

Article

UV-Crosslinking Effects on the Physicochemical and Rheological Properties of Fish Collagen Ink for 3D Bioprinting

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Abstract

Three-dimensional bioprinting revolutionized tissue and organ replacement by enabling the precise deposition of living cells and biomaterials, making it ideal for biomedical applications. Natural polymers are commonly used as bioink for their biocompatibility and bioactivity. Among them, type I collagen, the most abundant protein of extracellular matrix, is commonly used as bioink. However, mammalian-derived collagens raise concerns related to zoonotic disease transmission, religious restrictions, and immunogenicity. Fish-derived collagen represents a safer and more sustainable alternative, although its rapid degradation and limited mechanical properties remain significant challenges. In this study, the printability of a novel fish collagen ink was assessed for micropatterned scaffolding by extrusion. In order to overcome material-related challenges, the effect of UV-induced crosslinking was investigated. Morphological, rheological, and physicochemical characterizations—including thermal behavior, degradation resistance, exposed chemical groups, and roughness—were performed before and after UV treatment. Results demonstrated that UV crosslinking significantly improved the structural integrity and stability of the printed scaffolds. These findings support the potential of UV-crosslinked fish collagen as biomaterial ink for regenerative medicine and tissue engineering applications.

Keywords: 3D bioprinting; fish collagen; tissue engineering; UV crosslinking; micropatterned scaffolding

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1. Introduction

Over the past three decades, three-dimensional (3D) bioprinting started to be employed for creating functional substitutes of living tissues and organs by overcoming the limitations of conventional fabrication methods of 3D scaffolds. The origins of 3D bioprinting can be traced back to 1988 when Klebe modified a standard Hewlett-Packard inkjet printer to deposit cells through a process called cytoscribing [1]. Since then, numerous 3D printing technologies have been adapted for biomedical applications [2], mainly including extrusion-based, droplet-based, and energy-based technologies. Extrusion-based modalities utilize mechanical force (i.e., pneumatic pressure, piston, plunger, or rotating screw) to extrude a continuous stream of bioink from a nozzle onto a printing platform or into a liquid medium. Droplet-based methods (e.g., inkjet or laser-assisted

printing) generate and deposit discrete droplets of bioink, offering higher resolution but suffering from limited material compatibility. Energy-based systems use focused light (e.g., stereolithography or two-photon polymerization) to solidify photosensitive bioinks with ultra-fine precision. Currently, most 3D bioprinting methods rely on extrusion-based printed scaffolds, where cells and/or growth factors are embedded within ad hoc developed bioinks [3,4]. The development of new bioinks poses challenges as they have to serve multiple purposes, as they need to have an appropriate viscosity and maintain a defined geometry after printing, as well as allow cell growth and not hinder mass transport [5].

The most widely used biomaterials in 3D bioprinting are hydrogel-based inks. Both natural and synthetic polymers revealed to be promising inks thanks to the possibility to tune their printability and mechanical performance [6–9]. Among natural polymers, collagen, gelatin, alginate, hyaluronic acid, chitosan, dextran, and fibrin stand out as natural polymers suitable for bioprinting, combining structural properties with biocompatibility [10–13].

Among these materials, collagen is one of the most preferred. Type I collagen is the most abundant protein of the extracellular matrix (ECM) and plays a crucial role in maintaining the integrity and the architecture of connective tissues. With collagen accounting for a significant portion of the total protein in vertebrates, it possesses several desirable characteristics such as low immunogenicity, biocompatibility, biodegradability, and the ability to support cell adhesion, migration, and differentiation [14].

Native-like collagen is soluble in dilute acid and has the capability to self-assemble into fibrous hydrogels through noncovalent interactions including hydrogen bonding, electrostatic force, and hydrophobic interactions [15]. Consequently, type I collagen has found extensive applications in the preparation of hydrogel scaffolds for tissue engineering and repair. Despite the favorable biological properties of collagen scaffolds, their rapid degradation and poor mechanical strength impose limitations on their application. Additionally, its insolubility in physiological-like conditions hinders its applicative area because of the limited compliance with cell-based approaches. Moreover, commercially available collagen inks are primarily sourced from bovine and porcine tissues, mammals that present various concerns, including the potential transmission of prions, religious restrictions, and allergic reactions. In this circumstance, marine collagen represents a valid and promising alternative, because of its freedom from all aforementioned issues. However, although fish collagen has some undoubted advantages, it suffers from an even lower stability in terms of time and mechanical resistance.

In this work, a novel fish collagen ink was proposed as an alternative to conventional mammalian-derived collagen products. In particular, this study is part of a broader research line focused on the exploitation of fish industry waste for biopolymer extraction intended for biomedical applications (Figure 1) [16–20]. The prototypal biomaterial ink was developed starting from native type I collagen extracted from Tilapia skin, by means of a proprietary process. Advantages in aquaponic Tilapia skin use as raw material for the development of biomaterials were argued elsewhere [16,21]. A distinctive feature of this prototype is the ready-to-use hydrogel state of the reconstituted native collagen in physiological-like conditions as well as the absence of chemical agents for crosslinking, which are typically employed in biomaterial ink formulations.

To the best of our knowledge, no studies have reported the 3D bioprinting of marine type I collagen as a standalone material. Due to its inherently low mechanical stability and limited printability, marine collagen is typically processed in combination with other polymers or chemically modified to enhance its rheological and structural properties. Existing approaches frequently involve blending marine collagen with synthetic polymers such as polycaprolactone, hydroxyapatite, alginate or introducing functional groups—most commonly methacrylation—to enable photocrosslinking and improve shape fidelity

[22–27]. Consequently, the literature describes marine collagen predominantly as a component of composite or chemically modified inks, rather than as an unmodified, single-component printable biomaterial.

Within the overall project workflow, the present study is intentionally positioned at the material and process optimization stage, aiming to demonstrate the potential of a fish-skin-derived biomaterial ink for biomedical applications and to assess the feasibility of enhancing its structural integrity through zero-length, mild UV crosslinking treatments. Herein, efforts were made towards the investigation and optimization of UV crosslinking to further enhance the mechanical strength and stability of the biomaterial ink over extended periods of time for assuring its compliance with long-term applications. After the printability assessment of the biomaterial ink via extrusion-based 3D printing, a comprehensive investigation was performed on a two-layered model before and after UV exposure, including the examination of thermal stability, rheological properties, degradation resistance, and morphological characteristics, correlating these parameters with the printability of the biomaterial ink to provide a full understanding of its performance.

Thus, this work paves the way for the development of next-generation marine-derived biomaterial inks by demonstrating a strategy that combines biocompatibility, sustainability, and functional tunability. The integrated evaluation approach adopted provides valuable insights into the interplay between physicochemical properties and printability, offering a solid framework for the rational design of biomaterial inks suited for extrusion 3D bioprinting applications.

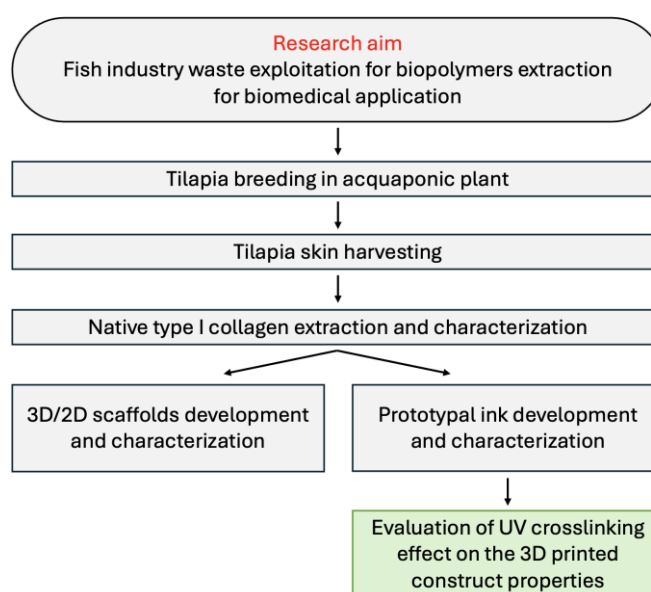


Figure 1. Flowchart of the research line focused on the exploitation of fish industry waste for biopolymer extraction intended for biomedical applications. The activities reported in this work are framed in green.

2. Materials and Methods

2.1. Materials

A prototypal type I collagen biomaterial ink from Nile Tilapia (*Oreochromis Niloticus*) skin was produced by Typeone Biomaterials S.r.l. (Calimera, Italy) by a proprietary process and provided in ready-to-use 1-mL syringes, dispersed in neutral buffered solution (0.01 M PBS, pH 7.4), at a concentration of $3.0 \pm 0.3\%$ (w/v). Tilapia fish of the same age (16–18 months) and comparable size (25×8 cm), reared in a pilot aquaponic plant (“Urban Farming Lab” of the Department of Innovation Engineering, University of Salento, Lecce, Italy) under identical environmental and feeding conditions (conventional feeding), were chosen for the collagenous biomaterial ink production (Figure 2) [16,20]. This approach was intentionally adopted to minimize biological variability at the raw-material level and to improve batch-to-batch consistency of the extracted collagen. All information about Tilapia fishes and breeding conditions were accurately detailed in a previous work [20]. All information about the properties of the type I collagen extracted from Tilapia skin that has been employed for the development of the prototypal biomaterial ink were accurately reported in a previous work [16]. If not otherwise stated, all chemicals were provided by Merck (Darmstadt, Germany).

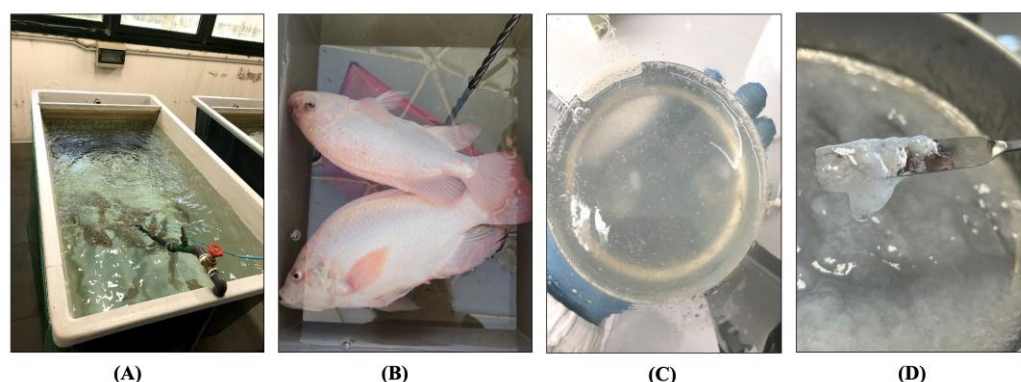


Figure 2. Overview of the biomaterial ink development workflow including: (A) the pilot aquaponic plant used for Tilapia breeding; (B) representative Tilapia specimen grown in the aquaponic system; (C) type I collagen extracted from Tilapia skin; and (D) prototypal collagen-based ink developed from the extracted material.

2.2. 3D Printing process of Biomaterial Ink

Two layered collagen scaffold prototypes with a thickness of 0.5 mm were printed at room temperature (25 ± 2 °C) and humidity ($40 \pm 10\%$) using a Anycubic i3 Mega S 3D printer (Shenzhen Anycubic Technology Co., Guangdong, China). A 18 G needle was used (Figure 3). Immediately after 3D printing, crosslinking of constructs was performed using a 1000 W UV light at 365 nm for 1, 2, and 3 min under open and ventilated conditions in order to limit heat accumulation. Crosslinking was performed at room temperature in order to mimic typical operating conditions for cell-laden biomaterial inks. The distance between the sample and the UV lamp was maintained at a constant 11 cm. Table 1 shows the sample codes acquired after varying UV irradiation times.

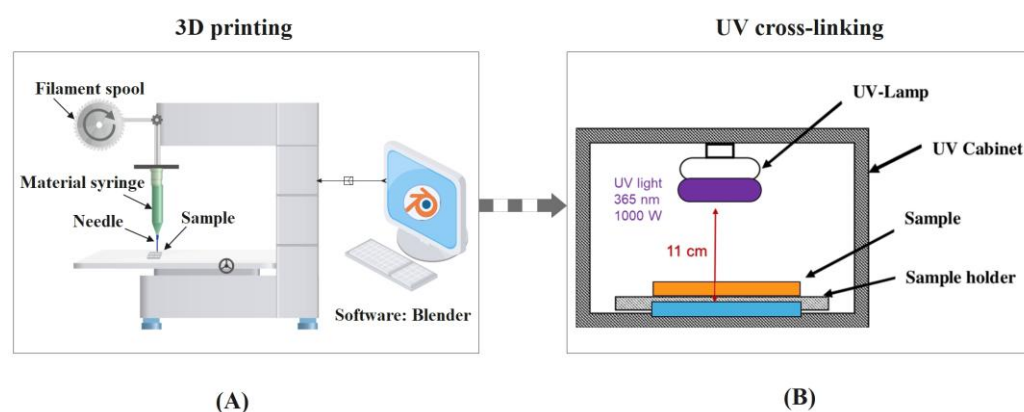


Figure 3. Schematic representation of (A) the 3D printing process and (B) the following UV crosslinking of the 3D printed collagen scaffolds.

Table 1. Sample description obtained from different UV irradiation times.

Sample Code	Description
0 min	Not crosslinked ink
1 min	Crosslinked ink by 1 min UV exposure
2 min	Crosslinked ink by 2 min UV exposure
3 min	Crosslinked ink by 3 min UV exposure

2.3. Filament Spreading Degree

Post-printing filament spreading is a parameter that can be used to compare the influence of bioink formulations and different crosslinking strategies on the printed construct architecture quality. To evaluate the shape fidelity retention of extruded filaments, the extent of filament spreading was assessed after 3D printing and UV crosslinking. Bio-material ink strands were printed in straight-line patterns (20 mm length) onto a flat substrate under controlled environmental conditions (25 ± 2 °C, $50 \pm 5\%$ RH). Images of the printed filaments were captured with a Dino-Lite digital microscope (AnMo Electronics Corporation, New Taipei City, Taiwan). Filament diameter (dp) was measured at 5 points per strand using ImageJ v1.54, (a three strands for condition were analyzed, 5 measurements were registered for each strand) after calibration with the Dino-Lite scale bar. The Filament Spreading Ratio (FSR) post printing was quantified as the ratio of the printed filament diameter and the nozzle diameter (dn) [28–31], as in the following equation:

$$FSR = \frac{dp}{dn} \quad (1)$$

A FSR near to 1 was considered indicative of good shape fidelity and minimal spreading for collagen hydrogels.

2.4. Rheological Properties

Rheological measurements of 3D printed and crosslinked hydrogels were conducted using dynamic mechanical analysis (DMA) with a parallel plate ARES rheometer (Rheometric Scientific, Piscataway, NJ, USA). Collagen hydrogels of 25 mm in diameter and 0.5 mm in thickness were placed between two parallel plates, each having a diameter of 25 mm, separated by a 0.5 mm gap. The upper plate was slowly lowered to the selected gap under controlled conditions, allowing the samples to fully adhere homogeneously to the plate without inducing pre-stress. Prior to testing, samples were allowed to equilibrate at the testing gap for 5 min. To determine the linear viscoelastic range (LVR) and the critical strain, oscillatory strain sweep measurements were performed at a frequency of 1

Hz, spanning a strain range from 0.01% to 100% [32]. Subsequently, frequency sweep tests within the LVR were conducted across frequencies ranging from 0.01 Hz to 16 Hz, maintaining a linear strain of 1% at a temperature of 20 ± 2 °C [33]. Steady-state rheological measurements were also performed, covering a shear rate range from 0.1 s^{-1} to 1000 s^{-1} , also at 20 ± 2 °C. For each type of sample, at least three independent measurements were carried out for each sample type to ensure the reliability and reproducibility of the data.

2.5. Morphological Properties

An EVO® 40 Scanning Electron Microscope (SEM) (Carl Zeiss AG, Jena, Germany) was utilized to examine the surface morphology and microstructure of the 3D-printed scaffolds. Immediately after being 3D-printed and UV-crosslinked, samples were freeze-dried by means of a LIO-5P freeze-drier (Cinquepascal s.r.l., Trezzano sul Naviglio, Italy) for 24 h ($P < 100 \text{ mTorr}$). Then, observations were performed at 20 kV accelerating voltage in VPSE mode to ensure optimal topographical contrast.

2.6. Surface Topography

Atomic force microscopy (AFM) was used to characterize the surface morphology of collagen samples through a MultiMode 8 AFM system (Bruker, Champs sur Marne, France) [34]. AFM analysis was performed with a silicon tip on a nitride lever cantilever having a spring constant of 0.4 N/m (2 nm tip radius, 115 μm length) in the Peak Force Quantitative Nanomechanical Mapping (QNM) mode with a scanasyst-air probe. The analysis was performed on air-dried samples that were dried for 72 h under flow hood at room temperature [35]. The scanning parameters were as follows: scan sizes of $1 \times 1 \mu\text{m}^2$ and $5 \times 5 \mu\text{m}^2$, scanning rates in the 0.640–0.574 Hz range, and resolution set at 512 lines per scan. ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA) and Nanoscope Analysis v.1.5 software were used to process AFM data and observe the ladder structure of collagen.

2.7. Spectroscopic Evaluation

Attenuated total reflectance spectroscopy (ATR) analysis was performed with a Jasco 6300 spectrometer (JASCO Corporation, Tokyo, Japan). After UV crosslinking, 3D-printed samples were let dry under flow hood for 72 h at room temperature before analysis in order to remove water molecule contributes. Infrared spectra were acquired in the wavelength range between 400 and 4000 cm^{-1} , 128 scans, and 4 cm^{-1} of resolution [36,37].

2.8. Thermal Properties

Biomaterial ink samples underwent thermal analysis using a Q2000 Series Differential Scanning Calorimeter (DSC) (TA Instruments, New Castle, Delaware, USA). Approximately 5 mg of samples were equilibrated in drilled sample pans at 10 °C for 5 min. They were then subjected to heating at a rate of 5 °C/min from 10 °C to 80 °C under nitrogen atmosphere [16,38]. For each sample, a minimum of three measurements were conducted. Denaturation temperature (T_d) was defined as the peak of the endothermic phenomenon observed in the heat flow versus temperature plot. Denaturation enthalpy (ΔH), which corresponds to the energy required for the transition, was also calculated relative to the collagen mass of the sample [39].

2.9. Swelling Degree

The construct ability to retain water and the influence of the crosslinking strategy on it was gravimetrically evaluated. Freeze-dried samples of approximately 20 mg (W_0) were accurately weighted and soaked in 1 mL of 0.01 M PBS. After the gentle removal of excess

surface water with filter paper at prefixed time points (W_t), the weight of samples was registered and used to calculate the % swelling degree ($S\%$) by the following equation [32,38]:

$$S\% = \frac{W_t - W_0}{W_0} 100, \quad (2)$$

The experiment was performed in triplicate for each sample type.

2.10. Resistance to Degradation

The degradation resistance of constructs was estimated in simulated physiological-like conditions. Samples of about 50 mg were weighted (W_0) before being soaked in 1.5 mL of 0.01 M PBS (pH 7.4) with 0.001% sodium azide (w/v) and incubated at 37 ± 1 °C. At fixed time points, 0.05 mL were withdrawn to determine the released protein content by means of the bicinchoninic acid assay kit (Merk). More in detail, 0.05 mL of supernatant were withdrawn from each sample and were diluted up to 0.150 mL with distilled water before being incubated with 0.150 mL of BCA-based solution prepared according the manufacturer instructions [32]. Samples were incubated at 60 ± 1 °C for 1 h. The absorbance of the purple-colored Cu^{2+} -BCA complex of the samples was acquired at 562 nm by means of the Envision® Multimode Plate Reader (PerkinElmer Inc., Waltham, MA, USA). A calibration line was prepared by serially diluting a 0.05 mg/mL bovine serum albumine solution and applying the Lambert–Beer law. The degraded sample amount was then determined by interpolation from the calibration line. Thus, the degradation resistance ($D\%$) of samples was expressed as the remaining weight % using the following equation:

$$D\% = 1 - \frac{W_0 - W_t}{W_0} 100, \quad (3)$$

where W_t is the esteemed remaining weight of the sample during time and W_0 is the dry initial mass. Each sample type was tested thrice.

2.11. Crosslinking Density

The content of free primary amine groups of uncrosslinked and UV-crosslinked samples was determined by means of the colorimetric TNBS assay following a protocol previously optimized for collagen-based substrates [32,40]. Approximately 3–5 mg of samples were equilibrated for 15 min in 0.5 mL of 4% (w/v) of sodium bicarbonate. Then each sample received an additional 0.5 mL of a freshly prepared solution of 0.05% (w/v) TNBS, before being hermetically closed and heated at 40 °C for 2 h. Subsequently, 1.5 mL of 6 M HCl were added, and samples were hydrolyzed at 60 °C for 90 min. Upon completion of the reaction, 2.5 mL of distilled water was added. Once the samples were cooled down to room temperature, absorbance was acquired at a wavelength of 320 nm by means of an Envision® Multimode Plate Reader (PerkinElmer Inc., Waltham, MA, USA). Control samples were prepared following the same protocol, with the exception that hydrochloric acid was added before the reactive, in order to prevent any reaction between TNBS and free amine groups. The linear calibration curve was constructed by correlating the absorbance values with the concentrations of a series of dilutions of glycine (0.1 mg/mL, w/v) in a 4% (w/v) sodium bicarbonate solution. Each measurement was performed in triplicate for each sample type, and the amount of free amino groups per 1000 residues was calculated accordingly. The untreated biomaterial ink was considered to represent 100% of the available free amine groups (Free $-\text{NH}_2$ i), and this reference value was used to determine the crosslinking degree ($CD\%$) in UV-exposed samples, expressed as the percentage of free amine groups remaining after the crosslinking process (Free $-\text{NH}_2$ f) [40].

$$CD\% = \frac{Free-NH_2i}{Free-NH_2f} 100, \quad (4)$$

2.12. Statistical Analysis

All data were reported as the mean $\pm$ standard deviation (SD) of at least three independent experiments. Statistical comparisons between groups were performed using the unpaired Student *t*-test. A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. 3D Printing of Biomaterial Ink

Two consecutive layers of the prototypal fish collagen-based ink were successfully printed following a standard grid-like geometry, confirming the printability and the feasibility of multilayer deposition. The resulting constructs (Figure 4) exhibited uniform layer deposition and well-defined geometrical features, indicative of good printability and precise shape fidelity. The reproducibility of the printing performance was preliminarily evaluated across independent collagen batches produced using the same extraction protocol. Importantly, collagen was extracted from fish of the same age and comparable size, all reared in a prototypal aquaponic facility under identical environmental and feeding conditions. This approach was intentionally adopted to minimize biological variability at the raw-material level and to improve the batch-to-batch consistency of the extracted collagen. However, a limited degree of batch-to-batch variability was observed, which is expected for naturally derived biomaterials. This variability was primarily addressed by fine-tuning the biomaterial ink concentration, which was systematically adjusted based on pre-printing viscosity measurements to ensure reproducible extrusion behavior. Through this optimization strategy, a working ink concentration of 3.0% ($\pm 0.3\%$) was identified as optimal for reliable printing across batches. By adjusting the concentration within this narrow range, consistent rheological properties and printing performance were achieved, effectively compensating for the minor batch-to-batch differences of collagenous materials.

No signs of filament spreading or collapse were observed during the deposition process, suggesting the adequate rheological properties of the material. A slight collapse was observed in the uncrosslinked sample (0 min), suggesting the need to adopt a crosslinking strategy for structural stability. Thus, after printing, scaffolds were immediately subjected to UV irradiation. Theoretically, UV exposure is expected to induce network stabilization through the generation of free radicals on aromatic amino acid residues, such as tyrosine and phenylalanine, which can form intermolecular bonds via their delocalized π -electron systems [41,42]. However, due to the non-specific nature of radical-mediated reactions, a defined chemical crosslinking pathway cannot be unequivocally identified. Notably, the overall architecture of the constructs remained unchanged following exposure, confirming that the ink maintained its structural integrity throughout the crosslinking process. No macroscopic signs of thermal damage were observed after UV exposure at the maximum investigated time. The effect of UV irradiation was clearly appreciable, with an exposure time dependent increased maintenance of the geometry. In particular, after 1 min of UV irradiation, only a slight improvement in structural stability was observed, with the printed constructs resembling the uncrosslinked condition in terms of filament spreading and partial loss of geometry. In contrast, the extension of the UV exposure to 2 min significantly enhanced the preservation of the printed shape, resulting in well-maintained filaments and clear inter-filament spacing. Interestingly, a further increase to 3 min of UV exposure did not lead to a noticeable improvement. On the contrary, the overall geometry appeared comparable, if not slightly compromised, compared to the 2 min condition. This

suggests that prolonged exposure does not necessarily correlate with better crosslinking performance and may even affect the construct's microstructure or introduce unwanted effects such as material properties loss. Indeed, it is known that UV radiation can disrupt the hydrogen bonds within and across the collagen matrix [41,42]. Additionally, a partial denaturation due to heat generation after 3 min exposure should not be excluded.

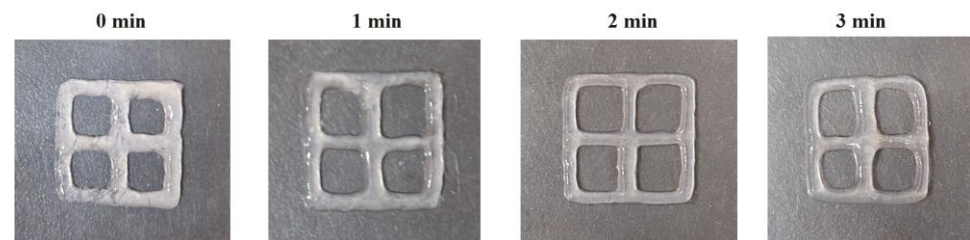


Figure 4. Macroscopic images of 3D-printed collagen scaffolds obtained via extrusion-based bi-printing. Uniform layer deposition and well-defined geometrical features are evident, demonstrating the good printability and structural integrity of the biomaterial ink. After printing, scaffolds were subsequently crosslinked under UV irradiation.

3.2. Filament Spreading

The ink's ability to retain its shape after printing was evaluated by means of FSR degree calculation. As is known, values approaching 1 indicate optimal structural fidelity retention ability and optimal printing conditions. When the deposited filament closely matches the nozzle diameter and minimal lateral spreading occurs, a favorable balance between ink viscoelasticity and early-stage network stabilization is obtained. In this study, FSR values were found to be higher than 1 in all conditions and in line with the literature data about collagen-based inks [29,31]. The uncrosslinked sample (0 min) revealed to have a FSR of about 2.8 ± 0.5 , confirming its printability and acceptable structure retention ability. UV exposure effectively reduced filament spreading, with a time-dependent effect. After 1 min of UV light exposure (1 min), FSR decreased to 2.4 ± 0.3 , with no significant change compared to the 0 min sample ($p = 0.2$), suggesting that there was insufficient crosslinking to prevent hydrogel relaxation. After 2 min of UV-light (2 min), FSR was found to be 1.8 ± 0.1 ($p = 0.01$ compared to 0 min), indicating a significant stabilization of the printed matrix. Conversely, 3 min of UV exposure were found to not significantly improve the hydrogel structural fidelity (FSR = 2.6 ± 0.1 , $p = 0.4$ compared to 0 min). A partial loss of fidelity occurred likely due to overcrosslinking or collagen denaturation due to heat generation.

3.3. Rheological Characterization

The rheological plot in Figure 5A showed that all the investigated samples exhibited a continuous decrease in viscosity with an increase in the shear rate at room temperature, indicative of a shear-thinning response typical of non-Newtonian fluids. This behavior was found to be typical of collagen-based inks [31,43,44]. The viscosity values measured at a shear rate of 1 s^{-1} at room temperature are reported in Table 2. These values were found to be similar to viscosity values of a fibrillar bovine collagen ink at 3.5% measured at 4°C [31], suggesting the higher viscosity of the fish collagen ink. The viscosity of the fish collagen ink was also found to be lower than a 2% fibrillar bovine collagen ink tested at room temperature, suggesting in this case the lower properties of the material object of this study [43]. However, because of missing data about the native structure preservation degree, comparison analysis could not be easily performed. In particular, after assessing the non-extrudability of basa skin collagen alone, in the work of Cavallo et al., basa skin collagen at 1–2% was mixed with 6% alginate, reaching viscosity values similar to that of

our fish collagen ink [24]. The shear-thinning behavior observed in these samples suggests that the internal structure of the materials undergoes alignment or reorganization under shear, reducing resistance to flow. This property is required in bioink for extrusion 3D printing, where ease of application and flow are critical.

Table 2. Shear moduli (at 1%), critical strain, and cohesion energy for crosslinked fish collagen ink obtained from strain sweep tests.

Sample Code	G' (Pa)	G'' (Pa)	G'_{crit} (Pa)	γ_{crit} (%)	E_c (KJ/m ³)	$\tan \delta$
0 min	112 ± 19	33 ± 6	101.19 ± 18	23.76 ± 0.25	28.6 ± 0.6	0.29 ± 0.01
1 min	108 ± 15	30 ± 4	99.70 ± 16	29.86 ± 0.22	44.5 ± 0.8	0.27 ± 0.02
2 min	251 ± 18	63 ± 2	227.09 ± 15	7.50 ± 0.05	6.4 ± 0.3	0.35 ± 0.01
3 min	128 ± 11	55 ± 5	115.06 ± 10	29.93 ± 0.18	51.5 ± 0.9	0.24 ± 0.02

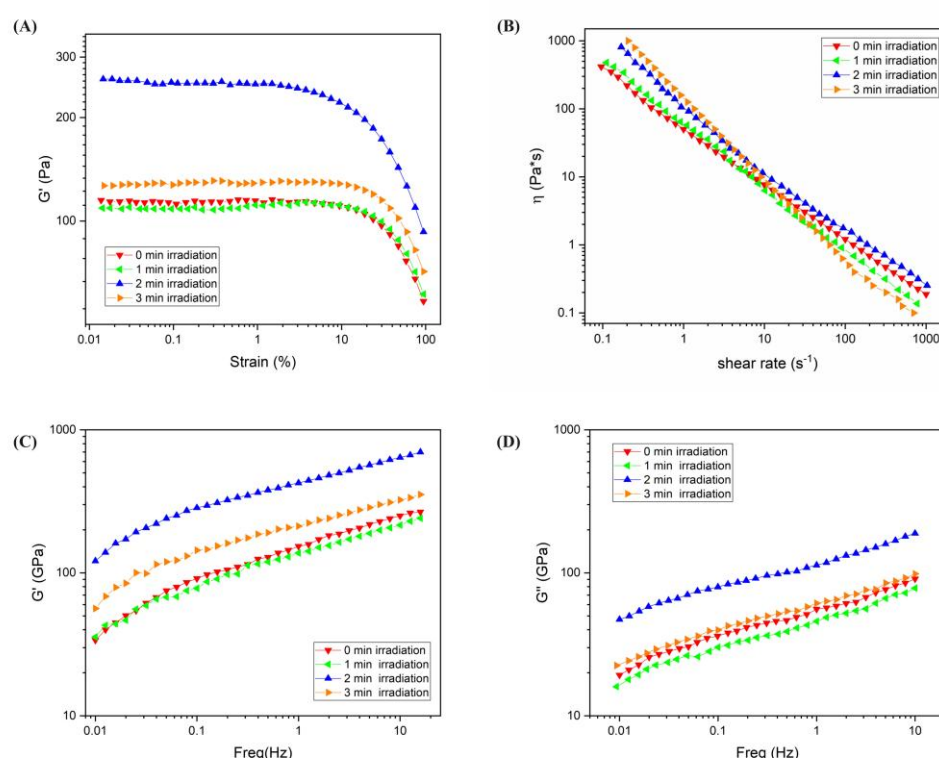


Figure 5. Rheological properties of uncrosslinked and UV-crosslinked fish collagen ink including (A) dynamic strain sweep at constant frequency (1 Hz), (B) viscosity measurement as a function of the shear rate, and G' (C) and G'' (D) in dynamic frequency sweep at constant strain (1%).

Figure 5B illustrates the evolution of the storage shear modulus (G') as a function of shear strain in a dynamic strain sweep test conducted at 1 Hz and 20 °C. At low strain values, G' was higher than the loss modulus (G'') for all tested samples, indicating a gel-like behavior in the linear viscoelastic region [31,43,44]. As detailed in Table 2, notable differences emerged among samples. The 0 min and 1 min samples exhibited a similar viscoelastic behavior, with the lowest initial G' values, approximately four times higher than the corresponding G'' values. Interestingly, the 2 min sample displayed a slight decrease in G' but a more pronounced reduction in G'' , suggesting a change in the internal network structure of the collagen gel influenced by UV irradiation. The effect of UV cross-linking was clearly observable and appeared to peak at 2 min, after which a decrease in both G' and G'' values was observed. Notably, the 2 min sample exhibited the highest viscoelastic properties, with G' exceeding G'' by more than fourfold. These findings

highlight the delicate balance between UV exposure time and the resulting rheological behavior of the biomaterial ink, which is crucial for optimizing its printability and mechanical stability in biomedical applications.

During the strain sweep test, the G' remained constant until the oscillation stress surpassed the intermolecular forces that define the linear viscoelastic region. The strain value at which G' decreased by more than 10% from its maximum initial value was designated as the critical strain (γ_{crit}). This critical strain marks the onset of nonlinear viscoelastic behavior in the polymer. The critical strain represents the maximum shear strain that can be applied to the system while maintaining linear viscoelasticity. Beyond this point, the material's response becomes nonlinear, indicating that the intermolecular network structure begins to break down or rearrange significantly. Identifying this threshold is crucial for understanding the mechanical limits of the material and for optimizing its performance in applications where maintaining linear viscoelasticity is essential. This insight helps in designing and tailoring polymer systems for specific mechanical and functional requirements in various industrial and biomedical applications. The γ_{crit} was used for calculating the cohesion energy (E_c) as follows:

$$E_c = \frac{1}{2} \gamma_{crit}^2 G_{crit}, \quad (5)$$

where G_{crit} represents the storage modulus at the γ_{crit} . The cohesion energy corresponds to the energy necessary for forming physical crosslinks between the polymer chains [45,46]. The critical strains for all analyzed samples are reported in Table 2. Notably, the 2 min samples exhibited the minimum critical strain of 7.5%, while the other samples showed critical strains around 29%.

Significant differences were also observed in the cohesion energy values, as detailed in Table 2. The 3 min sample exhibited the highest cohesion energy (51.5 kJ/m³), suggesting stronger and more distributed physical crosslinks between polymer chains. Conversely, the 2 min sample—despite displaying the highest storage modulus (G')—showed the lowest cohesion energy (6.4 kJ/m³). This apparent cohesion inconsistency can be explained by recalling that the storage modulus (G') and energy (E_c) describe different mechanical aspects of the network as follows: while G' reflects the stiffness of the material under small deformations, E_c accounts for the total energy that the network can absorb before structural breakdown, thus incorporating both stiffness and deformability. Although the 2 min sample exhibits the highest G' , it also shows the lowest critical strain, indicating that the network fails at relatively small deformations. This behavior suggests the formation of a highly rigid but brittle network, in which the increased crosslink density enhances stiffness but reduces the ability of the structure to dissipate energy under large deformations. The term “optimal” should thus be interpreted. In contrast, the 3 min sample may have formed a more ductile network with broader energy dissipation before failure, accounting for its higher cohesion energy despite a lower modulus. These findings highlight the complex interplay between crosslinking duration, network stiffness, and fracture behavior in collagen hydrogels. Understanding such relationships is essential for tailoring the mechanical and structural properties of bioinks for specific biomedical applications also taking into account that the optimal formulation for printing applications requires high elastic response and shape fidelity under small deformations (e.g., printability and dimensional stability), rather than maximum toughness or resistance to fracture [47,48].

Dynamic frequency sweeps were conducted at a constant shear strain within the linear viscoelastic region, where the material response is independent of the deformation magnitude. The DMA curves, shown in Figure 5C, reveal that the G' gradually increases with frequency. Additionally, G' remained higher than the G'' across the entire experimental frequency range, with G' to G'' ratios similar to those obtained in the strain sweep

tests. This indicates that all the samples behaved as self-standing hydrogels, with elastic behavior dominating over viscous behavior. However, significant differences were observed based on the UV crosslinking time of the samples. The sample subjected to 2 min of UV crosslinking demonstrated significantly higher elasticity, characterized by a G' value substantially greater than the G'' value. In contrast, other samples exhibited G' values that were four times lower than their corresponding G'' values, indicating a less pronounced elastic behavior. These findings underscore the impact of UV crosslinking time on the mechanical properties of the hydrogels. The enhanced elasticity observed in the 2 min UV-crosslinked sample suggests a more robust network structure, likely due to more effective crosslinking. This makes it particularly suitable for applications requiring high mechanical integrity and stability, such as in tissue engineering scaffolds and load-bearing biomedical implants. In contrast, the samples with lower elasticity may be better suited for applications where greater flexibility and deformation are advantageous, such as in wound healing dressings or drug delivery systems. Understanding these variations allows for the precise tuning of hydrogel properties to meet specific application requirements, ensuring optimal performance and functionality in various biomedical and industrial contexts.

3.4. Morphological Evaluation

SEM analysis was utilized to investigate the internal and superficial morphology of the freeze-dried constructs. According to operational requirements, SEM analyses were performed on dry samples, although it is generally acknowledged that the drying process may induce shrinkage or alterations in scaffold features, and therefore the observed morphology may not fully represent the hydrated state of the scaffolds. Acquired data should therefore be interpreted in a qualitative manner, focusing on relative morphological differences rather than absolute structural parameters. The transverse sectioning of the 3D-printed scaffolds allowed us to observe their internal structure, which was found to be characterized by an interconnected porous network, with randomly oriented pores of about $46 \pm 13 \mu\text{m}$ (Figure 6). No significant differences were evidenced among sample types. The porous structure derives from ice crystal sublimation that occurred during freeze-drying, which generates a three-dimensional network of voids that replicate the shape and arrangement of the original ice phase. This procedure is necessary to observe the samples. The observed open porosity with thick walls could be attributed to the high concentration of collagen in the ink formulation. This open and porous architecture is compliant with biomedical applications, as it can support some cell type adhesion and infiltration, besides nutrient and oxygen diffusion.

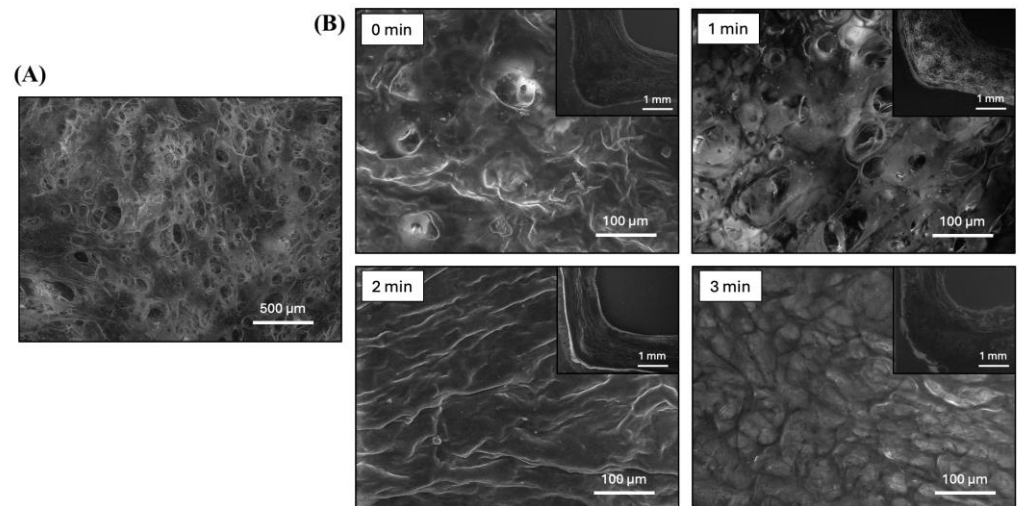


Figure 6. Representative SEM snapshot of 3D-printed scaffolds: (A) transversal section, and (B) images of the surface of the 3D-printed collagen scaffold after UV crosslinking at low magnification ($\times 50$) and high magnification ($\times 500$).

The SEM images displayed in Figure 6 also illustrate the scaffold surface at lower and higher magnifications. The as-produced scaffold (0 min) showed poorly defined filament edges and signs of collapse or merging, consistent with insufficient mechanical stability. The high-magnification image highlighted an irregular and rough surface. After 1 min of UV exposure, the overall morphology improved slightly, with increased compactness and less deformation at the edges. At higher magnification, an irregular and rough surface similar to the uncrosslinked condition was observed. After 2 min of UV exposure, the constructs exhibited a sharp and well-defined geometry at low magnification, with clear inter-filament separation and preserved corner fidelity. The high magnification image showed a smooth surface index of effective crosslinking. This condition appeared optimal in terms of macro-architecture preservation.

Interestingly, the overexposure to UV light (3 min sample) partially collapsed the filament organization. Scaffold edges appeared slightly less defined compared to the 2 min condition, and the high-resolution image showed a wrinkled texture, suggesting overcrosslinking and possible contraction or chain degradation [44,45]. A partial degradation of the triple-helical structure could also have occurred due to heat generation.

3.5. Surface Properties

AFM analysis provided superficial nanoscopic structural information of the 3D-printed fish collagen scaffolds with UV-induced crosslinking (0, 1, 2, and 3 min) in dry state (Figure 7). According to the operational requirements of the technique, AFM analyses were performed on dried samples [36]. As is known, AFM analysis could not be performed on hydrogel because of their excessive softness. However, the analysis of dried surfaces could give important information about the effects of the treatment on collagen supramolecular organization, although it does not fully reflect the hydrated state of the scaffold. Consequently, the acquired AFM data should be interpreted in a qualitative manner, with the aim of highlighting relative differences in surface morphology and roughness rather than providing absolute topographical or structural parameters. In the uncrosslinked sample (0 min), the surface displayed randomly oriented collagen fibers within a slightly rough surface. The 1 min condition was found to be very similar to 0 min, with no statistically significant differences. Contrarily, the 2 min UV-irradiated scaffold displayed a stiffer surface, characterized by a higher roughness. These results suggested

that fibrils could be more tightly bound. The AFM data were found to be consistent with rheological and thermal analyses demonstrating increased G' , critical escape strain, and temperature stability. This suggests that the matrix has stronger hydrogen bonding and cohesive interactions, allowing for improved structural fidelity [4,5,49].

Nonetheless, the surface morphology changed after 3 min of UV crosslinking and progressed to a more aggregated surface, characterized by a smaller number of intact fibers. This loss of resolution, or surface detail, may be the result of overcrosslinking that caused fiber unfolding and local network collapse [50,51]. Beyond exposure, it generally results in reductions in the mechanical and thermal properties, even if it may be apparent that densification has occurred. Overall, AFM data allow for the conclusion that UV exposure for 2 min is the optimum and most structurally preferred network which can maintain an appropriate or ideal balance of crosslink density and fiber definition—an important aspect in the bioprinting space which has demonstrated a need for both printability and fidelity of printing and post printing [2,3,52].

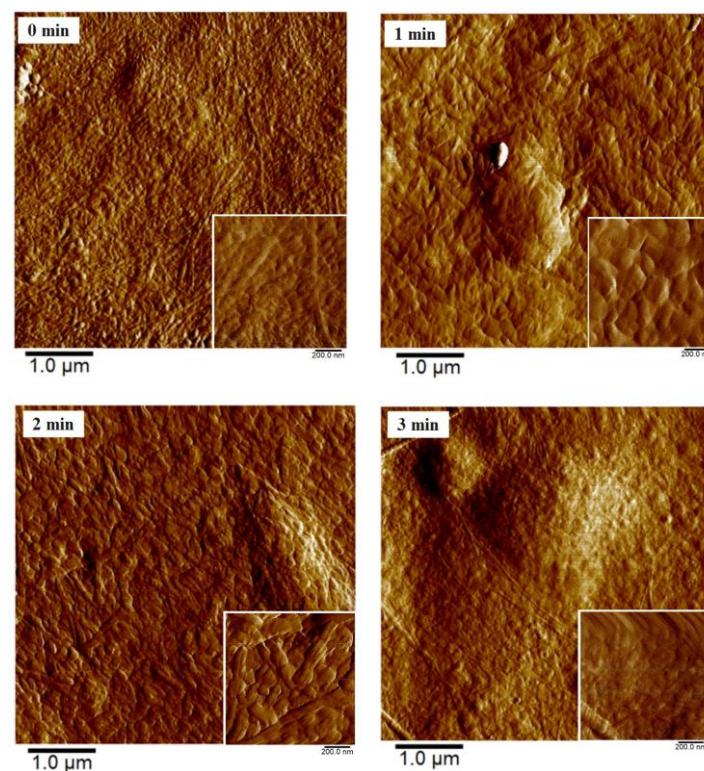


Figure 7. AFM images ($1 \times 1 \mu\text{m}^2$) of collagen scaffolds crosslinked under different UV irradiation times: 0 min, 1 min, 2 min, and 3 min. Insets show zoomed-in regions.

3.6. Spectroscopic Analysis

ATR analysis was carried out before and after the UV crosslinking process. All samples displayed the characteristic spectra of type I collagen (Figure 8). In particular, the peaks of amide I, amide II, and amide III of type I collagen were identified, as well as of amide A and amide B (Table 3). At about 1630 cm^{-1} , the amide I band, attributed to C=O hydrogen-bonded stretching, was detected. The amide II band, resulting from C-N stretching and N-H in-plane bending of the amide linkage, peaked at about 1550 cm^{-1} . The amide III band, associated with N-H bending coupled with CH_2 wagging and C-N stretching, was observed at 1240 cm^{-1} . In addition to the three main spectral contributions, other notable peaks were observed, attributable to Amide A ($3290\text{--}3321 \text{ cm}^{-1}$), characterized by N-H stretching coupled with intramolecular hydrogen bonding, and Amide B ($3072\text{--}3080 \text{ cm}^{-1}$), defined by N-H bending. Additional peaks that are typically found in collagen were

also detected and reported in Table 3. The detection of these peaks, which are characteristic of type I collagen, affirmed the proteinaceous nature of the material as well as its purity and the partial preservation of its native conformation. Figure 8 onsets also shows that UV crosslinking induced very slight variations in peaks position and intensity. Very similar FTIR spectra were found in a calf skin-derived collagen ink [53].

Before crosslinking, fish collagen ink exhibited the characteristic absorption bands of native type I collagen. The ratio between Amide III and CH₃ higher than 1 (ratio $\text{AmideIII/CH}_3 = 1.3$) confirmed the preservation of collagen's triple-helical structure. Generally, the effectiveness of the crosslinking strategy was visible from the contributes' slight shifts. In particular, the blue shift of amide III, amide II, COOH, amide A, amide B, and CH₂/CH₃ bands and the red shift of O–H and N–H stretching band indicated an increase in protein structural order and a reduced flexibility due to the increase in hydrogen interactions and bonds. After 1 min of UV crosslinking, the absorbance spectrum of the biomaterial ink was found to be quite completely stackable to that of the uncrosslinked sample. The lack of significant shifts in the positions and intensities of the primary absorption bands indicated that 1 min of UV exposure was insufficient to induce effective collagen crosslinking. Additionally, the Amide III and CH₃ ratio higher than 1 (ratio $\text{AmideIII/CH}_3 = 1.1$) confirmed that the treatment did not compromise the protein's structural integrity. The second experimental condition (2 min) suggested that an increased degree of crosslinking occurred. A more pronounced reduction in the ratio of Amide III versus the CH₃ contribute to a value equal to 1 underlined the negative effect of the crosslinking treatment of collagen structural integrity. However, the selected exposure time allowed us to not deeply affect collagen arrangement. Conversely, very low was the effect of 3 min of irradiation on the collagen crosslinking degree visible from the main peaks' slight shifts. These results, together with a significant loss in collagen triple-helical structure detected by the Amide III and CH₃ ratio (ratio $\text{AmideIII/CH}_3 = 0.9$), prompted the exclusion of this condition. Thus, the presence of specific peaks and their shift suggested that while some alterations occurred, the triple-helical structure of collagen was preserved in the 2 min UV irradiated sample, balancing its biological functionality and structural performances.

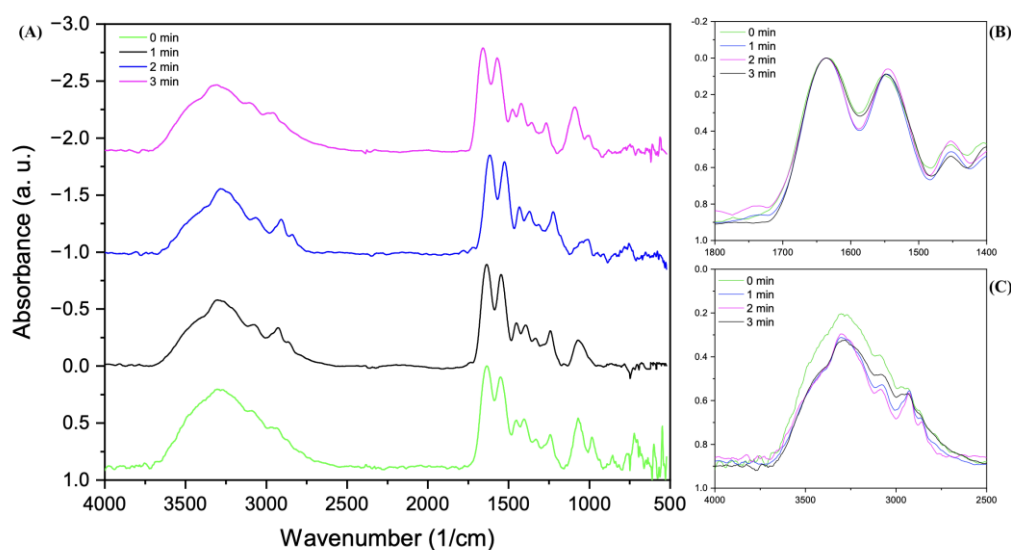


Figure 8. ATR spectra of uncrosslinked and UV-crosslinked ink samples (A) from 4000 to 400 cm^{−1} and comparative onsets of (B) amide I and II (1800–1400 cm^{−1}) and (C) amide A and B (4000–2500 cm^{−1}).

Table 3. ATR peak location (cm^{−1}) and assignment of 3D-printed samples of fish collagen ink.

Sample Peaks Assignment	0 min	1 min	2 min	3 min
C–C, C–O, and C–N deformation	984	989	977	987
C–O or C–N stretching	1069	1071	1041	1067
C–N stretching and N–H bending of amide III	1241	1241	1243	1244
COOH groups symmetric stretching	1404	1394	1390	1389
CH ₂ bending	1452	1452	1453	1452
N–H bending and C–N stretching of amide II	1550	1546	1545	1548
C=O stretching of amide I	1635	1636	1635	1636
CH ₂ /CH ₃ groups symmetric stretching	2874	2865	2858	2868
CH ₂ /CH ₃ groups asymmetric stretching	2938	2927	2927	2937
N–H stretching of amide B	3090	3079	3086	3088
O–H and N–H stretching band	3269	3269	3264	3227
N–H stretching of amide A	3302	3301	3304	3300

3.7. Thermal Stability

DSC is a powerful technique for evaluating the thermal stability of collagen. The thermal transitions of the collagen samples are shown in Figure 9A, and the corresponding data are summarized in Table 4. As expected, the Td of the fish-derived collagen ink was approximately 32–37 °C, which aligns with values reported in the literature [40]. An increase in Td was observed with UV irradiation time up to 2 min, followed by a slight decrease at 3 min ($p < 0.05$). This trend suggested that moderate UV exposure promotes intermolecular interactions, leading to improved thermal stability, while excessive irradiation may result in overcrosslinking or the partial degradation of the triple-helical structure, thereby reducing thermal integrity. These effects may arise from a combination of UV-induced photochemical damage and localized heat generation during prolonged exposure. Significant differences were also found in the ΔH , which reflects the energy required to disrupt hydrogen bonds within the collagen triple helix. Longer UV irradiation times generally increased ΔH ($p < 0.05$), indicating enhanced hydrogen bonding and more stable collagen networks. These results are consistent with the mechanical and rheological data, reinforcing the link between UV crosslinking duration and the structural organization of the collagen. Overall, these findings demonstrated that fine-tuning UV exposure can modulate the thermal and structural properties of collagen-based inks. Applications requiring higher thermal stability—such as in situ gelation or scaffolds exposed to physiological temperature—may benefit from optimized crosslinking protocols that maximize both Td and ΔH . DSC thus serves as a valuable tool for guiding formulation strategies aimed at enhancing the performance and reliability of biomaterial inks in biomedical contexts.

Table 4. Sample descriptions obtained from different UV irradiation times.

Sample Code	Td (°C)	ΔH (J/g)
0 min	36.4 ± 1.3	0.68 ± 0.16
1 min	38.1 ± 1.6	0.70 ± 0.10
2 min	44.7 ± 1.8	0.91 ± 0.15
3 min	40.4 ± 2.2	0.90 ± 0.17

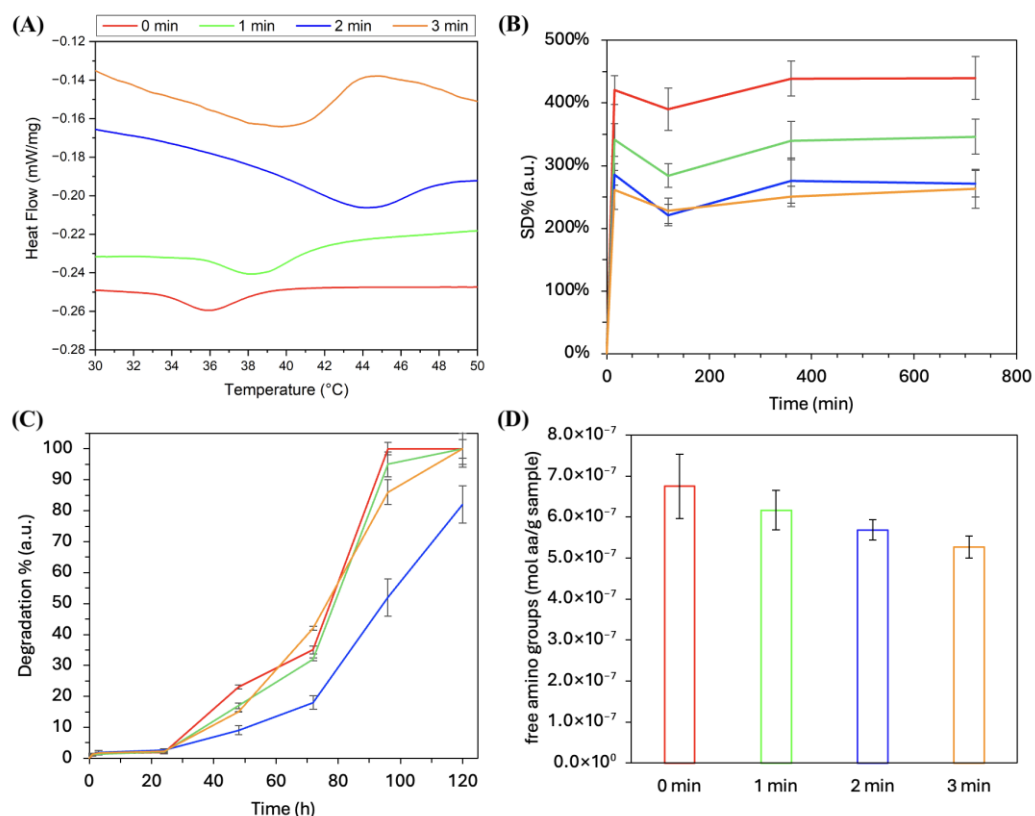


Figure 9. Physicochemical properties of printed samples subjected to increasing UV crosslinking exposure (0, 1, 2, and 3 min) in terms of (A) denaturation temperature, (B) swelling degree, (C) degradation resistance, and (D) free amino groups. Values are expressed as the mean $\pm$ standard deviation ($n = 3$).

3.8. Swelling Degree

The scaffolds' SD% is a useful tool for the evaluation of the efficacy of crosslinking treatments and their impact on their water retention ability. Figure 9B depicts the freeze-dried 3D-printed construct's behavior in physiological-like conditions across time. As expected, because of the high hydrophilic nature of fish collagen, all samples rapidly gained about 250–450% of their dry weight in water in the first 30 min of incubation. Then, the samples stabilized and reached a plateau after 6 h. The uncrosslinked samples (0 min) reached the highest SD% ($p < 0.05$), exceeding 400%. The application of 1 min of UV was found to decrease the SD% of about 1.3 times, with values that stabilized at about 300% ($p < 0.05$). In contrast, the application of 2 min or 3 min of UV crosslinking allowed us to obtain a sample with the lowest SD% across all time points, suggesting a more tightly crosslinked network that limits water uptake. No statistically significant differences were revealed among 2 min and 3 min samples ($p = 0.7$). These findings indicate that increasing the crosslinking time effectively reduces the hydrogel's swelling, with the 2 min samples emerging as the most efficient in restricting water uptake.

3.9. Degradation Resistance

The degradation behavior of the 3D-printed constructs until their complete dissolution is reported in Figure 9C. Although degradation in PBS is not fully predictive of in vitro and in vivo cell-mediated remodeling, which is governed by enzymatic activity and cell–matrix interaction, it was intentionally used as a simple and standardized method for the preliminary evaluation of the constructs in vitro half-life under controlled and physiologically relevant pH and ionic conditions, independently of the biological model. All

samples exhibited a progressive increase in degradation percentage, with a characteristic sigmoidal trend marked by an initial lag phase up to 24 h, followed by a sharp rapid degradation with a complete dissolution within 6–7 days for all conditions. Interestingly, while sample behavior at 1 min was similar to sample behavior at 0 min ($p > 0.05$), the sample crosslinked for 2 min showed a marked delay in the onset of degradation compared to the other groups, suggesting that this crosslinking condition effectively enhanced the structural integrity and resistance of the hydrogel matrix in physiological-like conditions. In contrast, samples exposed for 3 min exhibited faster degradation kinetics compared to 2 min ($p < 0.05$). This result is in line with data obtained from other analysis, where it seemed that 3 min of UV exposure partially compromises the protein structure and consequently the stability of the network, whereas an optimal crosslinking time (2 min) increased the construct properties. With respect to the degradation timescale, although it seems to be limited for some biomedical applications, it should be interpreted in the context of the intrinsic limitations of fish-derived collagen. Native fish collagen is well known not to be printable alone (it is always blended with other structural co-polymers) and to rapidly solubilize under physiological conditions, making the achievement of structurally stable printed constructs particularly challenging. In this framework, the ability to print a self-standing hydrogel construct and to achieve a degradation time of 5 days by means of a mild UV crosslinking step represents a significant improvement over the non-crosslinked system.

3.10. Crosslinking Degree

The extent of crosslinking could be esteemed by quantifying the amount of free amino groups before and after UV exposure. As shown in Figure 9D, the moles of free amino groups per gram of collagen progressively decreased slightly with an increase in crosslinking time. The non-crosslinked sample (0 min) exhibited the highest concentration of free amino groups (6.7×10^{-7} mol aa/g sample), consistent with the absence of crosslinking reactions. Upon UV exposure, a gradual reduction in available amino groups was observed, with values decreasing by about 10% in the 1 min sample, 15% in the 2 min sample, and 20% in the 3 min sample (5.2×10^{-7} mol aa/g sample). Although the difference in sample types is not significant ($p > 0.05$), the decrease in the number of free amino groups confirmed the mild level of UV-induced formation of bonds.

4. Discussion and Conclusions

Native fish collagen is known not to be printable alone as a self-standing hydrogel and to rapidly dissolve under physiological conditions, making the achievement of structurally stable printed constructs particularly challenging. In this study, a prototypal type I collagen-based ink from Tilapia skin was successfully 3D-printed by extrusion. Additionally, a fundamental and materials-focused investigation of the effects of UV-induced crosslinking on the printability, structural integrity, and short-term stability of the fish-collagen ink was performed.

The UV exposure time effect was investigated as a method for the mild tuning of scaffold structural and mechanical properties. All of the above-mentioned material properties were best increased at exposure times of 2 min, at which denaturation of the collagen triple helix was minimized, and thermal stability and dynamic viscoelastic behavior improved, in comparison to all other exposure times tested. These results demonstrated that by controlling exposure time using UV light, biomaterial ink properties can be tailored to fit the demands of soft tissue engineering.

Overall, the proposed biomaterial ink holds strong potential for applications in tissue engineering and regenerative medicine, particularly for soft tissue repair or in vitro models. Its tunable crosslinking behavior, combined with its sustainable sourcing and

advantages (lower risk of zoonotic transmission and absence of cultural and religious constraints), positions this biomaterial as a promising alternative biomaterial ink in the rapidly expanding field of marine-derived biomaterials. Although the degradation timescale may appear limited for certain biomedical applications, it should be interpreted in light of the intrinsic challenges associated with fish-derived collagen. In this context, the ability to print self-standing hydrogel constructs and to achieve a controlled degradation of approximately 5 days using only a mild UV crosslinking step represents a substantial advancement over non-crosslinked systems.

Importantly, this work should be regarded as a foundational study, aimed at elucidating the interplay between printability, UV-induced network stabilization, and the structural integrity of a prototypal fish-collagen-based ink. The adoption of a deliberately low-intensity crosslinking approach was chosen to preserve cytocompatibility and matrix permissiveness, while providing sufficient stability for early-stage scaffold handling and cell interaction. These findings lay the groundwork for the future further optimization of the system, including the fine-tuning of degradation kinetics, the integration of additional crosslinking mechanisms, or the modulation of biomaterial ink composition to address application-specific requirements. Future studies will be performed to assess the biological response of the UV-crosslinked constructs and to validate their suitability for regenerative applications.

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Data Availability Statement: Dataset available upon request from the authors.

Conflicts of Interest: Luca Salvatore was Chief Operating Officer and Technical Operator of the Typeone Biomaterials Srl Company. Author Nunzia Gallo was employed by the company Typeone Biomaterials Srl. Luca Salvatore and Nunzia Gallo were co-inventors of the patented technology used for the development of the biomaterial ink. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors declare that this study received funding from the Italian Ministry of University and Research and Regione Puglia through the prementioned projects. The funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

Abbreviations

The following abbreviations are used in this manuscript:

3D	Three-dimensional
ECM	Extracellular matrix
DMA	Dynamic mechanical analysis
LVR	Linear viscoelastic range
SEM	Scanning electron microscope
AFM	Atomic force microscopy
QNM	Quantitative nanomechanical mapping
ATR	Attenuated total reflectance spectroscopy
DSC	Differential scanning calorimeter
Td	Denaturation temperature
ΔH	Denaturation enthalpy
SD%	Swelling degree
W_t	Sample wet weight
W_0	Sample dry weight
W_a	Sample esteemed remaining weight
SD	Standard deviation
G'	Storage modulus
G''	Loss modulus
γ_{crit}	Critical strain
E_c	Cohesion energy
G'_{crit}	Storage modulus at the critical strain

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