 0.1 kPa was applied to ensure the full contact of the plate to the sample surfaces. The compression modulus (E) was calculated as the slope of the elastic linear region of the stress-strain curve.

2.5. Cell-loaded hydrogel preparation and sterilization

SilMA and SilMA-KI cell-loaded hydrogels were prepared according to the previously described protocol. All materials were sterilized prior to use, and the sterility was maintained throughout the procedure by performing all steps within a biosafety cabinet. SF solution was filtered with a 0.22 μ m filter and then placed in a vial under UV radiation (VL-6.LC; Vilber Deutschland GmbH, Eberhardzell, Germany) at 254 nm with a distance of 15 cm, resulting in an exposure of 0.13 mW/cm², for 30 min. LAP solution, KI powder, and cylindrical silicon molds were sterilized by autoclave (a cycle of 30 min at 120 °C). To facilitate handling and ensure accurate formulation, the required amount of KI for each composition was pre-weighed and transferred into individual vials prior to sterilization. Immortalized chondrocyte human cell line C-28/12 [35,36] was used to assess the biocompatibility of the biomaterials and the biological behavior at early and late timepoints, that is 1 day (d), 3d, 7d, and 14d. The cells were maintained in culture in complete DMEM, and trypsin/EDTA (Trypsin 0.05%–EDTA 0.02% in PBS, Euroclone, Milan, IT, ECB3052D) was used for passaging cells at 70% confluence. Cells were maintained for all the duration of the experiments in incubator at 37 °C, 5% CO₂ and 95% humidity.

For the preparation of cell-loaded hydrogels, cells at 70% confluence were detached by trypsinization, and a suspension of $15 \times 10^6/500 \mu$ l C28 cells was prepared in complete DMEM, and 150 μ l of cell suspension was added to 2.5 mL of SilMA, SilMA-KI12.5 and SilMA-KI25 precursor solutions. The mixtures were gently homogenized manually, avoiding bubble formation. Then, 200 μ l of cell loaded solution was poured in cylindrical mold (8 mm diameter, 5 mm height) and crosslinked under UV light, as previously described. Cell-loaded cross-linked hydrogels were covered with complete DMEM and maintained in incubator: for each type of biomaterial, three samples were prepared and analyzed at 1d, 3d, 7d and 14d of culture. It is important to observe that this sample size, despite being small, is commonly used for initial studies concerning hydrogels [37–39]. 150 μ l of the same cell solution were also added to the bottom of tissue culture polystyrene (TCPS) 24 well plate, allowed to adhere with the addition of complete DMEM, and used as 2D control in cell viability assay.

2.6. Assessment of cell viability by lactate dehydrogenase assay

To evaluate cell metabolic activity and quantify potential cytotoxicity, Lactate Dehydrogenase (LDH) Colorimetric Activity Kit (Roche, 11644793001) was used following manufacturer's instructions. To better monitor the potential cytotoxic activity of the biomaterials on cells, early and late timepoints were considered: 1d, 3d, 7d, and 14d. The samples' absorbance was quantified on three different replicates for each experimental sample by using a microplate spectrophotometer (Infinite F200 PRO, TECAN, Mannedorf, Switzerland) at 490 nm wavelengths. LDH raw values were recorded and reported in graph, and, in parallel,

Table I

Assignment of the secondary structures of fibroin based on the peak positions within the Amide I region (1580–1720 cm⁻¹).

Assignment	Peak Position (cm ⁻¹)
Side chain	1597–1609
β -sheet	1610–1625
β -sheet	1626–1636
Random coil	1637–1655
α -helix	1656–1662
β -turns	1663–1696
β -sheet	1697–1703

the cytotoxicity was expressed as a percentage relative to the positive control (i.e., cells seeded in TCPS and treated with 1% Triton-X solution in culture medium).

2.7. Evaluation of extracellular matrix production by histology and transmission electron microscopy (TEM)

Cell morphology and the potential production of extracellular matrix (ECM) were evaluated by histological staining, including hematoxylin and eosin (H&E) and Safranin-O-Fast Green (S-O-FG), and by means of TEM. For histological staining, 1d, 7d and 14d timepoints were considered, while for TEM analysis only early and late timepoints, i.e. 1d and 14d.

To perform the histological staining, cell-loaded biomaterials samples were OCT (Tissue Tek BAKURA, 4583)-embedded and underwent frozen sectioning using a cryostat. Briefly, a small amount of OCT was placed at the bottom of biopsy molds, avoiding bubbles, and samples were placed in the center of the mold and other OCT was added on top to completely cover the samples. Then, the molds containing the samples were foil-wrapped and snap-frozen in liquid nitrogen, and after 1 min the frozen OCT molds were stored at -80°C . Successively, samples

underwent frozen sectioning using a cryostat (Leica, mod. CM1950) to obtain 7 μm -thick sections, which were stored at -80°C until staining.

H&E and S-O-FG staining were performed as follows: sections were left 10 min at room temperature, fixed with 3.7% paraformaldehyde solution (Sigma Aldrich, P6148) for 20 min, and washed with DDW. For H&E staining, sections were stained with Gill Hematoxylin 1:2 (BIO OPTICA, 05-06015/L) for 1 min, and wash in DDW several times and in tap water for 10 min. Then, sections were immersed in eosin (BIO OPTICA 05-M10007) for 8 min and then washed in DDW for several times to remove the excess of staining solution. Successively, sections were dehydrated in ethyl alcohol (VWR BDH Chemicals, 20821.330) solutions (70% for 3 min, 80% for 3 min, 90% for 3 min, and 100% twice for 3 min) and then immersed in xylol (VWR BDH Chemicals, 28975.325) solutions twice for 5 min, and cover slip was mounted in non-aqueous mounting medium (Merck Life Science, 1.00579). For S-O-FG staining, after Gill Hematoxylin, sections were stained with 0.02% Fast Green (KALTEK, 1277) for 40 min, and then rapidly immersed three times in 1% acetic acid (CARLO ERBA REAGENTS, 401422). Then, sections were immersed in 0.1% Safranin for 10 min and washed three times in DDW for 1 min each. Successively, sections were dehydrated, and cover slip was mounted.

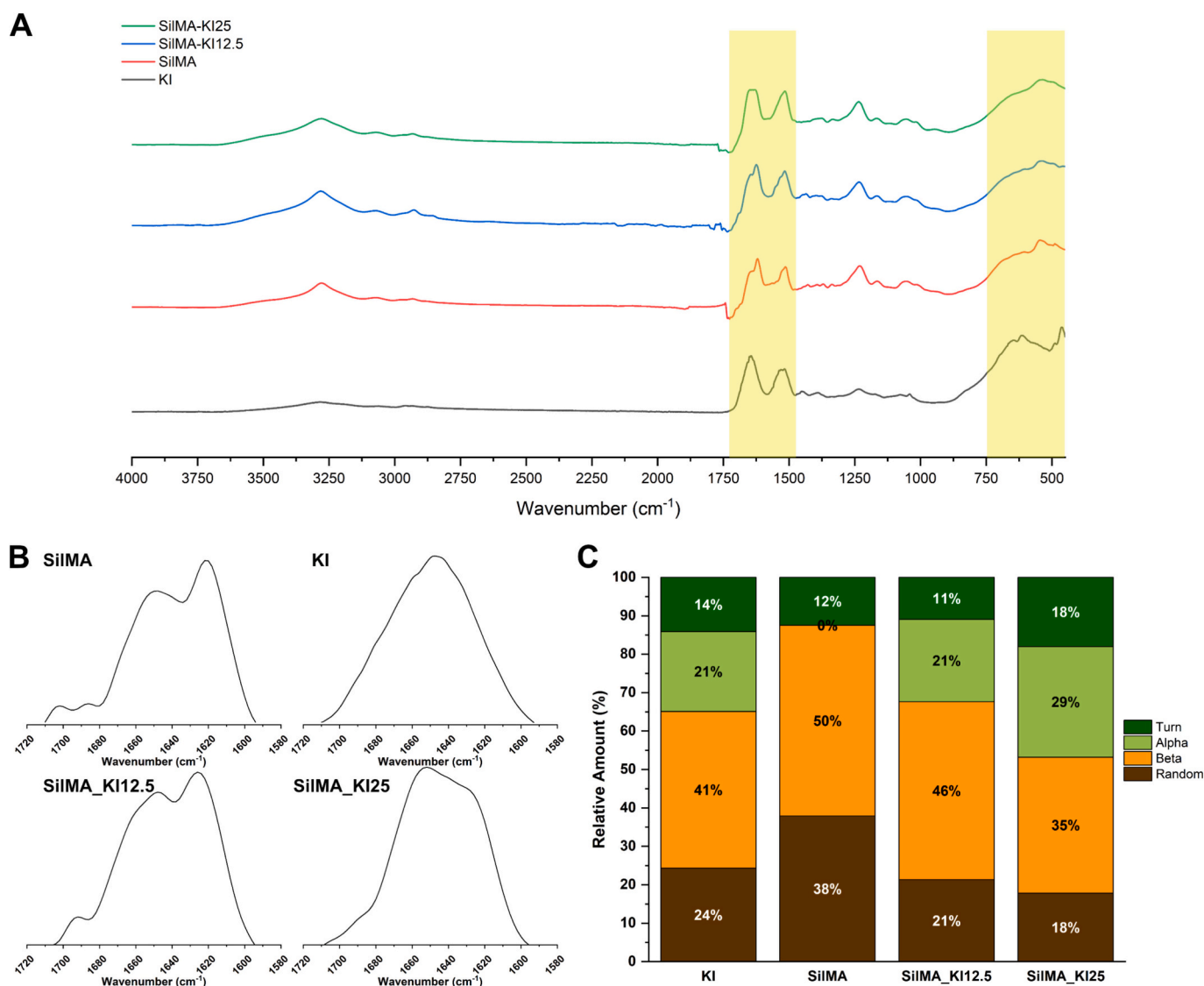


Fig. 1. ATR-FTIR analysis of KI, SiIMA, and SiIMA-KI composites. (A) Full spectra highlighting KI-diagnostic regions: amide I–II ($1700\text{--}1500\text{ cm}^{-1}$) and fingerprint ($\approx 700\text{--}520\text{ cm}^{-1}$). (B) Amide-I ($1720\text{--}1580\text{ cm}^{-1}$) showing a clear shift in the spectrum. (C) Secondary-structure quantification reveals KI-driven reduction in β -sheets and enrichment of random coil/turn content with increasing KI.

All images were captured using a Nikon Eclipse 90i microscope equipped with Nikon Imaging Software elements (Nikon Corporation, Minato, Tokyo, JP).

2.8. Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD). GraphPad Prism 10.1.1 was used to perform statistical analysis and plot the data. An ordinary two-way ANOVA followed by a Tukey post hoc test was performed to understand the impact of the KI concentration and hydration state of the hydrogel (Dry or Wet) on the compression modulus. To analyze LDH assay data, two-way ANOVA followed by Tukey's multiple comparisons test was performed to assess differences among groups and timepoints. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. FTIR analysis

In Fig. 1A, two windows are diagnostic and visible in the blends: within amide I–II ($1700\text{--}1500\text{ cm}^{-1}$) the random-coil amide I band at $\sim 1648\text{ cm}^{-1}$ grows at the expense of the β -sheet band at $\sim 1620\text{ cm}^{-1}$, and in amide II the KI-like feature at $\sim 1538\text{ cm}^{-1}$ becomes more prominent relative to the silk-like band at $\sim 1514\text{--}1518\text{ cm}^{-1}$; in the fingerprint region ($\approx 700\text{--}520\text{ cm}^{-1}$) the cystine C–S ($\sim 620\text{ cm}^{-1}$) and S–S ($\approx 536\text{--}540\text{ cm}^{-1}$) vibrations (hallmarks of KI) modify the SilMA-KI12.5 and SilMA-KI25 spectra as visible by comparison with the SilMA spectrum. First, within amide I–II ($1700\text{--}1500\text{ cm}^{-1}$) the random-coil amide-I band at $\sim 1648\text{ cm}^{-1}$ grows at the expense of the β -sheet band at $\sim 1620\text{--}1628\text{ cm}^{-1}$, and the KI-like amide-II feature at $\sim 1538\text{ cm}^{-1}$ becomes more prominent relative to the silk-like $1514\text{--}1518\text{ cm}^{-1}$ component. Second, in the fingerprint window ($\approx 700\text{--}520\text{ cm}^{-1}$) we detect cystine vibrations characteristic of KI: a C–S stretch at 620 cm^{-1} and an S–S stretch at $536\text{--}540\text{ cm}^{-1}$. Peak positions extracted directly from the dataset confirm these assignments in all samples: amide I (random coil) 1648 cm^{-1} ; amide I (β -sheet) 1620 cm^{-1} in SilMA shifting toward the KI position in the blends (1624 cm^{-1} in SilMA-KI25; 1628 cm^{-1} in SilMA-KI12.5 and KI); amide II (KI-like) 1538 cm^{-1} ; amide II (silk-like) $1514\text{--}1518\text{ cm}^{-1}$ (SilMA 1514 ; SilMA-KI12.5 1516 ; SilMA-KI25 1517 ; KI 1518); amide III (disordered/coil) 1240 cm^{-1} ; C–S 620 cm^{-1} ; S–S $536\text{--}540\text{ cm}^{-1}$ (SilMA-KI12.5 at 536 , SilMA-KI25 at 539 , KI at 540 cm^{-1}). KI fingerprint markers are measurable in the blends and strongest in KI (C–S peak intensity ≈ 0.071 in SilMA versus 0.073 in SilMA-KI12.5/25 and 0.101 in KI; S–S ≈ 0.087 in SilMA, 0.088 in SilMA-KI12.5, 0.085 in SilMA-KI25, 0.088 in KI). These dataset-level findings align with the amide-I deconvolution.

In Fig. 1B the primary amides clearly show a shift toward higher wavenumbers from SilMA to SilMA-KI12.5 and SilMA-KI25. This reflects the results of the I Amide deconvolution shown in Fig. 1C, starting from a β -sheet-dominated SilMA ($\approx 50\%$ β , $\approx 38\%$ random coil, $\approx 12\%$ turns,

$\alpha \approx 0$), the KI-containing samples move toward lower β -sheet fractions and higher non- β component with the appearance of an α contribution. There is an increase in the α contribution which is in the same direction as the increase in the KI addition. It should be noticed that being a dispersion the composition is not the same in overall sample so the outcome of the deconvolution should be taken more as a trend than as representative of the whole sample.

3.2. Morphological and mechanical characterization

SilMA-KI hydrogels were produced successfully (Fig. 2) by exposure to UV light lamp with a 365 nm wavelength for 10 min (intensity of 4.58 mW/cm^2).

The incorporation of wool KI powder into SilMA hydrogels led to the formation of a composite system where KI appeared as darker inclusions uniformly embedded within the brighter SF matrix (Fig. 3). It should be noticed that SilMA alone tends to form a fibrous structure, which is symptomatic of the presence of β secondary structures. As the KI percentage increased, the black fibrous KI domains became progressively more abundant. Despite this increase in concentration, the KI particles remained evenly distributed within the matrix, without evidence of macroscopic phase separation or clustering that could compromise material homogeneity. Higher magnification views confirmed the intimate contact between KI inclusions and the surrounding SF, highlighting a stable integration of both components.

The rheological characterization (Fig. 4A, on the left) shows flow curves of shear stress and viscosity as functions of shear rate for hydrogels with 0% , 12.5% , and 25% KI. All formulations displayed an approximately linear increase of shear stress with shear rate. The viscosity plots showed a slight initial decline at low shear rates (weak shear-thinning) but quickly plateaued across most of the range tested. Higher KI concentrations resulted in proportionally higher viscosity and stress responses, as shown in Fig. 4A.

Fig. 4A (on the right) shows the viscosity versus shear rate curves for SilMA, SilMA-KI12.5 and SilMA-KI25. All samples demonstrate relatively flat curves at higher shear rates, with a very slow decrease in viscosity at the increase in share rate, with a mild shear-thinning at the lowest rates. Higher KI concentrations consistently yield higher viscosities across the shear rate range.

Mechanical characterization (Fig. 4B and quantified in Fig. 4C) was performed to evaluate the influence of KI content and hydration state on the compressive properties of hydrogels with varying concentrations of KI (0% , 12.5% , and $25\%_{w/w}$) and LAP, under both dry and pseudo-physiological (wet) conditions.

The compression stress-strain curves for all samples, irrespective of KI concentration and hydration state, exhibited a clear hysteresis loop, indicative of energy dissipation during loading and unloading cycles (Fig. 4B). All the tested conditions were able to recover the initial shape with only minor deformations (as shown in Fig. 1S). The compressive elastic modulus (*E*), calculated from the linear elastic region of the curves, varied distinctly across different KI concentrations (Fig. 4B).



Fig. 2. SilMA and SilMA-KI hydrogels crosslinked by UV rays' exposure.

Transmission electron microscopy

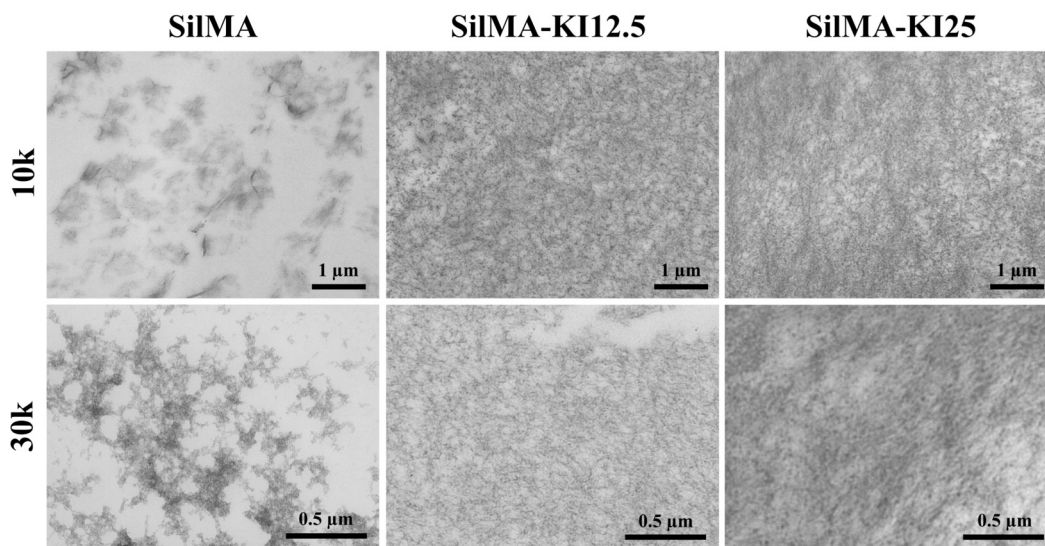


Fig. 3. Microstructural analysis of SF hydrogels blended with wool KI powder at different concentrations and magnifications, after 1 day of culture, in absence of cells. The bright background corresponds to the SF matrix, while the dark regions represent KI inclusions.

Table II summarizes the descriptive statistics for the compressive modulus (obtained by linear regression in the 10%–20% strain region, **Fig. 2S**) across all tested conditions. Under dry conditions, SilMA-KI12.5 demonstrated the highest mean modulus (12.79 ± 0.86 kPa), slightly surpassing SilMA-KI25 (11.55 ± 2.15 kPa). Conversely, samples without KI exhibited significantly lower modulus values (7.91 ± 1.58 kPa). A similar trend was observed under wet conditions, with SilMA-KI25 showing the highest modulus (13.42 ± 2.20 kPa), followed by SilMA-KI12.5 (10.95 ± 1.31 kPa) and SilMA alone (8.06 ± 0.85 kPa). The two-way ANOVA results (**Table III**) highlighted KI concentration as the dominant factor significantly affecting the elastic modulus ($p = 0.0007$), accounting for approximately 64% of total variation. Interestingly, the hydration state (dry versus wet) showed no significant impact ($p = 0.9354$), contributing only minimally (0.015%) to the observed variance (**Fig. 4C**). Additionally, no significant interaction between KI concentration and hydration state was found ($p = 0.1714$).

3.3. Cell viability

Cell viability analysis demonstrated that hydrogels containing 0%, 12.5%, and 25% w/w KI supported cell viability up to 14d of culture.

LDH assay results are presented in **Fig. 5**. Graph A shows the raw absorbance values for all the three hydrogel formulations overtime. Using the positive control (i.e. lysed cells sample) as reference, all hydrogels exhibited lower cytotoxicity at every considered timepoints. Higher LDH levels were observed at 1d compared to subsequent timepoints, likely reflecting transient cellular stress associated with handling and encapsulation procedures. Indeed, LDH levels progressively decreased at later timepoints.

In **Fig. 5 B**, cytotoxicity values normalized to the positive control are shown as a percentage of cytotoxicity. Although cytotoxicity was elevated at day 1 (reaching up to 70%), it markedly decreased over time. Statistical analysis revealed significant differences over time within each biomaterial formulation, whereas no significant differences were detected among the different hydrogel compositions. In particular, the two-way ANOVA (**Table IV**) shows that time accounted for the majority

of the observed variance (79.41%) and was the only statistically significant factor ($p < 0.0001$), indicating that LDH levels changed over time during culture. In contrast, the percentage of keratin incorporated in the SilMA hydrogel contributed minimally to the total variance (0.91%) and did not significantly affect LDH release ($p = 0.4904$). The interaction between time and keratin content was also not significant ($p = 0.2879$). These results indicate that increasing KI content in the biomaterial formulation does not induce additional cytotoxicity (**Table IS** for multiple comparisons).

3.4. Evaluation of extracellular matrix production by histology and TEM

The presence of cells within the biomaterials was confirmed by H&E staining, which revealed cells embedded in the hydrogel matrix (**Fig. 6**, see arrows). Cells were observed within the characteristic bubble-like structures of the hydrogel network, as indicated by arrow.

An initial production of ECM components was evidenced by S-O-FG staining after 14d of culture, with discrete positively stained spots (**Fig. 6**, S-O-FG). Consistent with previous observations in primary chondrocytes cultured in 3D conditions, the appearance of proteoglycans visualized on thin sections by TEM was granular [34], while the keratin is present as dark aggregates of small fibers. The TEM images (**Fig. 7**) highlight how proteoglycans (granular appearance, indicated with asterisks) are distributed throughout the extracellular environment in a rather diffuse manner under all conditions, except in panel A, where a reduced presence of proteoglycans can be observed at higher magnification. Conversely, panels D and F show a marked increase in the number of proteoglycans, which are homogeneously distributed around the cells, particularly evident in panel F. In panel D, well-structured fibers with varying thickness can also be clearly distinguished (indicated arrows). In panels B and E, the intracellular proteoglycan component remains confined within lacunae. Ultimately, the results from TEM analysis reinforced the understanding that C28/I2 cells generated proteoglycans, most notably at the 14-day mark and with the highest KI concentration (SilMA-KI25).

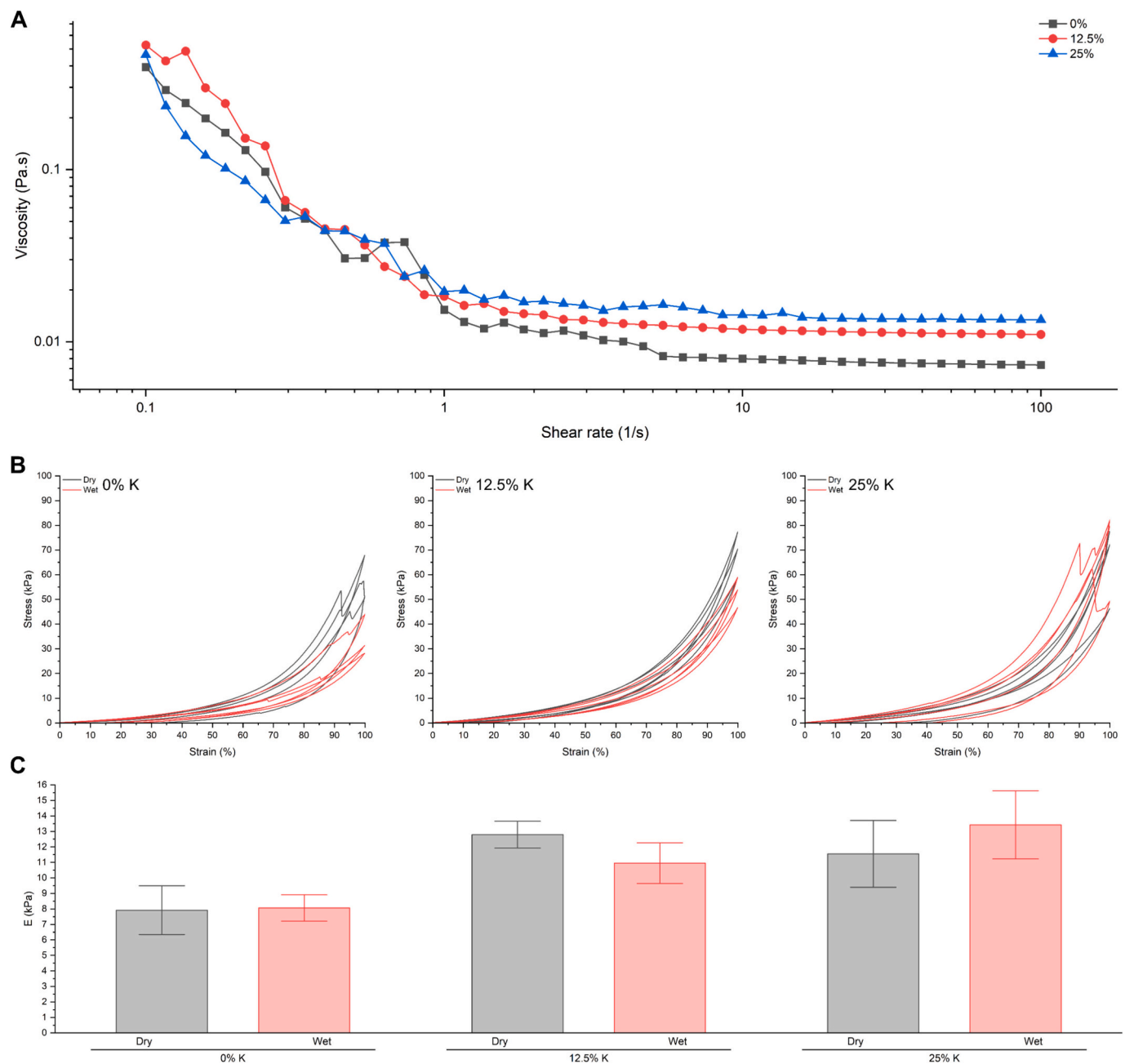


Fig. 4. (A) Rheological curve showing viscosity as functions of shear rate for SilMA/KI/LAP hydrogels with 0%, 12.5% and 25% KI. All formulations display near-Newtonian behavior with mild shear-thinning at low shear rates and consistent viscosity at higher shear rates (cross-fluid behavior). Increased KI concentration results in higher overall viscosity, confirming its reinforcing effect on flow properties; (B) Mechanical curves of compression loading and unloading of dry and wet samples (24 h in PBS at 37 °C) of samples with different KI percentages (0%, 12.5% and 25%). All the mechanical curves present hysteresis; (C) Elastic modulus determined by compression in each condition (data presented as mean $\pm$ StD).

Table II

Descriptive statistics on the Elastic modulus determined by compression reporting mean, standard deviation (StD), Minimum (Min) and Maximum (Max) values, Median, first (Q1) and third (Q3) interquartile, and the interquartile range (IQR).

KI %	State	Mean kPa	E StD kPa	Min kPa	Q1 kPa	Median kPa	Q3 kPa	Max kPa	IQR kPa
0	Dry	7.91	1.58	6.09	6.09	8.80	8.84	8.84	2.75
12.5	Dry	12.79	0.86	11.91	11.91	12.82	13.64	13.64	1.73
25	Dry	11.55	2.15	9.46	9.46	11.42	13.76	13.76	4.30
0	Wet	8.06	0.85	7.45	7.45	7.71	9.03	9.03	1.59
12.5	Wet	10.95	1.31	9.45	9.45	11.54	11.86	11.86	2.41
25	Wet	13.42	2.20	11.64	11.64	12.75	15.88	15.88	4.24

Table III

Two-way ANOVA of Compressive Young's modulus. The sole Significant factor is the Amount of KI included. The state (Dry or Wet) did not significantly impact the compression modulus.

Source of Variation	% of total variation	P value	Significance
Interaction (KI*State)	9.235	0.1714	ns
KI (%)	63.73	0.0007	***
State	0.0154	0.9354	ns

4. Discussion

The acetabular labrum is a fibrocartilaginous structure essential for hip joint stability and lubrication, primarily composed of type I collagen fibers and some fibro-chondrocytes. Labral degeneration, frequently associated with hip pathologies such as femoroacetabular impingement, is a significant source of pain, impairs joint function, and significantly increases the risk of early-onset osteoarthritis, ultimately leading to a decline in life quality. In this context, there is an urgent clinical need to develop and fabricate biomimetic scaffolds able to support labral tissue regeneration, acting as implantable graft substitutes.

Building on recent advances in tissue engineering and hydrogel-based strategies for cartilage regeneration, this study developed and fabricated SilMA-based and composite hydrogels as candidate biomaterials for acetabular labrum regeneration.

Three different hydrogel formulations were fabricated and evaluated: SilMA alone, and SilMA incorporating 12.5% or 25%_{w/w} KI. The successful incorporation of wool-derived KI powder into SilMA hydrogels was confirmed by morphological analysis, which revealed uniformly distributed darker KI inclusions within the brighter silk fibroin matrix. Even at higher KI concentrations, no macroscopic phase separation or clustering were observed, indicating excellent material homogeneity.

Spectroscopic analyses confirmed the presence of cystine-rich KI inclusions within the composite hydrogels. Deconvolution of the amide I band demonstrated uniform KI dispersion within the SF matrix, and revealed the emergence of α -helical structures, absent in pure SilMA. The intensity of these α -helical signals increased proportionally with KI content, indicating that KI not only integrates effectively into SilMA but also introduces distinct secondary structural motifs, thereby modulating the molecular organization of the hybrid material.

Rheological analysis confirmed a near Newtonian behavior for all the three composite uncrosslinked hydrogels, as evidenced by the linear increase of shear stress with shear rate. This suggests overall a cross-fluid

Table IV

Two-way ANOVA of LDH assay data. The sole significant factor is the time. The percentage of KI included in the biomaterial formulation did not significantly affect the cytotoxicity assay. Indicating that KI is safe even in high percentages.

Source of Variation	% of total variation	P value	Significance
Interaction (Time*KI)	4876	0,2879	ns
Time (days)	79,41	<0,0001	****
KI (%)	0,9062	0,4904	ns

behavior (shear-thinning at low shear rate and then Newtonian), which is compatible with previous reporting [40]. The initial shear thinning behavior is an important characteristic for extrusion-based 3D bio-printing applications, potentially enabling the fabrication of multilayered scaffolds. Nonetheless, the solutions' low viscosity would only permit fabrication with a supporting bath (Freeform Reversible Embedding of Suspended Hydrogels, FRESH), as previously documented for SilMA [33]. The incorporation of KI increased the overall viscosity (resistance to flow while maintaining Newtonian like profiles), likely due to the fibrous and structural nature of the KI [41]. This is an essential feature of hydrogels, indicating the injectability potential. All the hydrogels, regardless of KI content or hydration state, exhibited a pronounced hysteresis loop, indicating effective energy dissipation during cyclic loading and unloading. This behavior is likely attributable to the internal frictional forces or viscoelastic behavior characteristic of the polymeric network and interactions within the hydrogel matrix.

Successively, compressive properties were evaluated for all three hydrogels to preliminarily characterize the developed formulation and evaluate the structural behavior rather than replicating the full mechanical performance of native labral tissue.

The addition of KI to SilMA hydrogel modified distinctly the compressive properties in terms of compressive elastic modulus (E), with non-significant differences between dry and wet conditions. Under dry conditions, the 12.5% KI hydrogel demonstrated the highest mean modulus, followed by 25% and 0% KI, while under pseudo-physiological (wet) conditions, the 25% KI hydrogel showed the highest modulus, followed by 12.5% and 0% KI. Thus, the addition of KI significantly affected the elastic modulus of the hydrogels, maybe due to the reinforcement effect provided by KI fibers and its intrinsic fibrous nature, enhancing the stiffness and mechanical integrity of the composite hydrogel. The slight decline in modulus observed at the highest KI concentration under dry conditions may reflect the aggregation or incomplete dispersion of KI fibers, possibly leading to structural heterogeneity. Indeed, the extraction process of KI requires hard conditions, and the fibrous structure can be fragmented, and the fibers network can

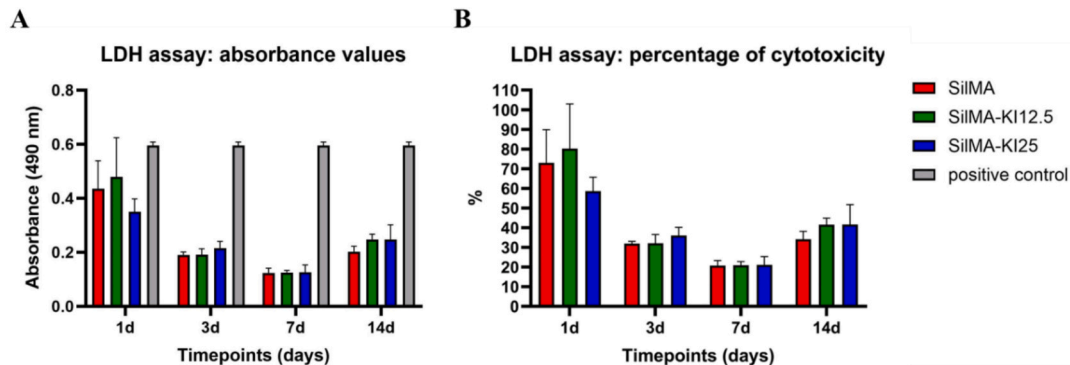


Fig. 5. Results of the LDH assay: (A) raw absorbance values measured at 490 nm for each experimental sample; (B) corresponding cytotoxicity levels normalized to the positive control.

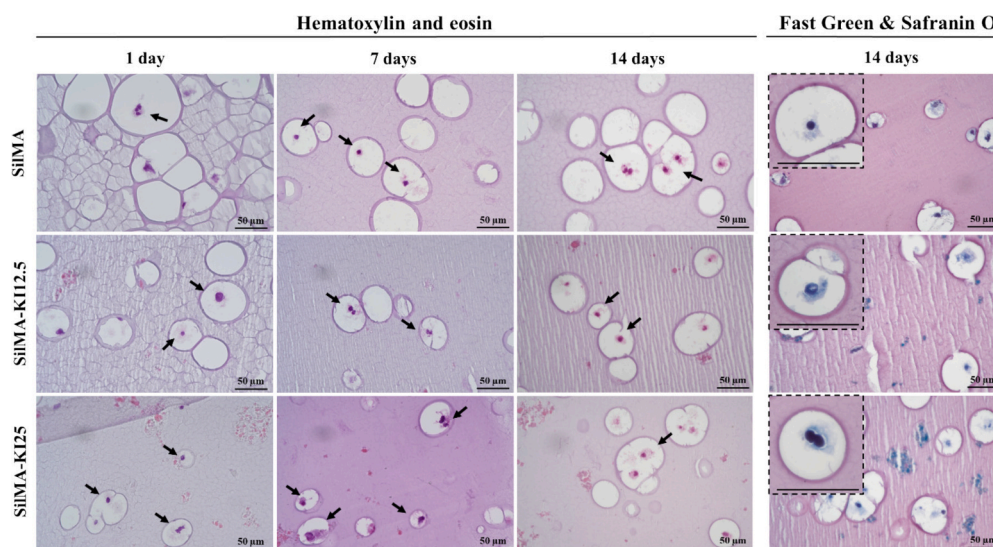


Fig. 6. Hematoxylin and Eosin and Safranin-O-Fast Green staining of SilMA-KI hydrogels at 1d,7d, and 14d. Scale bar 50 μm (the arrows indicate the cells embedded in the hydrogel matrix).

be damaged [42,43]. Our best finding in wet conditions (0.01342 ± 0.0220 MPa) differ significantly from the compression modulus reported in literature for human acetabular labrum (31.75 ± 16.7 MPa) [44]. However, the test we carried out was not confined, whereas the result on the human labrum was from a confined test, and this could have a significant impact on the result. In fact, the only other unconfined compression test mentioned in the literature involved the bovine acetabular labrum, which had a lower compression modulus (0.157 ± 0.057 MPa) [45].

Although the measured values remained an order of magnitude higher than our initial findings, they were more closely aligned with our results.

This discrepancy can also be attributed to the intrinsic mechanical limitations of our bioengineered construct, which is composed of a soft biopolymeric matrix embedded with living cells. Such materials typically exhibit substantially lower stiffness than purely synthetic polymers or native fibrous tissues, as they are designed to support cellular viability and remodeling rather than bear high mechanical loads.

Additionally, the hydrogel samples used for the tests were small constructs that do not reproduce the anisotropic, fiber-reinforced architecture of native labral tissue.

Further investigation, after this initial assessment, will focus on developing prototypes that more closely mimic native tissue in terms of geometry and structure, enabling functional mechanical validation.

Indeed, the native acetabular labrum is primarily subjected to circumferential tensile loading in vivo, and evaluation under tensile, dynamic, or cyclic conditions is critical to assess suitability for labral replacement. At this stage, however, the mechanical tests performed in our study represent a preliminary characterization of the developed hydrogel formulations, intended to evaluate the baseline viscoelastic and structural behavior rather than fully replicating the mechanical performance of native labral tissue.

Biological evaluation using the human chondrocyte cell line C28/I2 demonstrated similar data on cytotoxicity for all the three hydrogels, regardless of the content of KI and its concentration. LDH release, an indicator of cytotoxicity and membrane damage, was found elevated at

1d, probably due to the initial stress from cell encapsulation and handling. Indeed, from 3d of culture up to 14d, the release of LDH was notably lower, especially when compared to positive control, suggesting sustained cell viability and minimal cytotoxicity. Indeed, a significant decrease in LDH release was detected overtime for all the three formulations of biomaterials. These results are consistent with previous studies demonstrating a good biocompatibility of SF and KI, also thanks to KI intrinsic expression of cell attachment sequences such as LDV and RGD [46,47], making our hydrogels good candidates for tissue engineering applications [48].

Further investigations are required to include additional analyses (i.e., metabolic activity assays, live/dead staining) to provide a more comprehensive biological characterization, even if obtaining high-quality cryostat sections of these highly hydrated hydrogel constructs was technically quite challenging due to their soft and water-rich nature. However, considerable optimization will be required to obtain reproducible sections for additional histological or immunofluorescence analyses.

Notably, by the later timepoints of culture, cells exhibited early ECM production, as detected by histological and TEM analysis. Initial production and deposition of proteoglycans were observed inside cells at high resolution, indicating that the composite hydrogel supports chondrocyte biosynthetic activity and may promote the development of fibrocartilage-like tissue.

Overall, the data demonstrated that SilMA-KI hydrogels possess favorable rheological and mechanical properties, as well as biocompatibility. The reinforcement provided by KI fibers contributes to the enhancement of the mechanical integrity and potential functionality of the SilMA hydrogels under physiological conditions, highlighting the potential as scaffold materials for labral tissue engineering and offering a first step toward biomimetic regeneration of fibrocartilaginous structures.

Future studies will focus on long-term evaluation of cell-matrix interaction and on the application of these hydrogels in additive manufacturing platforms to generate anatomically relevant, multilayered constructs for clinical translation.

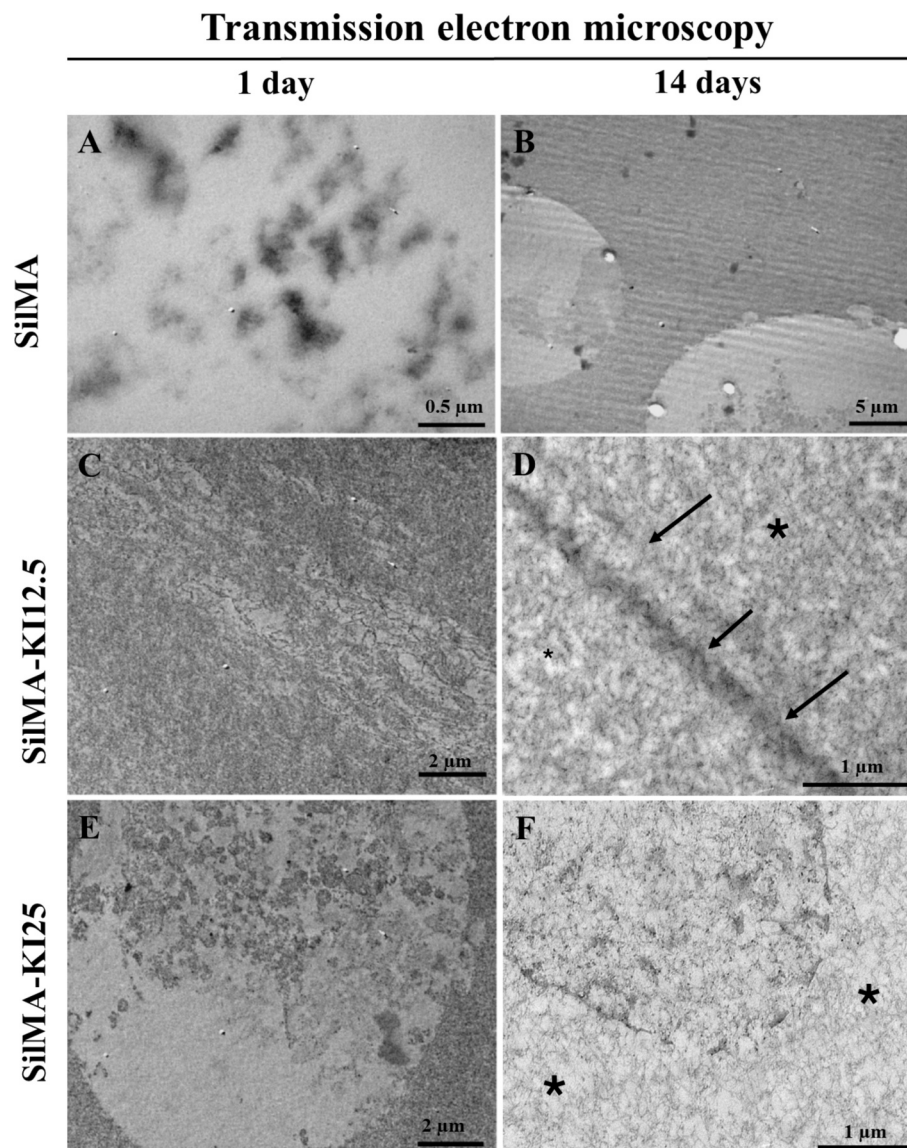


Fig. 7. TEM microphotographs of SilMA-KI hydrogels highlight the production of proteoglycans at 1d and 14d. Asterisks indicate proteoglycan and arrows indicate keratin fibers.

5. Conclusion

This study presents the successful development and characterization of SilMA-based hydrogels, incorporating KI, as promising biomimetic materials for acetabular labrum regeneration.

SilMA-KI hydrogels exhibited near Newtonian rheological behavior and favorable injectability profiles, supporting their suitability for extrusion-based 3D bioprinting. The incorporation of KI significantly modulated the mechanical properties of the hydrogels under both dry and wet conditions, enhancing the elastic modulus. In addition, the hydrogels showed successful fluid flow and biocompatibility with human chondrocyte cell lines, and early signs of ECM production.

These findings highlight the potential of SilMA-KI composite hydrogels as functional scaffolds for labral tissue engineering.

Further investigations are required to assess the potential fabrication of scaffolds and their performance in anatomically relevant microenvironment, that is essential to advance clinical translation.

CRediT authorship contribution statement

Giorgia Borciani: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Alessio Bucciarelli:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Michela Battistelli:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Amber van der Beek:** Investigation. **Eugenia Spessot:** Writing – review & editing, Investigation. **Devid Maniglio:** Writing – review & editing, Funding acquisition. **Keita Ito:** Writing – review & editing. **Brunella Grigolo:** Writing – review & editing, Resources. **Eleonora Olivotto:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Brunella Grigolo reports financial support was provided by Italian Ministry of Health. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioadv.2026.214843>.

Data availability

Data will be made available on request.

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