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ARTICLE

Valorization of chicken feathers using aqueous solutions of ionic liquids

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The poultry-processing industry generates large quantities of waste rich in keratin, a fibrous protein representing around 90 wt % of chicken feathers, which is currently disposed of in landfilling or incinerated. Keratin is commonly recognized as a renewable biopolymer resource used for biomaterials preparation (e.g., films and hydrogels) with interest for biomedical applications. Even though research on keratin recovery from chicken feathers started many years ago, very few keratin materials from this source have been developed due to the low keratin solubility in most common solvents and poor protein recovery yield. Although ionic liquids (ILs) have been reported as alternative solvents with high dissolution capability for several biopolymers, keratin recovery from chicken feathers using aqueous solutions of ILs was not investigated up to date. Considering the Green Chemistry Principles (especially the first one: zero waste) and Circular Economy concepts, in this work, we show that chicken feathers can be effectively dissolved in an aqueous solution of 1-butyl-3-methylimidazolium acetate (80 wt %), further allowing the keratin recovery and preparation of keratin biofilms. The keratin recovery from the IL aqueous solution was optimized considering the coagulant type, solution:coagulant weight ratio, temperature, and time, being the coagulant type the variable with higher influence in the recovery process. Under the best conditions (ethanol, 1:2 w/w, 5 °C, and 1 h), 90 wt % of keratin was recovered. The IL recovery and reuse were also evaluated, still allowing 82 wt % of recovery yield at the end of the third cycle. The recovered keratin was characterized, confirming the required physicochemical properties. A keratin film was finally prepared and characterized through cell viability, oxidative stress and wound healing assays, opening the path for the use of keratin films in biomedical applications.

Introduction

Chicken is one of the most consumed meats in the world, with an annual consumption of 65 million tons worldwide. Its high consumption produces a large amount of feathers waste, corresponding to around 7 wt % of the total mass of an adult chicken^{1–6}. Currently, this waste is disposed of through incineration or landfilling, contributing to environmental pollution and highlighting the necessity to develop sustainable and profitable ways of waste valorization^{1,7}, while being connected to the biorefinery concept⁸. On this subject, it is crucial to highlight the circular economy concept, considered a top priority in the EU Sustainable Development Goals (SDGs)⁸. On a dry weight basis, the highest component in chicken feathers is keratin (around 90 wt %), with the rest consisting of fibers, ash, and fat⁹. Keratin is a structural and fibrous protein, and the third most abundant biopolymer in the environment, after cellulose and chitin^{10–13}. Keratin is also the main component of hair, wool, nails, hooves, and horns. Nevertheless, the potential production

of this protein from chicken feathers is 2.5 times higher than the current output of wool (the main source investigated in the literature)⁶. Due to its properties, keratin has applications in the biomedical field and tissue engineering¹⁴, encouraging some researchers to investigate its use for biomaterials preparation (e.g., films and hydrogels)^{15–17}.

Despite its relevance, due to the inter and intramolecular disulfide bonds in sulfur-containing amino acids, keratin presents low solubility in common organic solvents and has a poor recovery yield^{10,13,18,19}. Furthermore, commonly reported methods (e.g., chemical hydrolysis, microwave, and steam exposure techniques) provide negative impacts on the protein structure and respective properties. For instance, elevated temperatures used in the chemical hydrolysis method promote amino acid degradation, and methods using chemical compounds (e.g., thiols and 2-mercaptoethanol) are time-consuming and present environmental harm, as reviewed by Chilakamarry et al.¹¹ and Shavandi et al.²⁰. To overcome this concern, several studies have been conducted to identify efficient and greener alternatives to completely dissolve biomass rich in keratin, while allowing an efficient keratin recovery. Among the alternatives reported, the use of ionic liquids (ILs) - salts composed of large organic cations and organic or inorganic anions - should be highlighted^{10,21–27}. If properly designed, ILs allow complete biomass dissolution without requiring high temperatures and long-time processing. Another disadvantage

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overcome by the use ILs is in the keratin recovery process. Traditionally, when conventional dissolution (e.g., using urea and sodium metabisulfite) is carried out, dialysis is necessary to recover keratin²⁸, or the adjustment of the isoelectric point using hydrochloric acid accompanied by sodium sulfate^{6,12}. When using ILs for biopolymers recovery, coagulant solvents, such as water²² or ethanol²⁹, can be used.

In 2005, 1-butyl-3-methylimidazolium chloride ([C₄C₁im]Cl) was used to dissolve wool and regenerate wool keratin for the first time²⁵, and since then ILs have demonstrated excellent results with this type of biomass when properly selected^{20,23,29,30}. Due to the wide variety of ILs and the complexity of keratin (which does not have regular repeating units), it is not easy to identify the best IL for keratin dissolution. To illustrate it, Liu et al.¹⁰ screened 621 ILs for three keratin models dissolution using COSMO-RS and validated it with experimental results. According to the authors, 1-ethyl-3-methylimidazolium acetate ([C₂C₁im][C₁CO₂]) was one of the best ILs for protein dissolution, in accordance with the results reported by Zhang et al.³¹ that investigated the regeneration of wool keratin using this IL. The results showed that when the same cation is used, the anion [C₁CO₂]⁻ presents a higher ability to dissolve keratin than Cl⁻ and Br⁻ due to its stronger hydrogen-bonding acceptor ability. Despite these promising results^{10,31}, keratin recovery from biomass using [C₁CO₂]-based ILs has been scarcely addressed in the literature. Wool has been the focus of most research involving ILs^{17,22,25,26,32}, and chicken feathers have not been efficiently used^{23,33}. Wang et al.³³ reported the use of a hydrophobic IL, 1-hydroxyethyl-3-methylimidazolium bis(trifluoromethanesulfonyl)amide, and sodium bisulfite, for chicken feathers dissolution, achieving a maximum yield of keratin of 21 wt %. Latter, Ji et al.²³ investigated the use of some imidazolium-based ILs and sodium sulfite for keratin recovery from duck feathers, achieving a yield of 75.1 wt %. In both works, sodium sulfite salts were used to improve biomass dissolution, and no pure ILs or their aqueous solutions have been reported for chicken feathers dissolution and keratin recovery.

In this study, the performance of ILs and their aqueous solutions for chicken feathers dissolution and keratin recovery was investigated. The keratin recovery conditions were optimized by varying the coagulant type, solution:coagulant weight ratio, temperature, and time. The physicochemical properties of recovered keratin samples were determined. The IL recovery and reuse were also evaluated, aiming for the development of a process more interesting in terms of economic and environmental factors. Using the optimal conditions, a keratin film was prepared. Its mechanical properties and biological activity were evaluated, foreseeing biomedical applications, and opening the path to more research in this field.

Materials and methods

Materials

Chicken feathers were collected from the Campoaves company in Oliveira de Frades, Portugal. The ILs used, 1-butyl-3-methylimidazolium acetate ([C₄C₁im][C₁CO₂]) (>98 wt %), [C₂C₁im][C₁CO₂] (>98 wt %), [C₄C₁im]Cl (99 wt %), 1-ethyl-3-methylimidazolium chloride ([C₂C₁im]Cl) (99 wt %), 1-butyl-3-methylimidazolium bromide ([C₄C₁im]Br) (99 wt %), 1-butyl-3-methylimidazolium thiocyanate ([C₄C₁im]SCN) (>98 wt %) and 1-butyl-1-methylpyrrolidinium ([C₄C₁pyrr]Cl) (99 wt %), were purchased from IOLITEC. Ethanol (99.8 wt %) and acetone (100 wt %) were acquired from Fisher Scientific and Valente & Ribeiro Lda, respectively. Chemical reagents used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, including urea, hydrochloric acid, tris (hydroxymethyl)-aminomethane (99 wt %) and Page Ruler Low Range Unstained Protein Ladder, were purchased from Thermo Fisher Scientific. Sodium dodecyl sulfate (SDS) and glycerol were from PanReac AppliChem and Biochem Chemopharma, respectively. TEO-Tricine Precast Gels-Run Blue™ (12 wt %, 12-well, 10x10 cm) was purchased from Abcam. The compounds used for biological analysis, including Roswell Park Memorial Institute (RPMI) 1640 Medium, penicillin, streptomycin, lipopolysaccharide (LPS) from *Escherichia coli* (serotype O26:B6), propidium iodide (PI), and resazurin were obtained from Sigma Chemical Co. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) were obtained from Thermo Fisher Scientific. cellQART® tissue culture treated 12-well and 24-well inserts PET membranes pore size 1 mm were acquired from SABEU GmbH & Co. KG. Anti-human CD54-APC (clone 1H4), and CD86-FITC (clone BU63) were purchased from ImmunoTools GmbH, and anti-human CD83-APC (Clone HB15e) was from BD Biosciences.

Chicken feathers pre-treatment

The collected feathers were washed three times with soap and dried in an air oven (Carbolite Gero) at 50 °C for 72 h. Then, the feathers were milled and immersed in ethanol 99 % for 24 h to remove the fatty matter. The cleaned wet feathers were dried at 50 °C for 24 h and then stored in plastic bags at 5 °C until further use.

Chicken feathers dissolution and keratin recovery

ILs screening for chicken feathers dissolution

Seven ILs, namely [C₂C₁im]Cl, [C₂C₁im][C₁CO₂], [C₄C₁im]Cl, [C₄C₁im][C₁CO₂], [C₄C₁im]Br, [C₄C₁im][SCN], and [C₄C₁pyrr]Cl, were used in an initial screening to verify the ILs' dissolution capability for chicken feathers. The ILs selected comprise the combination of different cations and anions, taking into consideration the ILs that have been reported in the literature for the effective dissolution of different biopolymers from biomass^{29,31,34–37}. Nevertheless, to the best of our knowledge, the selected ILs still have not been evaluated for chicken feathers dissolution. The dissolution assays were carried out at 100 °C for 4 h in a solid:liquid (chicken feathers:solvent) ratio of 1:20 w/w

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in a carousel (Carousel Tech, Radleys). These conditions were established according to previously reported data^{11,23,38}. Treated chicken feathers were used, as previously described. After identifying the best IL, the IL concentration effect was evaluated by using aqueous solutions of [C₄C₁im][C₁CO₂] at 100 wt %, 80 wt %, and 60 wt %.

Keratin recovery

Aqueous solutions of [C₄C₁im][C₁CO₂] at 80 wt % were prepared and heated at 100 °C. When this temperature was achieved, treated feathers were added to the IL solution, in a solid:liquid ratio of 1:20 w/w. The dissolution was conducted at 100 °C for 4 hours. Then, the solution was moved to a centrifuge tube. The coagulant solvent (water, ethanol, water:ethanol mixtures or acetone) was added at solution:coagulant ratio of 1:1, 1:2, 1:3 or 1:5 w/w and stored at different temperatures (-20 °C, 5 °C or 25 °C) during different time periods (from 1 h to 24 h) to induce keratin precipitation. Posteriorly, the solution was centrifugated for 20 min at 25 °C and 4000 rpm in a refrigerator centrifuge machine (Neya 16 R, Remi Elektrotechnik Ltda), promoting the separation of the precipitated keratin. The obtained protein was washed with water to remove IL residues and centrifugated at the previously described conditions. Finally, the recovered keratin was dried at 50 °C for 48 h.

The keratin recovery yield (RY%) was determined according to Equation 1. Contrarily to what is commonly observed in the literature (keratin yield based on the total biomass mass), the yield of the recovered keratin was obtained considering the amount of protein available in chicken feathers (90 wt %) ⁹. All experiments were conducted at least in duplicate, with the average values and respective standard deviation being presented.

$$RY\% = \left(\frac{m_{\text{keratin}}}{m_{\text{feathers}} \times 0.9} \right) \times 100 \quad (1)$$

where m_{keratin} represents the mass of keratin recovered and dried and m_{feathers} the mass of chicken feathers used at the dissolution step.

Keratin characterization

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

4 mg of the recovered keratin were dissolved in 1 mL of buffer solution (0.05 g·mol⁻¹ Tris-HCl pH 8.0, 8 g·mol⁻¹ urea, and 0.01 g·mol⁻¹ DTT) and stirred for 2 h. The keratin solution was dissolved in a running buffer (0.5 g·mol⁻¹ Tris-HCl pH 6.8, 20 w/w glycerol, 4 w/w SDS, and 0.01 g·mol⁻¹ dithiothreitol (DTT)) and then heated at 90 °C for 5 min to complete denaturation. The protein marker (3.4 × 100 kDa) and the samples were loaded into the polyacrylamide gels and submitted to a run at 80 V for 1 h, followed by 1 h at 120 V. Then, the proteins were stained with Coomassie Brilliant Blue G-250 overnight at room temperature.

Fourier transform infrared attenuated total reflectance (FTIR-ATR)

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The FTIR-ATR spectra of chicken feathers and recovered keratin samples were acquired in a FTIR system Spectrum BX, PerkinElmer, equipped with a single horizontal Golden Gate ATR cell and a diamond crystal. The analysis of functional groups available was performed at room temperature with controlled air humidity. All data were recorded in a frequency range of 4000-400 cm⁻¹ by accumulating 32 scans with a resolution of 4 cm⁻¹ and an interval of 1 cm⁻¹. The acquired spectra were recorded as absorbance values.

Thermogravimetric analysis (TGA)

Thermogravimetric analyses were carried out in a differential thermogravimetric analyzer Hitachi STA300. Approximately 5 mg of keratin samples were placed in an aluminum pan and further analyzed under a nitrogen gas blanket using a flow rate of 1 mL·min⁻¹. The samples were heated at a rate of 10 °C·min⁻¹ with a temperature range of 30-900 °C.

IL recovery and reuse

To achieve the IL recovery and possible reuse, after the centrifugation step, the supernatant (solution composed of IL + water + coagulant) was collected and transferred to a clean 50 mL round bottom, previously weighed. The volatile compounds were removed from the solution using a rotatory evaporator composed of rotavapor R-10, heating bath B-491, vacuum pump V-700 and vacuum controller V-850 (all from Buchi, Switzerland). The obtained solution was dried in a vacuum line for 5 days at room temperature to remove any traces of water and coagulant. The IL acquired was weighed, and the IL recovery yield was determined knowing the dry IL mass and the initial IL mass used for the feathers dissolution. The IL reuse was evaluated for a total of 3 dissolution and recovery cycles.

Keratin film processing

The keratin film processing was evaluated using the best conditions for keratin recovery. After the washing step, a keratin solution (15 wt %) was prepared with distilled water. The mixture was mixed under constant magnetic stirring at 60 °C for 30 min. Then, the solution was cast on silicone molding and placed in an air oven at 50 °C for 24 h.

Keratin film characterization

Scanning electron microscope (SEM)

SEM was performed using a high-resolution field-emission (HR-FESEM) Hitachi SU70 microscope. Keratin film was coated with a thin carbon layer prior to the sample analysis to ensure its conductivity. Images were obtained using an accelerating voltage of 10 kV and a working distance of 15 nm.

Tensile strength

Tensile strength was conducted using Lloyd EZ 50 testing machine. Film samples were cut into strips 1 cm wide and 4 cm long, and at least two replicates were performed.

Contact angle



Contact angles were determined to assess the hydrophilicity of the film using a semi-automatic wettability analysis with high dosing precision (DSA25S, Krüss). Multiple measurements were realized by adding a drop of 7 μL of solvent (water or ethylene glycol) at a rate of 7 $\mu\text{L}\cdot\text{s}^{-1}$ on the film deposited on a rigid substrate.

Biological activity

Cell culture

Murine Raw 264.7 macrophages (ATCC TIB-71) were cultured in DMEM containing 4.5 $\text{g}\cdot\text{L}^{-1}$ glucose, 0.004 $\text{mol}\cdot\text{L}^{-1}$ L-glutamine, 1500 $\text{mg}\cdot\text{L}^{-1}$ sodium bicarbonate and supplemented with 10 % non-inactivated fetal FBS, 100 $\text{U}\cdot\text{mL}^{-1}$ penicillin and 100 $\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin. The human keratinocyte cell line HaCaT (DKFZ, Heidelberg, Germany) and the mouse fibroblast cell line 3T3 (ATCC CRL1658) were cultured in the same DMEM medium containing heated inactivated FBS. These adherent cells were incubated at 37 °C in a humidified atmosphere (95 % air and 5 % CO_2) and were used after reaching 70 % - 80 % confluence, which occurs approximately every 3 days after each passage. The THP-1 human monocytic cell line (ATCC TIB-202) was cultured and maintained at a cell density between 0.2 $\times 10^6$ and 1 $\times 10^6$ $\text{cells}\cdot\text{mL}^{-1}$ in RPMI 1640 supplemented with 10 % inactivated FBS, 0.025 $\text{g}\cdot\text{mol}^{-1}$ glucose, 0.010 $\text{g}\cdot\text{mol}^{-1}$ HEPES, 0.001 $\text{mol}\cdot\text{L}^{-1}$ sodium pyruvate, 100 $\text{U}\cdot\text{mL}^{-1}$ penicillin and 100 $\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin. Cells were subcultured every 3 or 4 days and kept in culture for 2 months.

Cell viability assays

To investigate the biocompatibility of keratin film, its effect on the viability/metabolic activity of monocytes, macrophages, keratinocytes, and fibroblasts cells was assessed by flow cytometry through analysis of PI exclusion or by the resazurin assay³⁹. For monocytes, 0.3 $\times 10^6$ THP1 cells were plated in 0.5 mL of medium per well of a 24-well plate, and then the keratin film was put into contact with cell cultures for 24 h by means of transwell inserts with 1 μm PET membranes. At the end of exposure, cells were collected, washed twice with PBS, and then resuspended in 400 μL FACS buffer (PBS + 2 % FBS). 1 μL of 1 $\text{mg}\cdot\text{mL}^{-1}$ PI was added to each condition, and cells were acquired in an Accuri C6 flow cytometer (BD Bioscience). Dead cells were identified by including PI and increased fluorescence in the FL2 channel (585/40 nm filter). For adherent cell lines, 0.5 $\times 10^6$ Raw 264.7, 0.1 $\times 10^6$ HaCaT or 0.1 $\times 10^6$ 3T3 cells were plated per well of a 24-well plate in 500 μL medium and let to stabilize overnight. Then, keratin film samples were put into contact with cell cultures using transwell inserts. After 24 h of incubation, resazurin was added to cells (final concentration of 50 μM) during the last 2 h of incubation. Finally, 200 μL from each condition were transferred to a 96 wells plate, and the absorbance of resorufin (the product of the resazurin reduction) was measured at 570 and 600 nm in a Tecan Infinite M200 Pro spectrophotometer (Tecan Trading AG). The data are the average of three biologically independent experiments conducted in duplicate for each condition, and the results were expressed as

the average cell viability \pm SD. The keratin film used in this study was sterilized by exposure to UV for 45 min. DOI: 10.1039/D2GC04477C

Oxidative stress evaluation

To evaluate if exposure to keratin film triggers oxidative stress, ROS formation was analyzed with ROS indicator CM-H2DCFDA. Briefly, 0.75 $\times 10^6$ Raw 264.7 cells were plated per well of a 12-well plate and let to stabilize overnight, followed by co-culture with keratin film. As a positive control for oxidative stress induction, cells were treated with 100 $\text{ng}\cdot\text{mL}^{-1}$ LPS. After 24 h exposure, cells were washed with PBS and then loaded with 2 μM CM-H2DCFDA in HBSS (in $\text{mol}\cdot\text{L}^{-1}$: 0.0013 CaCl_2 , 0.0005 MgCl_2 , 0.0053 KCl , 0.138 NaCl , 0.00044 KH_2PO_4 , 0.0042 NaHCO_3 , and 0.00034 Na_2HPO_4 , pH 7.4) for 30 min at 37 °C in the dark. Cells were washed three times with HBSS, and images were acquired with an EVOS M5000 Imaging System (Thermo Fisher Scientific) at 20x magnification (scale bar 100 μm).

Wound healing assays

The effect of keratin film on the spreading and migration capabilities of HaCaT keratinocytes and 3T3 fibroblasts was assessed using a scratch wound assay which addresses the expansion of a cell population on surfaces. HaCaT or 3T3 cells were seeded into 12-well tissue culture dishes at a density of 0.3 $\times 10^6$ $\text{cells}\cdot\text{mL}^{-1}$ and let to stabilize in a medium containing 10 % FBS until they reached a confluent cell monolayer. Then, a linear wound was generated in the monolayer with a sterile 200 μL or 1000 μL plastic pipette tip for HaCaT or 3T3 cells, respectively. Any cellular debris was removed by washing the wells with PBS. At least five images of the scratched area of each condition were taken at this 0 h time point using an EVOS M5000 Imaging System at 4x magnification (scale bar 200 μm). Exposure to keratin film was performed in a medium containing 2 % FBS. Medium containing 10 % FBS or Cytochalasin D were used as positive and negative controls, respectively. After 16 h, at least five images of the scratched area of each condition were taken and compared to the corresponding 0 h time point.

Results and discussion

ILs screening for chicken feathers dissolution

Most of investigated ILs showed no ability to achieve the complete dissolution of chicken feathers at the ratio 1:20 w/w, with the exception of the acetate-based ILs ($[\text{C}_4\text{C}_1\text{im}][\text{C}_1\text{CO}_2]$ and $[\text{C}_2\text{C}_1\text{im}][\text{C}_1\text{CO}_2]$) that allowed the complete chicken feathers dissolution at 100 °C and 4 h. This is in accordance with results previously reported in the literature since the acetate anion is a strong hydrogen bond acceptor with high hydrogen-bond basicity³¹, thus allowing the dissolution of biopolymers that can establish hydrogen-bonding, such as proteins. Passos et al.⁴⁰ reported some ILs' hydrogen-bond basicity by estimating their hydrogen bond interaction energies (E_{HB}) with COSMO-RS. ILs with more negative values of E_{HB} present higher hydrogen bond basicity. The following order is observed for the ILs here investigated: $[\text{C}_2\text{C}_1\text{im}][\text{C}_1\text{CO}_2]$ ($E_{\text{HB}} = -20.21 \text{ kJ}\cdot\text{mol}^{-1}$) >



$[C_4C_1im][C_1CO_2]$ ($E_{HB} = -19.83 \text{ kJ}\cdot\text{mol}^{-1}$) > $[C_2C_1im]Cl$ ($E_{HB} = -14.99 \text{ kJ}\cdot\text{mol}^{-1}$) > $[C_4C_1im]Cl$ ($E_{HB} = -14.52 \text{ kJ}\cdot\text{mol}^{-1}$) > $[C_4C_1im]Br$ ($E_{HB} = -11.66 \text{ kJ}\cdot\text{mol}^{-1}$) > $[C_4C_1im][SCN]$ ($E_{HB} = -8.20 \text{ kJ}\cdot\text{mol}^{-1}$) > $[C_4C_1pyrr]Cl$ ($E_{HB} = -6.99 \text{ kJ}\cdot\text{mol}^{-1}$). Only ILs with the acetate anion are able to completely dissolve chicken feathers at the studied conditions. Furthermore, aqueous solutions of acetate-based ILs are alkaline ($pH \cong 10.4$, as determined by us), in contrast with aqueous solutions of $[C_2C_1im]Cl$ ($pH 4.77$) and $[C_4C_1pyrr]Cl$ ($pH 5.69$). These results additionally suggest that alkaline aqueous solutions display a better performance for chicken feathers dissolution, which has also been observed for cellulose⁴¹. Taking these results into account and considering the similar performance of both acetate-based ILs for chicken feathers dissolution, the following studies were carried out with $[C_4C_1im][C_1CO_2]$ aqueous solutions.

The IL concentration effect was evaluated using the pure IL and aqueous solutions of $[C_4C_1im][C_1CO_2]$ at 80 wt %, and 60 wt %. According to the results obtained, adding water up to 20 wt % promotes viscosity reduction, accelerating the mass transfer process and facilitating the feathers' dissolution. However, when the water concentration increases from 20 wt % to 40 wt %, the solution cannot completely dissolve the added chicken feathers (1:20 w/w) since water starts to compete with the hydrogen bonds responsible for dissolution, as previously reported⁴². In summary, the maximum dissolution of chicken feathers was obtained with an aqueous solution of $[C_4C_1im][C_1CO_2]$ at 80 wt %.

Keratin recovery

The effect of coagulant solvent and solution:coagulant ratio was evaluated for the keratin recovery from the IL aqueous solution, while keeping time and temperature as constants at 1 h and 5 °C, respectively. The results obtained are presented in Fig. 1. Among the studied coagulants (water, ethanol, acetone, and water:ethanol mixture), acetone results in a lower keratin recovery yield, ranging from 7.74 to 10.93, while ethanol presents the best results, with recovery yields ranging from 16.62 to 90.17. Between these solvents, water resulted in a range from 28.85 to 52.64, and water:ethanol mixture presented yields ranging from 21.45 to 48.82. Accordingly, it seems that the interactions established between the studied IL and ethanol are more efficient than between IL-water and IL-acetone, leading to a more efficient protein precipitation. This is due to the hydrogen bonds formed between the hydrogen atom (-OH) of ethanol with the IL cation (H-bonding donor) and the IL anion (hydrogen-bond acceptor)⁴³.

Considering ethanol as the best coagulant agent and evaluating the solution:coagulant ratio, it is possible to observe that by increasing it from 1:1 to 1:2 w/w, the keratin recovery yield increases from 16.62 to 90.17 wt %. However, no significant differences in the keratin recovery yield were observed above this ratio.

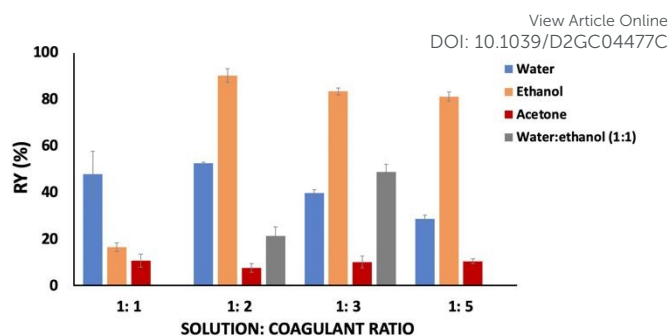


Fig. 1 Coagulant and solution:coagulant ratio effect on the keratin recovery yield.

The temperature effect was also considered on the recovery yield of keratin, whose results are provided in Fig. 2. Water and ethanol were used as the coagulant agents for 1 h and at a solution:coagulant ratio of 1:2 w/w. Concerning the results obtained with ethanol, no significant differences were observed in the keratin recovery yield (90.17 - 92.86 wt %) when the temperature was changed from -20 °C to 25 °C. Nevertheless, evaluating the temperature effect for water, there is an increase of ~8 wt % (from 44.45 up to 52.64 wt %) in the keratin recovery yield when the temperature decreases from 25 °C to 5 °C.

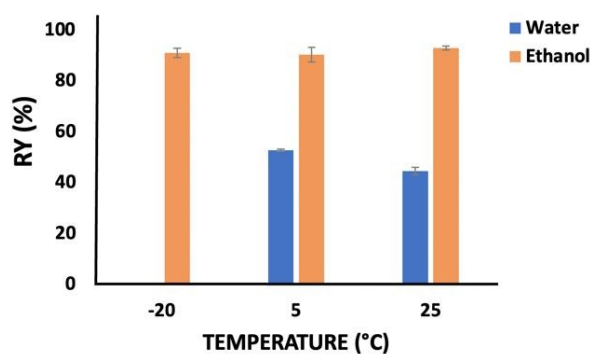


Fig. 2 Temperature effect on the keratin recovery yield.

Fig. 3 presents the behavior of the keratin recovery yield with time when water (5 °C) and ethanol (5 °C or -20 °C) were used as coagulant agents at a solution:coagulant ratio of 1:2 w/w. Considering the use of ethanol, no significant differences were observed from 1 h to 24 h, independently of the temperature. Thus, one hour was enough to achieve a high keratin recovery yield, i.e. 90.17 wt %. On the other hand, when water was used as a coagulant agent, time shows to be an important factor, with the keratin recovery yield significantly increasing (~20 wt %; from 47.93 to 69.54 wt %) during the first six hours. Nevertheless, no significant differences were observed at time periods higher than 6 h.



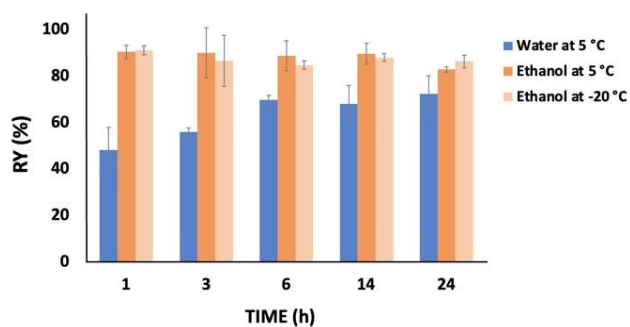


Fig. 3 Time effect on the keratin recovery yield.

All the previous results show that ethanol is the best coagulant agent, under the following operating conditions (time, temperature, and solution:coagulant ratio): 1 h, 5 °C, and 1:2 w/w. Under these conditions, the keratin recovery yield was 90 ± 3 wt %, which is a high yield, obtained using an amenable solvent and coagulant. Maity et al.⁴⁴ used 1 mL of an aqueous solution (25 wt %) of tetramethyl ammonium hydroxide (TMAOH) to gradually dissolve chicken feathers (10 to 600 mg), achieving a partial dissolution (~ 60 wt %) in 6 h. The keratin recovery yield was 72 ± 2 wt %, achieved by adding acetic acid and acetone (1:4)⁴⁴. Furthermore, Pourjavaheri et al.¹² achieved a keratin yield of ~ 88 wt % using sodium sulfide and ~ 66 wt %, using a solution of urea and L-cysteine, respectively. Fagbemi et al.⁴⁵ used sodium hydroxide (1.78 %) and sodium bisulfite (0.5 %), leading to a protein recovery yield of 65.21 %⁴⁵. Despite their lower recovery yields, it is relevant to highlight that these methods include dialysis for the keratin recovery^{1,15,28} and a long-time processing (~ 3 days), while being necessary to change the water 3 times a day.

Keratin characterization

Keratin samples obtained through the different recovery conditions were analyzed by FTIR, SDS-PAGE, and TGA to determine their physicochemical properties and possible IL contaminations.

From Fig. S1 it is possible to observe the essential peaks in the keratin samples obtained with different conditions (coagulant, solution:coagulant ratio, temperature and time), corresponding to stretching vibrations of O-H and N-H (Amide A) at $3650\text{--}2830$ cm^{-1} , C=O stretching (Amide I) at $1735\text{--}1600$ cm^{-1} , N-H bending and C-H stretching (Amide II) at $1600\text{--}1480$ cm^{-1} , and amide III ($1300\text{--}1200$ cm^{-1}), being in agreement with the literature^{46,47}. The same peaks are observed for the remaining samples with the remaining studied variables, except for the use of ethanol at 1:5 w/w solution:coagulant ratio. From the FTIR spectra investigating the influence of the amount of ethanol (Fig. 4), the absorption peaks obtained for chicken feathers, ethanol 1:1, 1:2, 1:3, and 1:5 correspond to 1627 cm^{-1} , 1640 cm^{-1} , 1620 cm^{-1} , 1642 cm^{-1} , and 1630 cm^{-1} for Amide I; 1616 cm^{-1} , 1550 cm^{-1} , 1554 cm^{-1} , 1550 cm^{-1} , and 1512 cm^{-1} for Amide II; and 1227 cm^{-1} , 1232 cm^{-1} , 1231 cm^{-1} , 1246 cm^{-1} , and 1226 cm^{-1} for Amide III, respectively. Significant peaks at 1390 cm^{-1} (O-H bending) and 1160 cm^{-1} (C-O stretching) are observed for the samples recovered with ethanol 1:1, 1:2, and 1:3 (– cf. Fig. 4), indicating the IL presence in the keratin sample. These results highlight that a higher amount of coagulant during the recovery step is more efficient for the IL removal.

1, 1246 cm^{-1} , and 1226 cm^{-1} for Amide III, respectively. Significant peaks at 1390 cm^{-1} (O-H bending) and 1160 cm^{-1} (C-O stretching) are observed for the samples recovered with ethanol 1:1, 1:2, and 1:3 (– cf. Fig. 4), indicating the IL presence in the keratin sample. These results highlight that a higher amount of coagulant during the recovery step is more efficient for the IL removal.

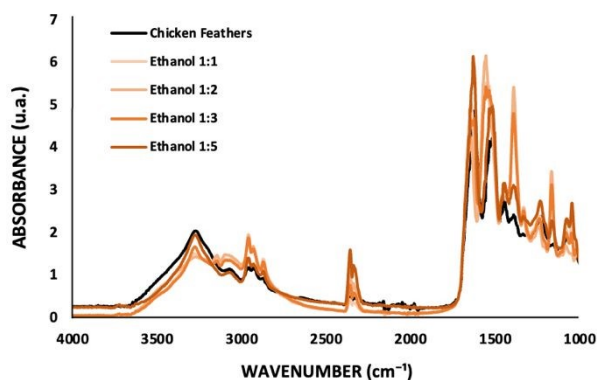


Fig. 4 FTIR spectra of chicken feathers and keratin samples recovered with ethanol at different solution:coagulant ratio.

The molecular weight of keratin samples obtained with different recovery conditions was analyzed by SDS-PAGE, showing a band around 5–12 kDa (cf. Fig. S2 in the ESI). This is in accordance with the results reported in the literature for keratin from chicken feathers using other processing methods^{3,12}. Nevertheless, when acetone was used as a coagulant agent, no protein bands were identified in the electrophoresis gel, suggesting that the protein was degraded into lower molecular weight fractions, which are not possible to identify in the gel.

A two-step degradation was observed in the TGA curves of both chicken feathers and recovered keratin samples (Fig. S3 in the ESI). The first degradation step corresponds to water loss, occurring between 25 and 100 °C. The second stage of degradation of keratin polypeptides corresponds to the decomposition of disulfide bonds, resulting in the release of hydrogen sulfide and sulfur dioxide. Overall, the thermal stability assays of chicken feathers and recovered keratin samples showed that these biomaterials are stable up to 215 °C. The TGA results indicate that the recovered keratin has a similar profile to the original keratin in the feathers, being in agreement with the literature^{21,48}.

In general, no significant differences were observed in the keratin properties with the different recovery conditions, except for the improvement of the IL removal using a higher amount of ethanol and no identification of the protein band with the use of acetone as a coagulant agent.

IL recovery and reuse

Aiming to develop a sustainable process, $[\text{C}_4\text{C}_1\text{im}][\text{C}_1\text{CO}_2]$ was recycled and reused three times with fresh chicken feathers. The results obtained are depicted in Fig. 5. All components of chicken feathers were dissolved into the IL aqueous solution (80 wt %, in



a solid: liquid ratio of 1:20 w/w, at 100 °C, during 4 h), indicating that what was not recovered could be retained in the coagulant solvent, yet without significantly affecting the dissolution of chicken feathers and keratin recovery performance. Approximately 82 % of the IL was recovered at the end of the third cycle, with the keratin yield decreasing only 4 % from the first to the third cycle. This indicates that the reused IL does not significantly compromise the chicken feathers dissolution and keratin recovery. As depicted in the ESI, in Figs. S5 to S8, ^1H and ^{13}C NMR spectra show that the IL maintains its structure, with no degradation, after the recovery step.

As shown in the FTIR results (– cf. Fig. 4), part of the IL is lost with the recovered protein. This may justify some of the IL losses observed here; however, it can also be related to losses during the whole process (e.g. during sample transfer between flasks). We would expect a higher recovery in a larger-scale industrial process, where surface losses are significantly lower. Besides, considering a higher volume of IL, we would expect more cycles to be successfully carried out. On the other hand, the washing step can improve the IL recovery, as reported by Zhang et al.⁴⁹, who used $[\text{C}_2\text{C}_{1\text{im}}]\text{Cl}$ for cellulose regeneration. The authors recovered 71 % of the IL after the first washing cycle, which was improved for ca. 98 % considering four washing cycles. In future work, we suggest an evaluation of several washing steps to improve the IL recovery and a techno-economic of this process applied at a large scale.

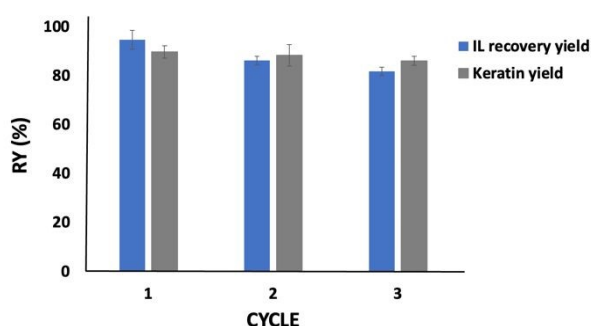


Fig. 5 IL and keratin recovery yield during three consecutive cycles.

Film preparation and characterization

The best conditions (1 h, 5 °C, and ethanol 1:2 w/w) were used for keratin recovery. After the washing step, an aqueous solution of keratin (15 wt %) was prepared and heated at 60 °C for 30 min. Posteriorly, the solution was dried in a silicone molding at 50 °C for 24 h. A homogeneous, opaque, and yellow thin film was obtained (Fig. 6) and stored at 5 °C for further characterization. Mechanical and biological tests were performed to validate their future applications. To the best of our knowledge, no previous results have been reported for keratin films obtained from chicken feathers processed with ILs. Therefore, these results can help to identify possible applications of this new biomaterial.



Fig. 6 Keratin film processed from chicken feathers waste.

Film morphology

SEM was used to characterize the surface morphology of the keratin film. The topography of the keratin film (Fig. S4 in the ESI) is smooth and homogeneous. The same morphology was observed by Nuutinen et al.⁵⁰, who investigated the use of deep eutectic solvents in the processing of keratin films from feathers.

Film tensile strength

The tensile strength of the produced keratin film is 0.1289 ± 0.01 MPa, indicating that the film is fragile. The poor mechanical properties of this biomaterial is expected due to the low molecular weight of keratin⁵⁰. Although not critical given the envisioned biomedical applications, to overcome this situation, cross-linking keratin (e.g., glycerol) have been proposed and providing excellent results⁵⁰. Another option successfully reported in the literature is the addition of a biocompatible biopolymer, creating a blend, allowing to improve the properties of the material³⁵. In this regard, He et al.⁵¹ reported that the film's tensile strength was improved around five times by incorporating sodium alginate into the keratin film.

Contact angle

Contact angle measurements were performed to assess the hydrophilicity of the prepared keratin film. When water was used as a solvent, the drop quickly spread across the film, being not possible to measure the contact angle. This happens because protein films are sensitive to water due to their hydrophilic nature⁵⁰. Considering the higher density of ethylene glycol, it was successfully used to realize this analysis. Keratin film presented a contact angle of $52.77^\circ \pm 1.52^\circ$, characterizing it as hydrophilic ($\leq 90^\circ$). This characteristic can be related to the presence of the polar group at the film surface and possibility of hydrogen-bonding. Analogously, Hamouche et al.⁵² prepared wool keratin film and reported a contact angle of 53.5° , while Nuutinen et al.⁵⁰ prepared feathers keratin films and reported a contact angle of $45.84^\circ \pm 4.57^\circ$. In the literature, hydrophilic films have been positively investigated for biomedical applications⁵³.

In vitro cytotoxicity

Cytotoxicity tests have been successfully used as an initial step for bio-safety testing and identification of the potential toxicity of different biomaterials⁵⁴. Aiming to evaluate the cytotoxicity of



the prepared keratin film, some cells, namely macrophages, monocytes, keratinocytes, and fibroblasts, were used. In Fig. 7, the cytotoxicity results obtained are presented. Compared to the control, no significant impact was observed in the samples, indicating that keratin film has no toxicity for these cells. Although there is no data in the literature about the cytotoxicity of keratin films from chicken feathers processed with ILs, the cytotoxicity of keratin from different sources and methods follows the findings of this work⁵⁵. Overall, our results confirm that $[C_4C_1im][C_1CO_2]$ is efficient and safe to be applied in keratin biomaterials processing.

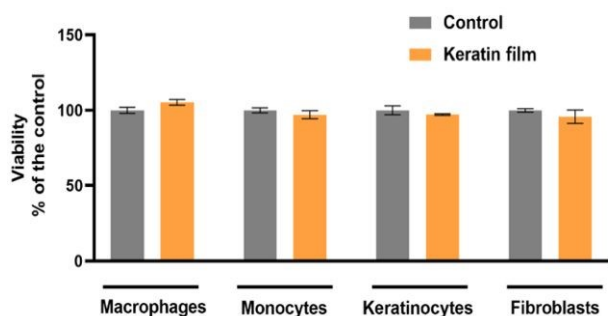


Fig. 7 Evaluation of keratin film's cell viability *in vitro* in macrophages, monocytes, keratinocytes, and fibroblasts cells.

Anti-Inflammatory and antioxidant effects of keratin film

As LPS promotes an increase in nitrites – a stable metabolite of NO – production in macrophages, it has been used for the identification of the anti-inflammatory potential of compounds⁵⁶. The main goal here was to identify if the macrophages' exposure to keratin film could induce NO production (Fig. 8A). Herein, keratin film produced very low levels of nitriles (4.29), showing no significant differences with the control (1.01). This result indicates that keratin film can inhibit nitrile production without affecting cell viability. In what concerns ROS, representative images of fluorescence microscopy (Fig. 8B) show that the LPS induced a significant increase in ROS production. Images were acquired with an EVOS M5000 Imaging System at 20x magnification (scale bar 100 μ m). Interestingly, in the presence of keratin film, similar levels of control were observed, indicating the antioxidant effect of this biomaterial.

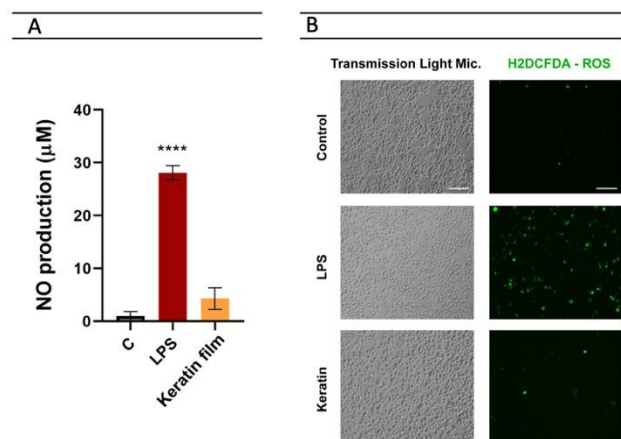


Fig. 8 Investigating the keratin film in the (A) NO production and (B) oxidative stress.

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Expression of keratin film on the surface of dendritic cells (CDs)

As CDs are crucial components that contribute to the immune response⁵⁷, we used CD54, CD83, and CD85 as activation marks and LPS as positive control to investigate if the exposition of monocytes to keratin film could promote any modifications in the immune response. According to the results presented in Fig. 9, the exposure of monocytes to keratin film promotes an increase in the expression of some markers, namely CD83 (an activation marker) and CD54 (a responsible molecule to promote adhesion between cells). Generally, it happens when cells are activated (pro-inflammatory stimulations). Considering this result, combined with the slight increase in NO observed in macrophages (Fig.8A), we can suggest that keratin film may promote a slight activation of the innate immune response.

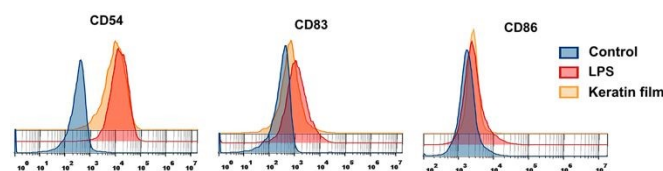


Fig. 9 Investigation of keratin film expression on different dendritic cells (CD54, CD83, and CD86).

In vitro wound healing

Aiming to evaluate the wound healing effect and having in mind that cell migration is essential for wound contraction and the later curing stages, we investigated the ability of the prepared keratin film on keratinocytes (principal cell of the epidermis) and fibroblasts (responsible cell for making the extracellular matrix) to speed up the closure of the wound. The results showed that keratin film improves the proliferation of the cells, accelerating wound healing at 16 h (Fig. 10).

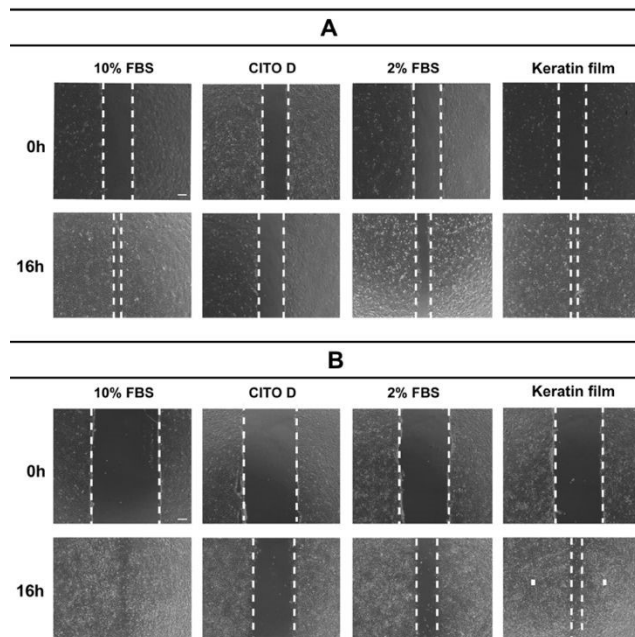


Fig. 10 The wound healing ability of keratin using (A) keratinocytes and (B) fibroblasts: an *in vitro* investigation.

The mechanism of the wound healing ability of keratin is associated with keratin-cell interaction and amino acid sequences of RGD (arginine-glycine-aspartic acid) and LDV (leucine-aspartic acid-valine). These amino acids promote cellular attachment, migration, and proliferation during the healing process; however, the definite molecular mechanism remains unclear^{58,59}. Future investigations are necessary to clarify the molecular mechanism and assess the possible use of keratin for biomedical applications. In addition, wound healing studies have been realized with a focus on human hair and wool keratins, remaining chicken feathers, which are a more abundant source than wool, and easier to control than human hair due to perming and coloring-dyeing,⁶⁰ underexplored.

Conclusions

Herein, the valorization of chicken feathers waste by keratin recovery with ILs was demonstrated. More precisely, an aqueous solution of [C₄C₁im][C₁CO₂] (80 wt %) was successfully used to achieve complete chicken feathers dissolution at a solid:liquid ratio of 1:20 w/w under a short dissolution time (4 h) at 100 °C. Optimum recovery conditions were identified at 1 h, 5 °C, and using ethanol in a ratio of 1:2 w/w, achieving a keratin recovery yield of 90.17 wt %, with good physicochemical properties.

The IL was successfully recycled and reused three times, allowing to recover ~82 wt % of the IL at the end of the third cycle. We expect a higher recovery in an industrial process, where surface losses are significantly lower and more cycles can be successfully realized.

Using the optimal operating conditions, a keratin film (15 wt %) was prepared, and its properties were evaluated. In summary, a hydrophilic and homogeneous film was successfully prepared. As a future work, the optimization of the keratin film processing, particularly to improve its tensile strength, is essential. Its cytotoxicity was investigated in macrophages, monocytes, keratinocytes, and fibroblasts. The keratin film did not present any cytotoxicity for these cells. Satisfactory results of anti-inflammatory activity were obtained. In addition, the *in vitro* wound healing study showed that keratin film improves the proliferation of the keratinocytes and fibroblasts, accelerating wound healing at 16 h.

The availability and low cost of keratin, when obtained from industrial waste, and its biochemical and physical properties are remarkable advantages. As future steps, evaluating the economic analysis and investigating the molecular mechanism of keratin film in promoting wound healing are essential. The biorefinery concept presented herein is sustainable and can be extended to other keratin-based waste.

Author Contributions

Conceptualization, C.P., H.P., J.A.P.C. and M.G.F.; methodology, C.P. and B.N.; writing – original draft preparation, C.P.; writing –

review and editing, H.P., B.N., J.A.P.C. and M.G.F.; supervision, H.P. and M.G.F.; funding acquisition, M.G.F.; project administration, M.G.F. All authors listed have made a substantial, direct, and intellectual contribution to the work and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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References

- 1 S. Isarankura Na Ayutthaya, S. Tanpichai and J. Wootthikanokkhan, *J Polym Environ*, 2015, **23**, 506–516.
- 2 B. Y. Alashwal, A. Gupta and M. S. B. Husain, *IOP Conf Ser Mater Sci Eng*, 2019, **702**, 1-7.
- 3 N. B. Kamarudin, S. Sharma, A. Gupta, C. G. Kee, S. M. S. B. T. Chik and R. Gupta, *3 Biotech*, 2017, **7**, 1–9.
- 4 T. Tesfaye, B. Sithole and D. Ramjugernath, *Sustain Chem Pharm*, 2018, **8**, 38–49.
- 5 T. Upcraft, W. C. Tu, R. Johnson, T. Finnigan, N. van Hung, J. Hallett and M. Guo, *Green Chem*, 2021, **23**, 5150–5165.
- 6 B. Mu, F. Hassan and Y. Yang, *Green Chem*, 2020, **22**, 1726–1734.
- 7 M. Zhan and R. P. Wool, *J Appl Polym Sci*, 2016, **44013**, 1-7.
- 8 A. R. Abouelela, S. Y. Tan, G. H. Kelsall and J. P. Hallett, *ACS Sustain Chem Eng*, 2020, **8**, 14441–14461.
- 9 T. Tesfaye, B. Sithole, D. Ramjugernath and V. Chuniilall, *Waste Manag*, 2017, **68**, 626–635.
- 10 X. Liu, Y. Nie, Y. Liu, S. Zhang and A. L. Skov, *ACS Sustain Chem Eng*, 2018, **6**, 17314–17322.



ARTICLE	Journal Name
11 C. R. Chilakamarry, S. Mahmood, S. N. B. M. Saffe, M. A. bin Arifin, A. Gupta, M. Y. Sikkandar, S. S. Begum and B. Narasaiah, <i>3 Biotech</i> , 2021, 11 , 1–12.	28 A. Aluigi, C. Tonetti, F. Rombaldoni, D. Puglia, F. Fortunati, I. Armentano, C. Santulli, L. Torre and J. M. Kenny, <i>J Mater Sci</i> , 2014, 49 , 6257–6269.
12 F. Pourjavaheri, S. Ostovar Pour, O. A. H. Jones, P. M. Smooker, R. Brkljača, F. Sherkat, E. W. Blanch, A. Gupta and R. A. Shanks, <i>Process Biochem</i> , 2019, 82 , 205–214.	29 C. Apostolidou, <i>ChemistryOpen</i> , 2020, 9 , 695–702.
13 E. M. Nuutinen, P. Willberg-Keyriläinen, T. Virtanen, A. Mija, L. Kuutti, R. Lantto and A. S. Jääskeläinen, <i>RSC Adv</i> , 2019, 9 , 19720–19728.	30 S. P. M. Ventura, F. A. E Silva, M. v. Quental, D. Mondal, M. G. Freire and J. A. P. Coutinho, <i>Chem Rev</i> , 2017, 117 , 6984–7052.
14 R.-R. Yan, J.-S. Gong, C. Su, Y.-L. Liu, J.-Y. Qian, Z.-H. Xu and J.-S. Shi, <i>Appl Microbiol Biotechnol</i> , 2022, 106 , 2349–2366.	31 Z. Zhang, Y. Nie, Q. Zhang, X. Liu, W. Tu, X. Zhang and S. Zhang, <i>ACS Sustain Chem Eng</i> , 2017, 5 , 2614–2622.
15 M. Borrelli, N. Joepen, S. Reichl, D. Finis, M. Schoppe, G. Geerling and S. Schrader, <i>Biomaterials</i> , 2015, 42 , 112–120.	32 S. Zheng, Y. Nie, S. Zhang, X. Zhang and L. Wang, <i>ACS Sustain Chem Eng</i> , 2015, 3 , 2925–2932.
16 N. Ramakrishnan, S. Sharma, A. Gupta and B. Y. Alashwal, <i>Int J Biol Macromol</i> , 2018, 111 , 352–358.	33 Y. X. Wang and X. J. Cao, <i>Process Biochem</i> , 2012, 47 , 896–899.
17 R. Li and D. Wang, <i>J Appl Polym Sci</i> , 2013, 127 , 2648–2653.	34 N. A. Samsudin, F. W. Low, Y. Yusoff, M. Shakeri, X. Y. Tan, C. W. Lai, N. Asim, C. S. Oon, K. S. Newaz, S. K. Tiong and N. Amin, <i>J Mol Liq</i> , 2020, 308 , 113030–113037.
18 B. Ma, Q. Sun, J. Yang, J. Wizi, X. Hou and Y. Yang, <i>Environ Sci Pollut Res</i> , 2017, 24 , 17711–17718.	35 C. Polesca, H. Passos, J. A. P. Coutinho and M. G. Freire, <i>Curr Opin Green Sustain Chem</i> , 2022, 37 , 100675–100683.
19 R. K. Donato and A. Mija, <i>Polymers</i> , 2020, 12 , 1–64.	36 A. Rivera-Galletti, C. R. Gough, F. Kaleem, M. Burch, C. Ratcliffe, P. Lu, D. Salas-De La Cruz and X. Hu, <i>Polymers</i> , 2021, 13 , 2911–2921.
20 A. Shavandi, T. H. Silva, A. A. Bekhit and A. E. D. A. Bekhit, <i>Biomater Sci</i> , 2017, 5 , 1699–1735.	37 Y. Hu, L. Liu, W. Dan, N. Dan and Z. Gu, <i>J Appl Polym Sci</i> , 2013, 130 , 2245–2256.
21 A. Idris, R. Vijayaraghavan, U. A. Rana, D. Fredericks, A. F. Patti and D. R. MacFarlane, <i>Green Chem</i> , 2013, 15 , 525–534.	38 A. Idris, R. Vijayaraghavan, A. F. Patti and D. R. Macfarlane, <i>ACS Sustain Chem Eng</i> , 2014, 2 , 1888–1894.
22 A. Idris, R. Vijayaraghavan, U. A. Rana, A. F. Patti and D. R. MacFarlane, <i>Green Chem</i> , 2014, 16 , 2857–2864.	39 J. O'Brien, I. Wilson, T. Orton and F. Pognan, <i>Eur J Biochem</i> , 2000, 267 , 5421–5426.
23 Y. Ji, J. Chen, J. Lv, Z. Li, L. Xing and S. Ding, <i>Sep Purif Technol</i> , 2014, 132 , 577–583.	40 H. Passos, T. B. V. Dinis, A. F. M. Cláudio, M. G. Freire and J. A. P. Coutinho, <i>Phys Chem Chem Phys</i> , 2018, 20 , 14234–14241.
24 Y. J. Yang, D. Ganbat, P. Aramwit, A. Bucciarelli, J. Chen, C. Migliaresi and A. Motta, <i>Express Polym Lett</i> , 2019, 13 , 97–108.	41 M. A. R. Martins, F. H. B. Sosa, I. Kilpeläinen and J. A. P. Coutinho, <i>Fluid Ph Equilib</i> , 2022, 556 , 113414–113423.
25 H. Xie, S. Li and S. Zhang, <i>Green Chem</i> , 2005, 7 , 606–608.	42 X. Zhang, Y. Feng and X. Yang, <i>Fibers and Polymers</i> , 2021, 22 , 3326–3335.
26 X. Liu, Y. Nie, X. Meng, Z. Zhang, X. Zhang and S. Zhang, <i>RSC Adv</i> , 2017, 7 , 1981–1988.	43 I. Khan, M. L. S. Batista, P. J. Carvalho, L. M. N. B. F. Santos, J. R. B. Gomes and J. A. P. Coutinho, <i>J Phys Chem B</i> , 2015, 119 , 10287–10303.
27 Z. Zhang, Y. Nie, Q. Zhang, X. Liu, W. Tu, X. Zhang and S. Zhang, <i>ACS Sustain Chem Eng</i> , 2017, 5 , 2614–2622.	44 T. K. Maity, N. Singh, P. Vaghela, A. Ghosh, S. Singh, P. B. Shinde, R. A. Sequeira and K. Prasad, <i>Sustain Environ Res</i> , 2022, 32 , 1–8.



- 45 O. D. Fagbemi, B. Sithole and T. Tesfaye, *Sustain Chem Pharm*, 2020, **17**, 100267-100278.
- 46 O. L. Shanmugasundaram, K. Syed Zameer Ahmed, K. Sujatha, P. Ponnmurugan, A. Srivastava, R. Ramesh, R. Sukumar and K. Elanithi, *Mater Sci Eng C*, 2018, **92**, 26–33.
- 47 B. Ma, X. Qiao, X. Hou and Y. Yang, *Int J Biol Macromol*, 2016, **89**, 614–621.
- 48 S. Alahyaribeik and A. Ullah, *Int J Biol Macromol*, 2020, **148**, 449–456.
- 49 H. Zhang, A. Ionita, P. F. Serifiñan, M. L. Ferrer, M. A. Rodríguez, A. Tamayo, F. R. Alons, F. del Monte and M. C. Gutiérrez, *Molecules*, 2022, **27**, 987-999.
- 50 E. M. Nuutinen, T. Virtanen, R. Lantto, M. Vähä-Nissi and A. S. Jääskeläinen, *RSC Adv*, 2021, **11**, 27512–27522.
- 51 M. He, B. Zhang, Y. Dou, G. Yin and Y. Cui, *J Appl Polym Sci*, 2017, **44680**, 1-8.
- 52 H. Hamouche, S. Makhlof, A. Chaouchi and M. Laghrouche, *Sens Actuators A Phys*, 2018, **282**, 132–141.
- 53 D. Ahmad, I. van den Boogaert, J. Miller, R. Presswell and H. Jouhara, *Energy Sources Part A*, 2018, **40**, 2686–2725.
- 54 F. Soheilmooghaddam, G. Sharifzadeh, H. Adelnia and M. U. Wahit, *J Polym Environ*, 2021, **30**, 613–621.
- 55 G. J. Dias, T. N. Haththotuwa, D. S. Rowlands, M. Gram and A. E. D. A. Bekhit, *Food Chem*, 2022, **383**, 132436-132468.
- 56 S. N. Pedro, M. S. M. Mendes, B. M. Neves, I. F. Almeida, P. Costa, I. Correia-Sá, C. Vilela, M. G. Freire, A. J. D. Silvestre and C. S. R. Freire, *Pharmaceutics*, 2022, **14**, 827-844.
- 57 E. S. Reis, J. A. M. Barbuto, J. Köhl and L. Isaac, *Mol Immunol*, 2008, **45**, 1952–1962.
- 58 Y. Chen, Y. Li, X. Yang, Z. Cao, H. Nie, Y. Bian and G. Yang, *Acta Biomater*, 2021, **125**, 208–218.
- 59 R. R. Yan, J. S. Gong, C. Su, Y. L. Liu, J. Y. Qian, Z. H. Xu and J. S. Shi, *Appl Microbiol Biotechnol*, 2022, **106**, 2349–2366.
- 60 J. Wang, S. Hao, T. Luo, Z. Cheng, W. Li, F. Gao, T. Guo, Y. Gong and B. Wang, *Colloids Surf B Biointerfaces*, 2017, **149**, 341–350.

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