Keratin gel from chicken feathers waste obtained by mercaptoethanol extraction

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Abstract

Protein gels prepared by keratin extracted from chicken feathers show potential applications as engineered materials. Feathers are an abundant waste material, whose principal component is keratin, which may have gelling properties not yet sufficiently studied so far which are strongly dependent on the extraction method adopted.

The aim of the study is to explore the properties of gels obtained through mercaptoethanol extraction and dialysis process and to evaluate their structural characteristics. The keratin hydrogels were characterised with Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy through which it was possible to identify the secondary structure of the protein on hydrated and dry gel. Moreover, the morphological analysis by scanning electron microscopy (SEM) combined with the rheological analysis showed how the consistency of the gels is maintained on a wide range of loads and frequencies.

Furthermore, the biocompatibility of the gels was investigated for the release of subcutaneous drugs using curcumin, an antioxidant polyphenol compound. The fastest release was obtained at pH 7.4, corresponding to skin conditions.

1.Introduction

The extraction of keratin, the most diffuse biopolymer, from various types of waste, has recently become a diffuse practice for upcycling by-product materials. The principal sources of keratin are human hair [1], wool [2], and chicken feathers [3]. Chicken feathers from slaughtering do represent a very large waste source, in particular due to the fact that poultry meat consumption did present a fivefold increase in half a century, reaching the amount of 17.2 kg/capita in 2015 [4]. Applications suggested for chicken feather waste have been various, spanning from the production of organic fertilisers [5], the traditional use in textiles or related fillers [6], to geotextiles [7], or polymer composites [8]. However, in most cases keratin extraction from feathers has been aimed at the production of biomaterials, for example for cosmetic and pharmaceutical studies [9], although in some cases in the form of protein hydrolysates [10]. The process to serve a more practicable and reliable drug release would conversely require the preservation of the secondary structure of the protein [11]. This appears less difficult to obtain in the case of chicken feathers, provided an adapted extraction procedure is applied to this aim [12] and also paves the way towards their blending with other biopolymers, such as poly(hydroxybutyrate) (PHB) [13].

A peculiar characteristic that offered much value to this extraction process is the various geometries in which chicken feather keratin can be processed, including films, sponges, fibres and hydrogels [14]. The latter has shown some promising evidence with respect to the other forms of keratin, namely as regards the prospective mechanical performance of the structure [15]. More specifically, a variety of chemical methods based on reduction for the extraction of keratin from chicken feathers have been experimented, such as sodium sulphite, sodium dodecyl sulphate, and 2-mercaptoethanol [16]. 2-mercaptoethanol keratin extraction offers a high yield, such as 84%, after a time of 2 hours [17]. Slightly inferior yield was obtained in [18], in the order, though confirming the experimental outcome of extensive protein aggregation, even if with diffuse presence of oligomers. The study was specifically

oriented to the production of glycerol-plasticized films, which indicated optimal mechanical performance at approximately 50% cysteine carboxymethylation [19].

To the best of our knowledge, keratin gels have never been obtained using 2-mercaptoethanol extraction. However, hydrogels obtained from extracted keratin have proven their potential e.g., in terms of wound healing in a blend with genistein [20]. An essential characteristic that is also sought for into biopolymer hydrogels, among which those based on keratin are very popular in various fields, is mechanical durability over time [21]. It is required therefore that the extraction method would provide long lasting and resistant materials.

This study derives from previous investigations on metabisulfite extraction on wool and chicken feathers waste, which led to evaluations of the effectiveness of the process to obtain, after dialysis, material geometries suitable for further application in the biomedical field [22]. It was noticed during these studies that also 2-mercaptoethanol extraction, namely on chicken feathers, might be likely to provide some merits as for yield and preservation of the chemical structure of keratin obtained. Further analysis of the characteristics of this keratinous extract constitute the core of this work, which is then focused on potential use for drug delivery. With this aim, preliminary tests for drug delivery from the obtained gel have been performed, more specifically studying the release of curcumin in different pH conditions. Curcumin is a well-known naturally occurring anti-cancer compound [23], which has a long history of applications in drug release from biopolymers [24].

2. Materials and methods

2.1 Waste materials

The keratin-based waste materials, in the form of white feathers from chicken, were obtained during the process of slaughtering and have been supplied by Fileni S.p.A. (Cingoli, MC, Italy).

2.2 Extraction method

According to literature the 2-mercaptoethanol method of extraction of keratin for feathers has been used [25]. First, feathers were washed with cold water and soap, followed by grinding and a second wash with ethanol at 50 °C for 2 hours to remove surface fats and waxes.

The extraction was performed using the following proportions for a quantity of 4 g of feathers: Urea = 31.8 g, Mercaptoethanol = 1.2 mL (14.2 M), EDTA = 130 mg, Tris(hydroxymethyl)aminomethane = 3.23 g, total volume of the solution was 100 mL. The reagents' temperature was kept at 70 °C for 24 h, and the solution was then filtered to remove the partially digested residue. Following this, the solution was dialyzed using a membrane with a diameter of 28.6 mm and a molecular weight cut-off (MWCO) of 12-14 kDa in water for about 3 days at room temperature changing water once a day.

2.3 Characterization of the keratin gel

2.3.1 Microscopy Techniques

SEM analyses were carried out using a Field Emission Scanning Electron Microscopy (FE-SEM, Sigma Family, Zeiss, Jena, Germany), equipped with a backscattered detector (BSD) to obtain high-quality microphotographs. Before being analysed, a slice of keratin gel was dehydrated by evaporation of water at room temperature under a hood.

2.3.2 Spectroscopy Techniques

Raman spectroscopy was performed with a HORIBA IHR320 micro-Raman Scattering system (Horiba, Palaiseau, France) equipped with an optical Microscope model Olympus BXF41 (with 5, 20, 50, 100 objectives) (Münster, Germany). The Raman spectrometer was operated at 532 nm (diode laser) with a power of 40 mW. Raman spectra are collected on gel dehydrated by evaporation of water at room temperature under the chemical hood for 12/14 hours and on wet gel.

IR spectra were recorded from 4000 to 400 cm^{-1} with a PerkinElmer Spectrum 100 FT-IR instrument (Waltham, MA, USA) by total reflectance on a cadmium selenide (CdSe) crystal. For IR spectra the gel samples were dehydrated in the same way as the samples for Raman.

To obtain information about the secondary structure of the protein, the spectra were fitted into a set of Gaussian functions using Fityk (version 1.3.1).

2.3.4 Rheological characterization

Rheological analyses were carried out using a stress-controlled rotational rheometer (Kinexus lab+, Malvern, UK) equipped with a C40/4 cone-plate geometry. Gel was analysed by stress

sweep and frequency sweep tests at 25 °C. Stress sweep analysis was performed in the range of 0.5–100 Pa and at a frequency of 1 Hz. For the frequency sweep tests, an increasing frequency in the range of 0.01–10 Hz was applied to the samples at a constant stress (1 Pa). The measured rheological parameters were the elastic modulus (G'), the viscous modulus (G"), and the complex modulus (G*). Analyses were performed on thin disks with a thickness of 5 mm and diameter of 28.6 mm.

2.4 Preparation of gel with curcumin and evaluation of curcumin release

Pure keratin gel was cut in small cylinders with a thickness of 10 mm and same diameter of membrane dialysis (28.6 mm) and were tested to evaluate the possible absorption of hydrophobic drugs using a solution of curcumin (Cayman Chemical, CAS registry n 458-37-7), dissolved in alcohol. Curcumin solutions for the experiment were prepared as follows: 5 mg of curcumin were dissolved in 40 mL of a 80/20 vol.% ethanol/water solution. For absorption by the gel, 16 ml of this solution was placed in contact with a cut cylinder of gel in a becker. The becker was placed in a shaker (Stuart SI20H Scientific Hybridisation Oven/Shaker, Cole Parmer) with oscillation of 15 strokes/min for 60 min at ambient temperature. After the absorption, the excess of curcumin was washed away using distilled water. Curcumin release tests were done in triplicate using a solution of 10X PBS (phosphate buffer saline) diluted 5 times with the addition of 2% TWEEN 80® polysorbate was used. Then, three solutions at different pH (7.4/5.8/1.2) with the use of hydrochloric acid (HCl) for acidification were prepared, and the curcumin release at different pH were calculated in %, considering as 100% the quantity of curcumin initially added. During the release time (72 hours at ambient temperature), several samplings were carried out by taking 1 mL of solution and the relative absorbance was measured at 450 nm using a Varian Cary 1 spectrophotometer. Subsequently, the 1 mL sample was reinserted into the initial solution not to change the release medium volume.

3. Results and discussion

Unexpectedly and repeatedly (not less than 10 experiments were performed), the extraction led to the formation of a very consistent pure keratin gel after dialysis without any further manipulation, which appears as self-standing and composed of approximately 98% water in weight. In Figure 1, images of the gel at different moments are depicted. In particular, Figure 1a shows the gel just after production. Figure 1b shows a cut piece of the gel, stored in distilled water, two years after its extraction, which does not show any significant change in appearance, consistency and colour. The recovered keratin gel from dialysis was stored in distilled water in the fridge to avoid desiccation, which would normally occur in around 12 hours. After the introduction of curcumin, the gels treated with curcumin appeared with an intense orange colour (Figure 2), a sign that the curcumin had solidly and uniformly bound to it.

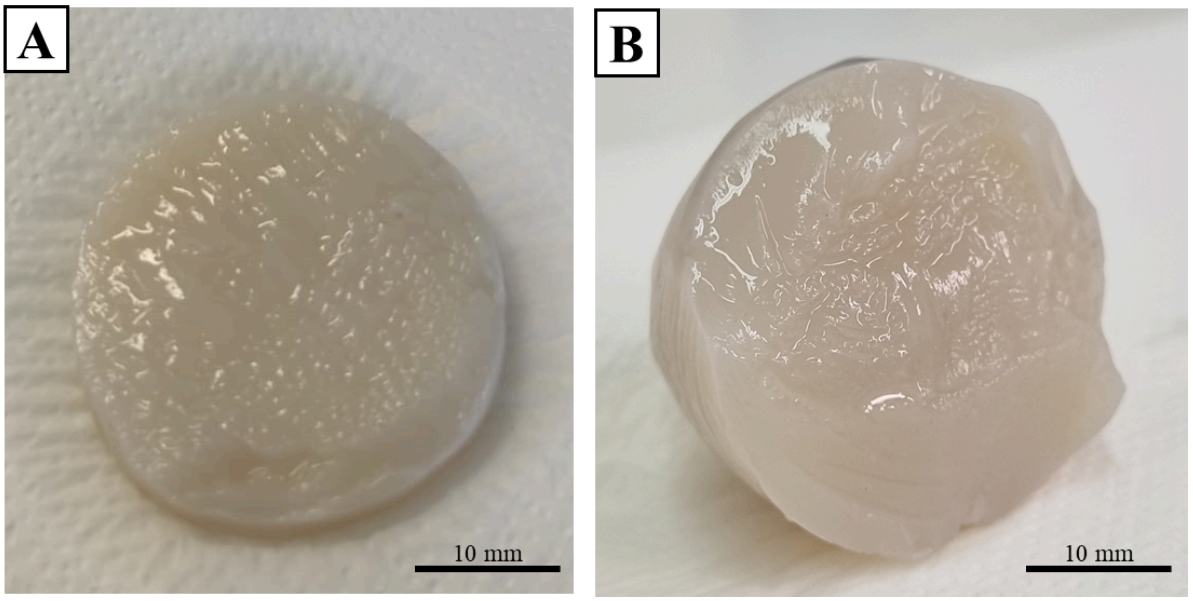

Figure 1: A) Sample of fresh keratin gel after extraction; B) Sample of keratin gel two years after its extraction.

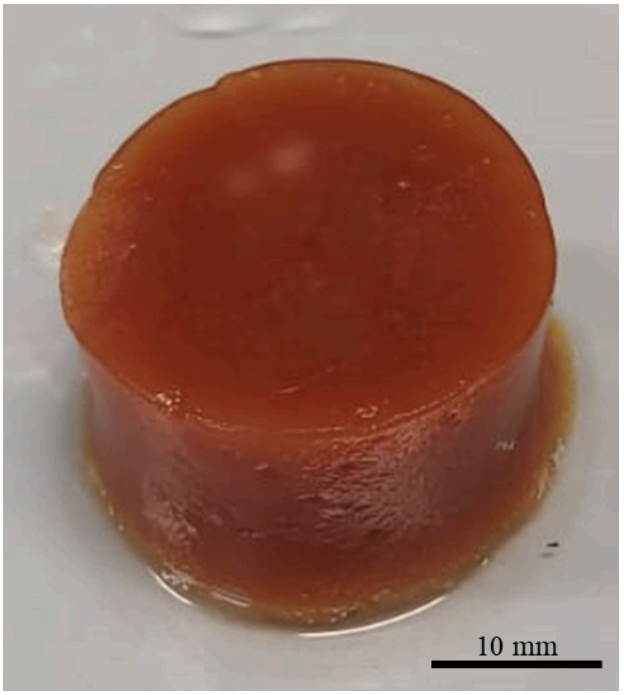

Figure 2: Cylindrical piece of keratin gel after curcumin absorption.

IR spectroscopy

The keratin gel was analysed using IR spectroscopy and the spectrum in Figure 3 shows the absorption bands of stretching vibration of N-H and OH bonds at around 3300 cm^{-1}, which are related to amide A [26]. Stretching vibrations of C=O bonds appear from 1600 to 1700 cm^{-1} and they are typical of the amide I band, which contains information about the secondary structure of keratin [27]. At 1520 cm^{-1}, the bending vibration of N-H of amide II is visible [28]. The stretching vibrations of C-N and C–H and bending vibrations of N-H and C=O at around 1220–1300 cm^{-1} are related to amide III. We also performed analysis on gel after the curcumin absorption and on pure curcumin for comparison, the spectra are reported in Figure 3. Curcumin spectrum is consistent with the one reported and extensively analysed by Fugita et al. [29].

In the spectrum of the gel containing curcumin, the contributions of curcumin peaks at 1282 cm^{-1} and 960 cm^{-1} were respectively attributed to the symmetric C-O-C stretch methoxy group [30] and to the trans-CH vibration of the benzoate [31].

Additionally, it was possible to obtain information about the composition of the secondary protein structure of the keratin gel by studying the deconvolution of the amide I band. In

Figure 4 is reported the contribution of three peaks at 1625 cm^{-1} for beta sheet, at 1650 cm^{-1} for alpha helix and at 1676 cm^{-1} for the disordered structure, respectively [32].

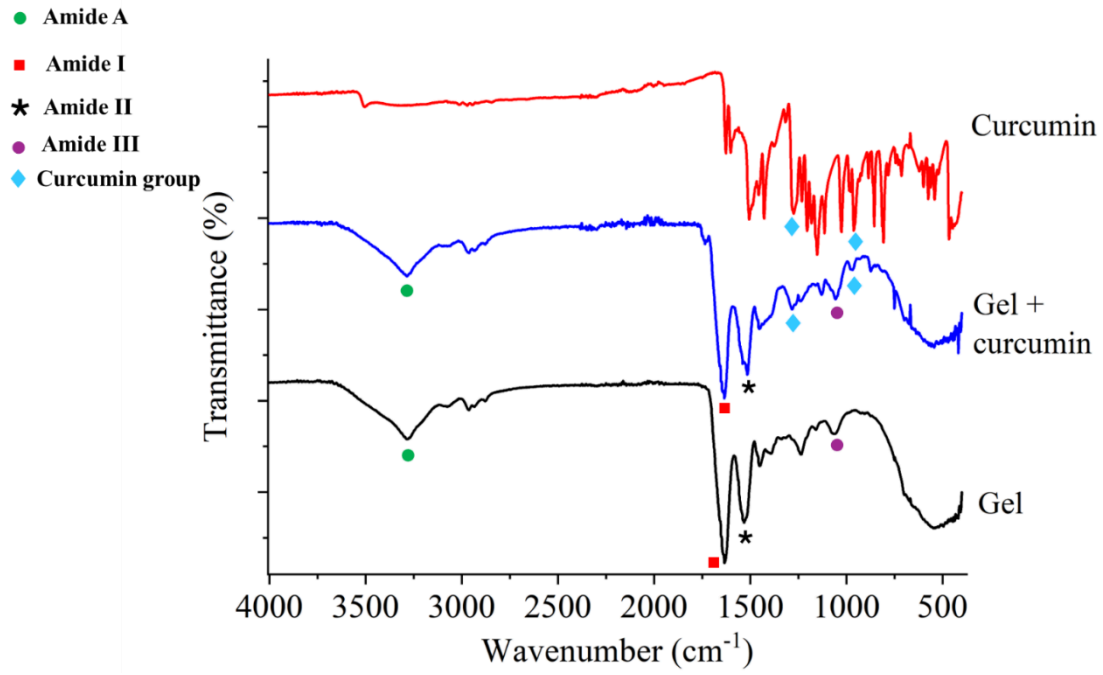

Figure 4: FTIR spectrum of pure keratin gel, curcumin and keratin gel after curcumin absorption.

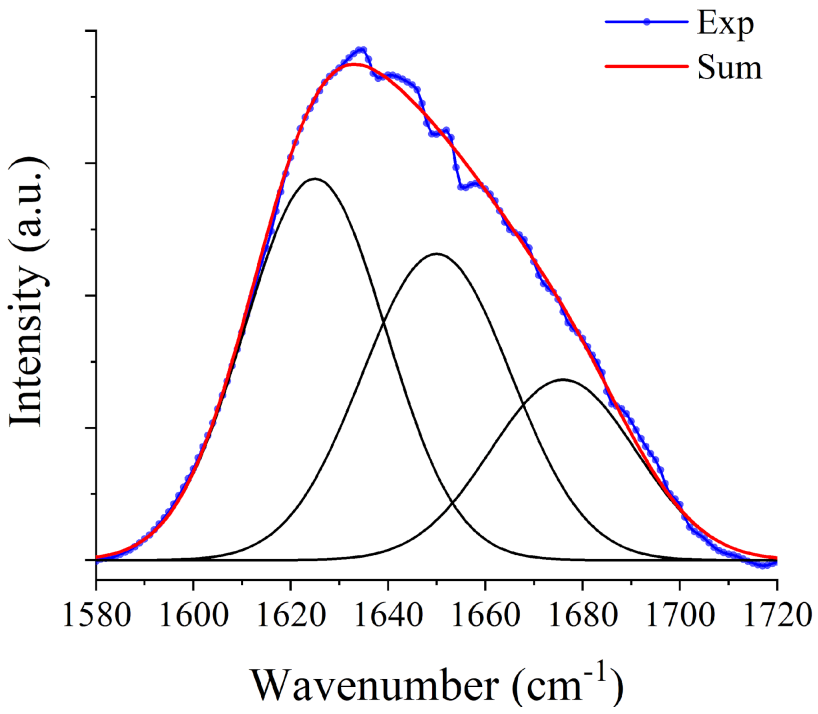

Figure 5: FTIR spectrum of amide I band for dry keratin gel. Black lines indicate the fitted peaks respectively for increasing wavenumber: beta sheet, alpha helix and disordered structure. Exp=experimental data and Sum=summation of the three contributions.

Raman Spectroscopy

The Raman spectra of keratin gel water-saturated and dry are depicted in Figure 6. The data show the characteristic bands of the proteins in position: 1200-1300 cm^{-1} for Amide III, 1600-1700 cm^{-1} for Amide I band and 1448 cm^{-1} for CH_2. The assignment of the vibration bands was done using previous literature as reference (see Table 1). The obtained spectra were similar in terms of the position of the peaks and do not exhibit distinct features, being the only difference in the noise of the signal that increases with the presence of water.

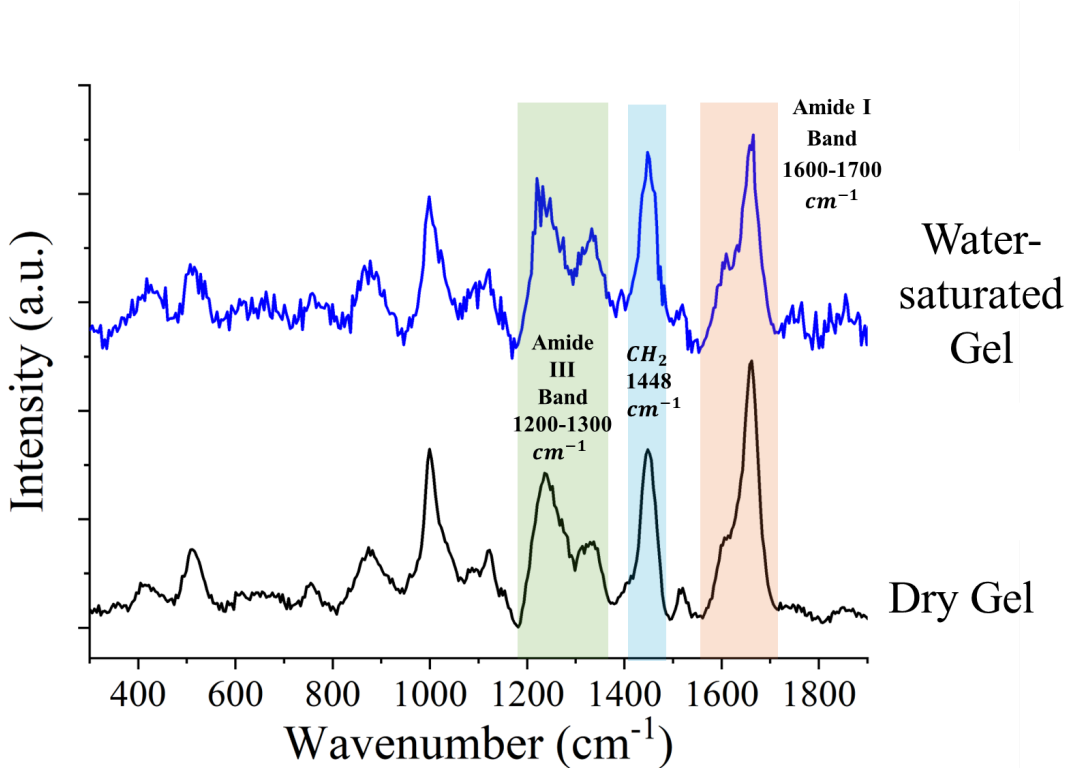

Figure 6: Raman spectra of dry (in black) and water-saturated (in blue) keratin gel with the main characteristic bands of the proteins underlined with coloured squares.

Peak position Raman (cm^{-1})	**Current Assignment**	**References**
521	SS (disulfide bridges)	Akhtar [33], Wang [34]
761	(CH_2)in-phase	Akthar [33]
866	CCH aromatic ring	Akthar [33]
1007	CC aromatic ring	Akthar [33], Wang [34]
1120	(CC) Skeletal	Akthar [33]
1153	(CC); (COH)	Akthar [33]
1238	Amide III	Talari [35]
1337	Amide III & CH_2 wagging Vibrations from glycine backbone & proline sidechain	Talari [35]
1448	CH_2	Akthar [33], Wang [34]
1511	C=C	Akthar [33]
1612	C=C tryptophan	Akthar [33], Skieresz-Szewczyk [36]

Table 1: Raman wavenumbers (cm $^{-1}$) and attribution of the vibrational modes according to the literature references

To study the secondary structure of proteins, the Amide I band was deconvoluted into its components. The deconvolution of Amide I band is shown in Figure 7, where the contributions are given by four components for dry (Figure 7a) and water-saturated gel (Figure 7b): 1576 cm^{-1} for guanine, 1601-1602 cm^{-1} for C=C (carbon–carbon double bond), 1623-1626 cm^{-1} for Tryptophan or beta sheet, 1658-1660 cm^{-1} for beta sheet structure and 1688 cm^{-1} for unordered coil. No particular differences have been noticed between the spectra, only the signal contribution of guanine, C=C and unordered structure increase in the presence of water (Figure 7b).

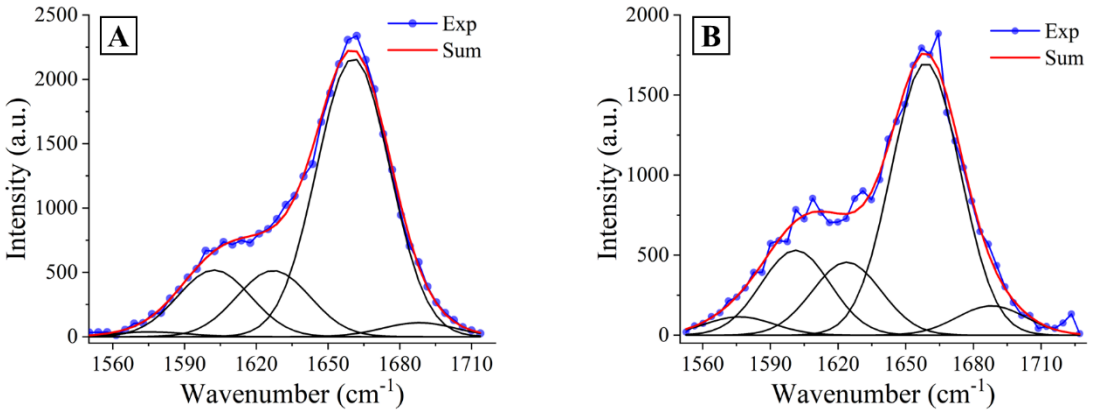

Figure 7: Raman spectra of Amide I band of dry (A) and water- saturated gel (B), the black lines indicate fitted peaks. Exp=experimental data and Sum=summation of the three contributions.

FTIR and Raman studies on the secondary structure of proteins are essential for understanding the formation of keratin gels. The secondary structure significantly influences the physical properties of these gels. Feather keratin, in particular, is mainly composed of ordered and rigid beta sheet structures that cannot be further stretched, resulting in mechanically robust hydrogels. Moreover, cysteine groups are located at the ends of keratin chains, acting as anchor points in the self-assembly of these chains (Figure 8). The strength and stability of keratin gels are primarily due to the formation of multiple covalent disulfide bonds between cysteine residues, enhancing the gel's mechanical properties.

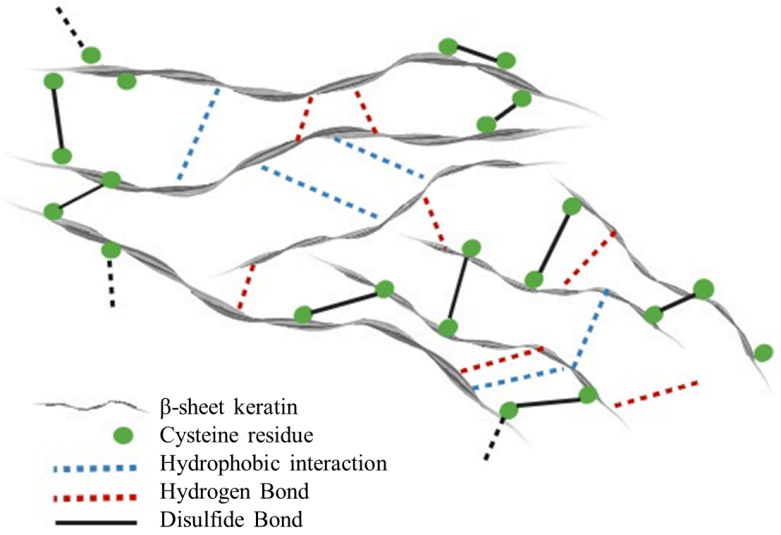

Figure 8: Illustration of the hypothesised self-assembly process of keratin hydrogels, highlighting key intermolecular forces: disulfide bonds, hydrophobic interactions, and hydrogen bonds. These forces drive the formation of stable molecular networks essential for hydrogel structure and function.

Scanning electron microscopy (SEM)

The analyses performed with a scanning electron microscope (SEM) showed structural features of the gels. Pure keratin gel (Figure 9a and 9b) showed a wound up structure due to dehydration, and the surface does not present voids. Bundles were also visible which suggest the preservation of the secondary structure of the protein (Figure 9b).

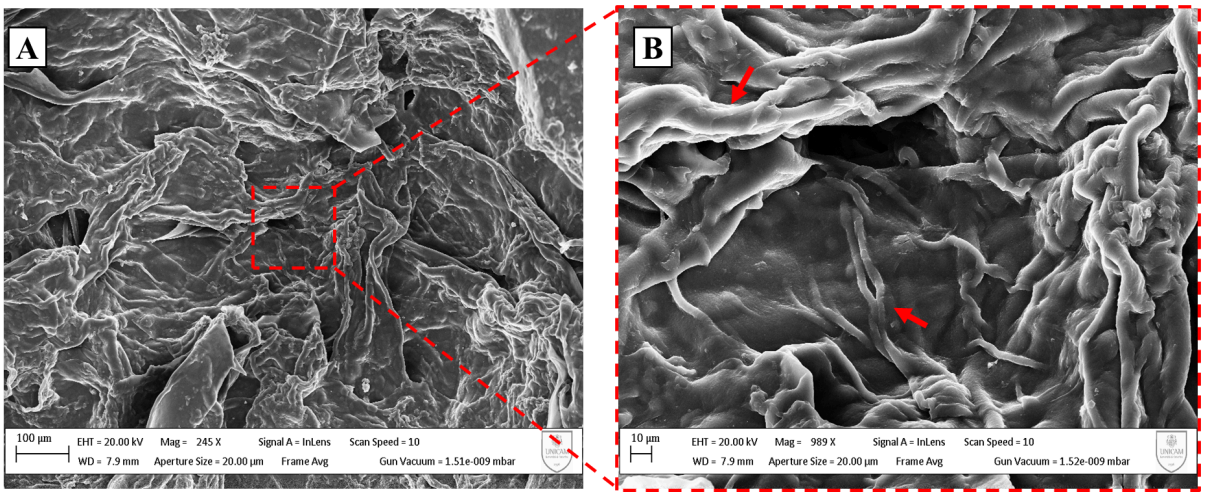

Figure 9: SEM images of A) and B) pure keratin gel at different magnifications. The arrows indicate the protein fibre bundles.

Analysis was performed also on keratin gel after curcumin absorption. Figure 10 shows a surface composed of bundles that form a lattice on which aggregates are visible, which can be reconnected with the presence of curcumin.

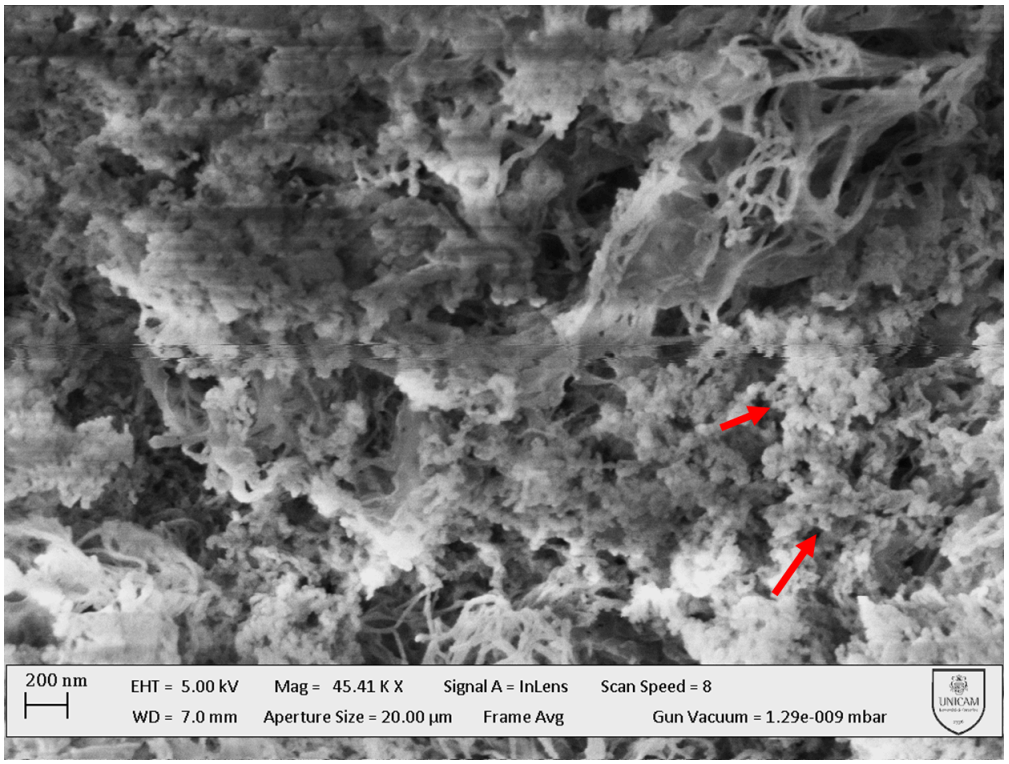

Figure 10: SEM images of keratin gel after curcumin absorption. The arrows indicate the curcumin aggregates.

Rheological Analysis

The keratin extracted using mercaptoethanol formed a gellified system that was easy to handle and to cut into the shape of discs. A rheological analysis has been performed to characterise the gel in terms of consistency and viscoelastic properties.

A stress sweep test at 25°C was firstly carried out (results are reported in Figure 11) to determine the consistency and linear viscoelastic region. In a stress interval up to 5 Pa, the values of the rheological moduli G' and G'' (both in the order of 10^3 Pa) and tan δ were quasi-constant as typical for structured gels, such as crosslinked poly(methyl methacrylate) (PMMA) [37]. Only a slight decrease of the G' modulus occurred at higher stresses applied, confirming the resistance of the gel to mechanical solicitations. ,

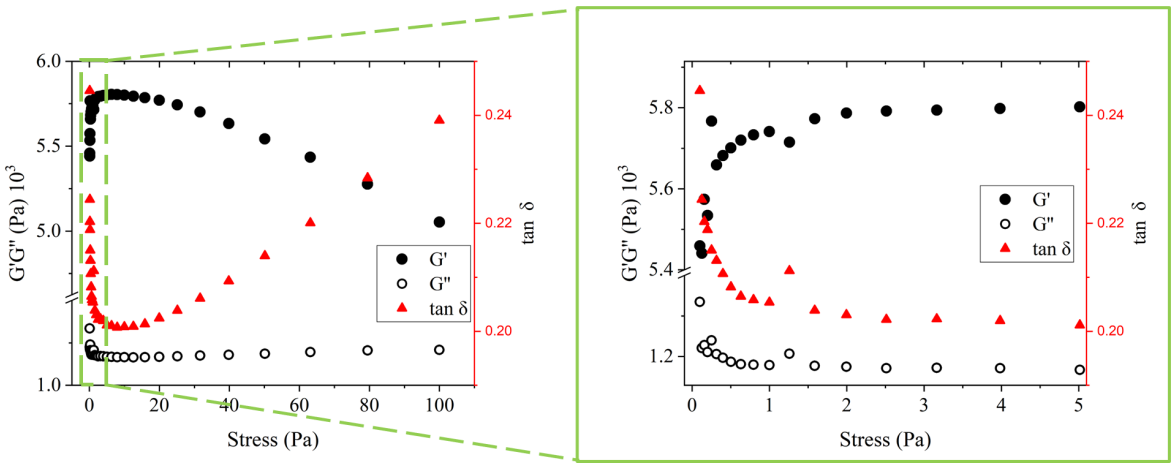

Figure 11: Stress sweep test of pure keratin gel. Black points indicate the values of G', white points indicate the values of G'' and red triangles indicate the tan δ related to the stress.

A frequency sweep test at 25 °C was also performed to confirm the solid-like behaviour of the gel (Figure 12). Indeed, for pure keratin gel, the value of the storage modulus (G', 12-14 kPa) was considerably higher than that of the loss modulus (G'') in the range of frequencies analysed. As compared with other biopolymer-based gels (e.g., liposome gels [38] and cellulose-derived ones [39]) already employed for drug release, this novel keratin-based gel appeared to be considerably more structural and sound. The high consistency on the one hand does not allow an easy injectability of the gels, but conversely does not necessarily require the addition of nanoparticles for higher compaction and hardening. A higher storage modulus (G′) is related to the ability of a material to store energy and return to its original shape after being subjected to stress. The substantial independence of tan δ from frequency identifies an elastic behaviour during shearing [40], which indicates that the gel may be particularly suitable for film casting at ambient temperature.

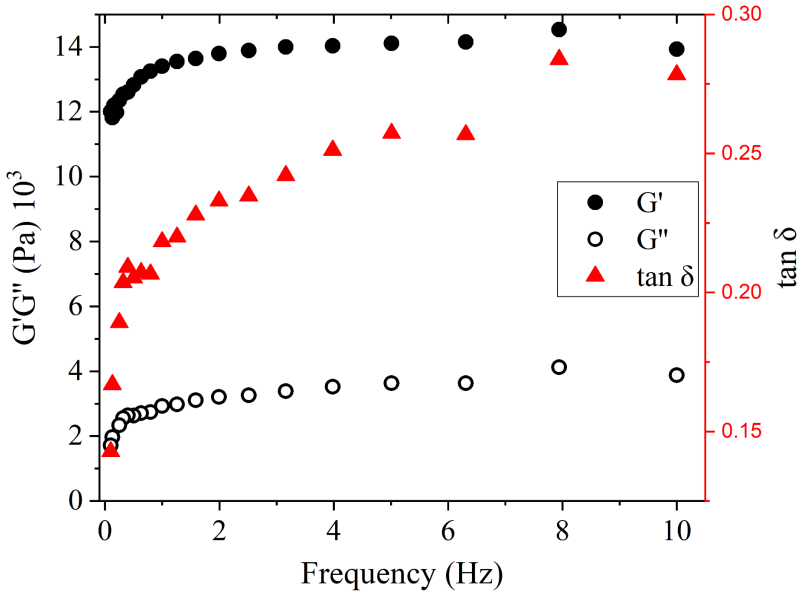

Figure 12: Frequency sweep test of pure keratin gel. Black points indicate the values of G', white points indicate the values of G'' and red triangles indicate the tan δ related to the frequency.

Drug delivery system

The release kinetics of curcumin at various pH was then investigated to evaluate any differences in the release rate related to pH in particular environments or conditions. For example, the pH of a typical mucous membrane, such as the colonic one (5.8) [41], that of the skin (7.4) [42] or the lower end of the stomach environment (1.2) [43]. For this purpose, similar sections of the curcumin-containing gels were incubated in phosphate buffered saline (PBS) solution, containing 2% polysorbate 80 as surfactant at various pH and the results are shown in Figure 12.

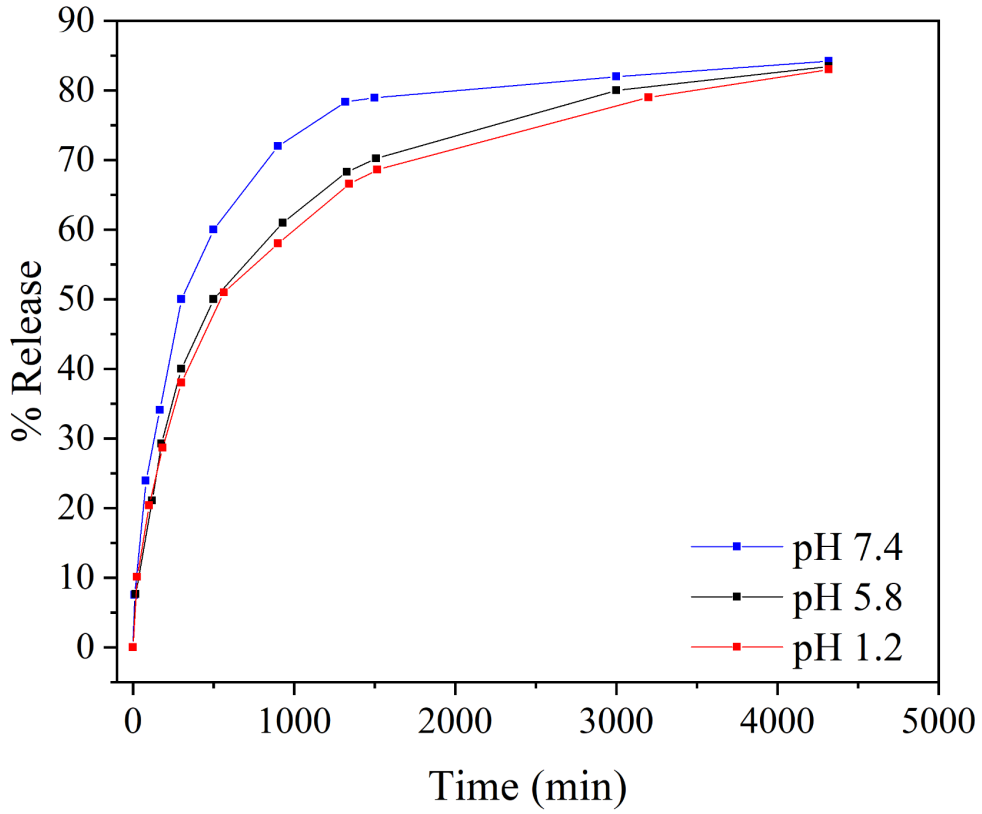

Fig. 12: Release of curcumin from Keratin gel at various pH

Data over the release of curcumin from the gel with time indicate that this reaches a plateau around 1700 minutes, therefore having a continuous release over a total of 24-28 hours. After 72 hours, the maximum release point for the various pH is always exceeding 80%, in particular 84.22% for pH 7.4, 88.45% for pH 5.8 and 83.01% for pH 1.2. However, the release is definitely more rapid in quasi-neutral conditions. The strength and elasticity of the gel obtained also indicates that there is no need for further blending of the gel with other biopolymers (e.g., cellulose-based ones), which has been revealed to considerably reduce the curcumin release [44].

Conclusions

The work demonstrated that gels obtained from chicken feathers through mercaptoethanol extraction The characterization of the gel highlighted the preservation of the secondary protein structure and the viscoelastic properties typical of structured gels. In addition to this,

the healing action of keratin could be also exploited for the purpose: due to the sufficient strength of the gel fabricated in a range of loads and frequencies corresponding to the most common synthetic hydrogels, this would be able to act as a multipurpose gel material. In particular, further development could be obtained by the addition of antibacterial nanoparticles, due to its functionality at different pH conditions. The release of curcumin did serve in effect as an indication for using mercaptoethanol-extracted keratin gel from chicken feather as a carrier for a multiplicity of natural healing and antioxidant agents.

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