MATERIAL KERATINIC CU POTENŢIALE BIO-APLICAŢII
KERATIN BASED MATERIAL FOR PERSPECTIVE BIO-APPLICATION

(Abstract)

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RECTORATUL

Către

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Chapter 1

1 Introduction

1.1 What is Keratin?

Keratins are a group of fibrous proteins produced in some epithelial cells of reptiles, birds, and mammals. These proteins are abundantly present in nature and constitute the major part of mammalian and reptilian nails and horns, claws, mammalian hair and hooves, wool, bird feathers, reptile and fish scales, bird beaks, and the outermost layer of skin in most animals. Over the years, keratin has been extracted from all low-value mentioned sources [1]. Renewable resources are mainly regarded as sources of biopolymers, which are gaining ground in the world of emerging materials due to their properties: availability, biodegradability, versatility, and similarity to synthetic polymers. Keratin waste from breeding, butchery or textile industries have been estimated worldwide to be more than 5,000,000 ton/year [2], [3], but due to its intrinsic insolubility and complicated morphological structure it is difficult to be extracted, purified and processed into useful forms. This is why keratins have not been exploited to the same extent as other proteins such as collagen or silk [4], [5]. The investigation of the structure and properties of keratins were very intense in the middle of the 20th century, and many works conducted in that period are considered to be definitive [6], [7]; many basic studies and practical procedures for keratin solubilization were established in the same period, and further studies brought slight modification of the original method [8].
1.2 Wool keratin

One of the most important sources of keratin is wool from the waste of the textile industries or coarse wool not suitable for spinning. Since the middle of the past century, when the synthetic fibers have begun their rise, wool has lost its importance in the textile sector and became a marginal fiber. High amounts of non-marketed raw wool have turned from an income source into a waste, and are burnt or landfilled as a waste stream.

Wool is a valuable natural fiber and has a long history as raw material for textile purposes. It was amongst the first commodities to be traded internationally and is still essential to the agricultural economies of many countries.

Washed wool contains dominantly pure protein, and it is formed by a histological structure which is made of three main morphological components, namely the cuticle, the cell membrane complex and the cortex. The cuticle of the wool fiber consists of a thin envelope of flat overlapping ‘cuticle cells’ arranged like roof tiles surrounding the cortex, which is made up of spindle-shape ‘cortical cells’ oriented parallel to the fiber axis. The cell membrane complex, sometimes referred to as intercellular cement, performs the function of cementing cortical and cuticle cells together. In Figure 1-1, a schematic of the morphological wool fiber structure is reported.
Keratin extracted from wool shows many interesting and peculiar properties, often depending on the extraction method used, that makes keratin an interesting material in many sectors like biomedical, filtering, cosmetic, agricultural, etc. [10]. As a biomaterial, wool keratin is biocompatible, biodegradable and able to support cell attachment and spreading [11].

There are different viewpoints to classify keratins. They have been classified as “hard keratin” and “soft keratin” according to their physical and chemical properties, particularly to the sulfur amount; type I or acidic keratin and type II or neutral-alkaline keratin on the basis on the predominant amino acids; α-keratin and β-keratin on the basis of their secondary structure. Keratin can also be distinguished according to their biosynthesis and their molecular weight [12]–[14].

1.3 Overview of chemistry and structure

Commonly, keratins are classified according to five criteria: [12], [14]: (i) the morphological entities of animal body which comprise them (intracytoplasmatic or “soft”, and morphologic or “hard” keratins), (ii) the primacy of biosynthesis (“primary” or normally synthesized in basal keratinocytes, and “secondary” or optionally synthesized), (iii) the predominant amino acids in their composition (“type I” or acidic, and “type II” or neutral-alkaline), (iv) the X-ray pattern (“alpha keratin” which includes α-helix-rich domains, “beta keratin” consisting in β-sheet packed macromolecules, and “gamma keratin” which represents the low molecular weight keratin-associated proteins (KAPs), which hold the keratin intermediate filaments (KIFs) together by embedding them in a protein matrix, and (v) the average molecular weight (MW): “low molecular weight keratins” (LMWK) with a molecular mass less than 40 kDa, “intermediate molecular weight keratins”(IMWK), of 40 to 57 kDa, and “high molecular weight keratins” (HMWK), of more than 57 kDa; the range of the MW values for α-, β- and γ-keratins are 40-68 kDa, 10-22 kDa and 10-18 kDa, respectively.
1.4 Solubilization pathways and methods

Due to keratins intrinsic hydrophobicity and presence of multiple interchain disulfide bridges that impart toughness to the keratinized entities and naturally evolved to be robust and resistant to degradation, which make these sources are practically insoluble in water and usual solvents.

Keratin can be extracted by: (i) unprotected solubilization, which unsystematically detaches keratins macromolecules or parts of them, by degrading both the backbone peptide bonds and the interchain disulfide bridges, and (ii) protected solubilization, which leaves keratin macromolecules almost intact, by preferential cleavage of the disulfide bridges, and by extensive disruption of the intermolecular hydrogen bonds. Except for severe hydrolysis treatments (when low molecular weight peptides and amino acids are obtained), both ways confront with the difficulty of maintaining the solubilized polypeptides in a colloidal state, as stable aqueous solutions. Freshly solubilized keratins are prone to precipitation because of their hydrophobicity and disulfide bridges tendency to reassemble from the newly resulted sulfhydryl groups. In an unprotected way, the isolation and soluble form of keratin can be achieved easily by conventional methods of hydrolysis, using acid, high-temperature water, alkali or enzymatic digestion of the peptide bonds to create a mixture of peptide and amino acids. In this way, none of the secondary or tertiary structure of the core protein has retained, and it considers it as harsh processing that destroys many unique characteristics of the protein. Keratin from greasy waste wool hydrolyzed with superheated water has been used for the preparation of organic fertilizers with a nitrogen release in the soil depending on the hydrolysis temperature and time [15], [16].

An outlook of the keratin solubilization pathways and methods, together with the nature of the mainly involved processes and the resulted keratin forms is given in Figure 1-2.
The feasible chemical methods for the extraction of quasi-native keratins are: reduction with thiols in denaturing media, which results in keratin forms known as “kerateines” [17], [18] sulfitolysis with alkali metal sulfites in denaturing media, which results in keratin forms known as “S-sulfokerateines” [19], [20] oxidation with peroxy compounds, which results in sulfonated keratins known as “keratoses” [6], [21]. The solubilization techniques were set up in the middle of the 20th century, but are still in use with minor improvements. Recently, an environmentally friendly method has been developed, which uses ionic liquids (ILs) as solubilizing agents and results in kerateine-like proteins [22] which can be regenerated into films [23], [24] or nanofibers [25].

By oxidative extraction using peracetic or performic acids, which oxidize cystine to cysteic acid, keratoses are obtained [6]. Keratoses are water-soluble and cannot participate in cross-linking reactions with the reformation of the disulfide bridges. Sulfitolysis describes the cleavage of a disulfide by sulfite to give a thiol and a S-sulfonate anion (or Bunte salt). While using the previous protein extraction methods, the molecular weight of extracted proteins remains unchanged; wool protein hydrolysis leads to the breaking of peptide bonds with the formation of low molecular weight peptides. Green processes, solvent-free, have been proposed for the hydrolysis of keratin. These treatments include hydrolysis with superheated water [26], [27] and enzymes [28]. In the case of less...
demanding applications, such as pharmaco-cosmetic or agricultural, nonspecific partial degradation provided by unprotected solubilization is acceptable.

1.5 Novel approaches to keratin solubilization

Environmentally friendly alternatives have been recently proposed for keratinous resources processing, of which hydrothermal and enzymatic hydrolysis have already been presented.

Preferential dissolution of the cortex is mainly assigned to the destruction of the hydrogen bonds, which are predominant in the α-helical domains of microfibrils [29]. The extended time required to dissolve the cuticle is linked to the high content of disulfide bonds, which are less prone to the IL attack. The increase of solubilization temperature promotes peptide bond hydrolysis, with significant impact on the physicochemical characteristics of the regenerated keratin-based materials [30].

1.6 Biomaterial application

In recent years, biopolymers consider it as a privileged material for the sustainable development and environmental sustainability concepts. Biopolymer material has found in the different feasible applications from biomaterial application to the industrial arena [31]. Biomaterials have been improved, and are continuing to be developed, along with the developing of new material to production processing, product quality and standardization in bio-application fields.

This significant amount of keratin sources in the environment is of notable for considering material as the proper potential for advanced technology. Different studies have carried out to investigate keratin sources physicochemical structure both in macroscopic and microscopic characteristics. The manipulation of keratin for considering in the advanced application is not straightforward, and this difficulty has been the main reason that keratin protein has not been commercially exploited as material in a way that other biopolymers such as collagens, cellulose have been. For developing keratin material beyond limited application, a series of process for isolation of keratin proteins that maintain core feature of the protein its structure needs to be improved. In this process, the hydrolyze do not occur on the peptide bonds and allow the keratin protein remain on native structure and form.
Keratin can be used for enhancing the repair of tissue for different organ of the body. Some studies have carried out to use keratin for providing better “healing properties” in bone damage. Bone mainly consists of fibrous protein, a mineral composites such as collagen and calcium, so using alternative protein fibroin and mineral composite for bone augmentation is quite reasonable as short time frame; [32]. Moreover, keratin has been counted as a proper candidate to apply in the soft tissue, such as to fill spaces for tissue enhancement or intervertebrate discs in the spine. In addition, considering keratin in other biological area has been interested. In particular, keratin characteristic has been studied and used as regenerative medicine, cell therapies, and regenerated tissue, in nerve generation.

Keratin forms which preserve the structural organization of the native keratin and are prone to suitable physical states, became attractive candidates for tissue engineering applications for the obtaining of scaffolds for tissue engineering.

Thus, biomaterials with different physical configurations and functions in relation to the living bodies can be fabricated, as given in Table 1-1.
<table>
<thead>
<tr>
<th>Solubilization method</th>
<th>Solubilization product/ physical state and characteristics</th>
<th>Application</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
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<tr>
<td>Keratine-sodium alginate/high porosity sponge</td>
<td>Cell culture</td>
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<tr>
<td>Multilayer films by the layer-by-layer method</td>
<td>Biocompatible surface for tissue engineering</td>
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<td>Keratine, sponge-like porous hydrogel</td>
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<td>Keratine/injectable and cytocompatible gel</td>
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<tr>
<td>S-carboxymethylated) keratine/PCL-keratin nanofibrous mats</td>
<td>Scaffold for vascular tissue engineering</td>
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<td>Keratose/hydratable fibers or powder</td>
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<td>S-sulfokeratine/ microparticles (~6 µm)</td>
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<td>S-sulfokeratine/photo-active film with antibacterial activity</td>
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<td>Biomimetic keratin/hydroxyapatite nanocomposite for osteoblast culture and bone tissue regeneration</td>
<td>[49]</td>
<td></td>
</tr>
</tbody>
</table>
### Ionic liquids

| Keratin-cellulose-chitosan composite films | Drug delivery substrate | [50] |
| α-keratin, powder | Electrospun nanofibers for bandages or hydrogels/ wound healing | [25] |
| Keratin-cellulose blend regenerated by casting | Scaffold for biomimetic hydroxyapatite mineralization | [51] |

### 1.7 Microcapsule and film

Encapsulation consists of surrounding a selected material, which constitutes the core, with some solid shell of a different compound, the nature of which is based on the proposed application. Examples of encapsulation purposes could be to protect materials inside the core against oxidation or reduction, to allow safe handling of toxic materials, to keep core materials from evaporation, or to mask unpleasant odors and flavors. In addition, many studies on microencapsulation have focused on the use of biodegradable and biocompatible synthetic or natural polymers and how their molecular structure could be tailored according to the desired applications. There is also an emerging interest in the encapsulation of hydrophobic molecules. Hydrophobic material with a protein carrier can be entrapped by sonochemical methods with significant dimension [52], [53].

Water-soluble keratin is one of the properties which can be quite helpful for applying in the biomedical application. Different studied presented application for keratones such as biocompatible fillers for implant application [40], matrix for peripheral nerve regeneration [42], hydratable powders for viscoelastic hydrogels that support cell adhesion and proliferation [41], wool-derived oxidized keratin kit for wound repair and burn healing [54], [55].

On the other hand, producing different blend materials from keratin have been reported. Some research related to the keratin film reported poor mechanical properties. Moreover, this is always concern about toxicity in the bio-field and food packing. Poly (vinyl alcohol) is synthetic hydrophilic and biodegradable polymer with a wide range of applications in the food packing, variety of coatings and pharmaceutical industries [56]. Recently PVA has been used in the biomedical field and for application such as drug delivery, wound healing, ultrafiltration and tissue engineering scaffolds. Because of its biocompatibility, PVA/protein films are produced for different application such as food
packing, tissue engineering, and drug delivery system. Various studies have been devoted to cross-link protein blend film or protein blend film/ PVA with various agents [31], [57].

Films produced from keratin have relatively poor mechanical properties and fragile structure causing application and process restriction. Several work has been developed to improve different properties of keratin films by blending keratin with the natural and synthetic polymers [58].
1.8 The Goals

The purpose of this study mostly dedicates in investigation physicochemical properties of keratin and compares them with previous studies. Part of the thesis focus on the different essential available pathways for the solubilization, extraction, and purification with highlighting on their pros and cons as well as some the mechanism of action and physiological feature of keratin. The effects of each method on the physicochemical structure have discussed and also the character of the different keratin-based material significantly reported how obtained keratins are affected by the each purification process. The achieved results were compared with earlier studies with the aim of emphasis possibility available in line with developing a bio-application approach.

The other section of the thesis, chapter 3, is specifically looking into the study on the particular application of keratin biomaterial in the shape of drug carrier and film. The keratoses, one of the high-quality obtained keratin, have been utilized for producing keratin-based microcapsules and film. The keratoses, with the high-cysteine keratin, readily underwent to oxidative polymerization to effort microcapsules and film. The newly developed microcapsules produced and characterized. Taking advantage of the high-cysteine content of keratoses, we had applied the sonication to produce microcapsules. On the other hand, keratoses film without any additives is quite fragile, and the biodegradable synthetic polymers have been widely used in the medical fields due to the biodegradability and high processibility into different shapes. In the continue in chapter 3, the new form of the keratoses blend film with PVA with various ratios was developed to overcome of physical limitation of the keratin-based film.
Chapter 2

2 Purification and Extraction of keratin

2.1 The purpose of the present chapter

This experimental section aims to extract keratin with different main methods: oxidation, reduction, sulfitolysis and superheated water hydrolysis. The produced keratin from critically characterized by various assays, as well as compared each physicochemical properties in details to suggest more suitable applications in the correlation of each extraction.

2.2 Preliminary discussion on extraction pathways

Wool is contained predominantly three-dimensional keratin structure which constituted with different amino acids. Wool structure is insoluble in water, weak acids, and alkali solution and has a structure with resistant to the majority of the chemical and physical substances. Keratin has a high amount of cystine, serine, glycine, and proline which significantly affect on physicochemical properties of wool keratin. The main pathways utilized to purify and extract from the keratinous material are oxidation, reduction, sulfitolysis and hydrolyzation which as it mentioned each method displays unique properties.
2.2.1 Oxidative methods

The basic recipe and working conditions for wool solubilization by organic peracids were established by Alexander et al. (1950) and Alexander and Earland (1952) [6], [59], and suffered slight modifications in current applications. As a definitive oxidation mechanism has not been yet established, the choice of an oxidation system is rather based on a pragmatic approach.

Most studies on oxidative solubilization of keratin are focused on wool and hair, for which several oxidizing agents are used: hydrogen peroxide, organic peracids, and inorganic persalts. At present, the most attractive seem to be the peracetic acid and peroxide inorganics (H₂O₂, perborates, percarbonates) /alkali system, which are efficient and environmentally friendly. Hydrogen peroxide decomposes without leaving unwanted secondary reaction products and acts as a “green chemical” [60].

Keratoses are water soluble, cannot participate in cross-linking reactions with the reformation of the disulfide bridges, and are susceptible to hydrolytic degradation due to the polarization of the backbone caused by the electrophilic properties of the cysteic acid. These characteristics lead to biomaterials that can degrade relatively fast in vivo as compared with kerateines, i.e., in the order of days to weeks [61].

2.2.2 Reductive methods

One of the benchmark reductive pathways for obtaining quasi-native keratins suitable for biomedical applications implies wool solubilization with 2-mercaptoethanol (2-ME), in solution buffered at pH 9.5, and 8 M urea as chaotropic agent; the alkali media induces partial unfolding of the highly packed keratin structure and favors the reduction reaction [18]. Further processing implies removal of reductant and denaturant from the reaction mixture by dialysis when unwanted aggregation between keratin peptides occurs. Cardamone (2010) studied the microstructure of keratin extracted from wool by reduction: from SDS-PAGE electrophoresis, protein homologs of molecular weight ≈ 40–60 kDa were isolated.

Several alternative techniques are presented in specialty literature with the aim of improving solubilization yield and kerateines stability. Thus, a stable aqueous solution of reduced keratins was prepared from wool, with an extraction yield of 48%, in the presence of additional SDS, in
neutral media (pH ≈ 6.7). At present, the Shindai method is considered the reference method for extraction of minimally denatured keratin fractions, both for analytical and preparative purposes.

Reduced keratins can be applied for producing films, sponges, high porosity scaffolds or bulk powders with valuable uses in regenerative medicine and tissue engineering; protein matrices are superior for tissue engineering, compared to matrices made from synthetic material due to their similarity to native extracellular matrices.

2.2.3 Sulfitolysis methods

As compared with reduction, sulfitolysis uses inexpensive, chemically benign and odor-free reagents. The attack is very specific to the cystine and cysteine residues in the protein, and hence the bioavailability of other essential aminoacids is preserved.

The dissolved S-sulfokerateines can be fabricated into different forms, with variable functionality. Biocompatible films from S-sulfokeratin were prepared by compression-molding [31] or by solvent casting [62] starting from wool solubilized by plain sulfitolysis in neutral denaturing media. The resulted S-sulfokeratin films supported the mammalian cell adhesion and proliferation, thus proving their biocompatibility. Blending keratin with another biopolymer can modulate the biological degradation rate [20].

2.2.4 Chemical-free hydrolysis

Lately, chemical-free hydrolysis is gaining ground, as a “green” or environmentally friendly alternative to conventional methods [16]. Moist heat and pressure in neutral media cleave the disulfide bridges but it also attacks the peptide backbone, so mixtures of oligopeptides and aminoacids will always result. Control of process parameters is difficult and determines a delicate balance between sufficient hydrolysis and over-processing. Two methods are in use for the chemical-free hydrolysis of keratinaceous materials such as a feather, wool or hair: steam hydrolysis/steam explosion, and superheated water.

Steam explosion is a process in which biomass is treated with hot steam (180 - 240 °C) under pressure (1 - 3.5 MPa) followed by an explosive decompression that results in an advanced rupture of the biomass structure. Wool subjected to steam explosion led to a mixture of water-
soluble peptides and free amino acids and a solid residue with advanced disruption of the histologic structure, associated with breaking of disulfide bonds and decomposition of the high-sulfur-content protein fraction [63]. Superheated water has been shown to be effective in the hydrolytic dissolution of keratinous materials.
Materials and methods

Wool fiber, 22 μm mean diameter, in the form of top (fiber sliver obtained from raw wool by scouring, carding and combing processes) was supplied from The Woolmark Co., Italy. All chemicals were of analytical grade and purchased from Sigma-Aldrich, except otherwise specified.

The fiber sample was cleaned by Soxhlet extraction with petroleum ether to remove fatty matter and surfactants, washed with Milli-Q water, conditioned at 20 °C, 65% R.H. for 24 h and cut into snippets around 2 mm length before further treatments.

Keratin extraction processes were carried out by oxidation [64], reduction [65], sulfitolysis [58] and superheated water hydrolysis [27].

2.3 Extraction pathways

In this section different extraction procedures of extracting of keratin from wool fiber has been reported.

2.3.1 Oxidative extraction

For the oxidative extraction 4 g of wool were put an aqueous solution of 2% peracetic acid for 24 h at room temperature (fiber to liquid ratio 1:50), then the oxidized sample was washed with Milli-Q water, dried, treated in a Linitest apparatus with an aqueous solution of tris(hydroxymethyl)aminomethane (Tris) 1M for 2 h at 37 °C and filtrated through a 120 mesh sieve. The filtrate was adjusted to pH 7, dialyzed in the cellulose tube (molecular weight cut off 12,000–14,000 kDa) for 2 days against the circulating system of distilled water, then centrifuged (12,000 rpm, 15 min) to remove the precipitate.
2.3.2 Reductive extraction

For the reductive extraction 4 g of wool were shaken in an aqueous solution (fiber to liquid ratio 1:25) of urea (8M), Tris (0.5M), dithiothreitol (DTT, 0.14M), and ethylenediaminetetraacetic acid (EDTA, 6mM) adjusted to the 8.6 pH with HCl in a Lintest apparatus for 2 h 30 min at 25 °C and then filtrated through a 120 mesh sieve. The filtrate was dialyzed in cellulose tube (molecular weight cut off 12,000–14,000 kDa) for 2 days against the circulating system of distilled water and filtered (5 μm pore size ) to remove insoluble parts.

2.3.3 Sulfitolysis extraction

4 g of wool fibers were treated with 100 mL of aqueous solution containing urea (8M), sodium metabisulfite (0.5M), adjusted to pH 7 with NaOH (5M) under shaking using a Lintest apparatus for 2 h and 30 min at 65 °C. The mixture was filtered through a 5 μm pore-size filter and dialyzed with the cellulose dialysis tube (molecular weight cut off 12,000–14,000 kDa) against distilled water in a circulating system for 2 days.

2.3.4 Superheated water hydrolysis

Keratin hydrolysate was prepared by treating 40 g of wool in 150 ml superheated water, for 30 min at 170 °C, using a 400 ml ceramic autoclave placed in a microwave oven Milestone Ethos 1600 (Milestone S.p.A., Bergamo, Italy). At the end of the process, the autoclave was cooled, and the resulting product was filtered with a 120 mesh stainless steel sieve. The liquid phase was further filtered by a 0.65 μm pore size, tangential flow filter (Millipore Pellicon XL).

All keratin obtained with different extraction methods were freeze-dried for successive characterizations.

2.4 Characterization

In this part characterization of extracted keratin from the different methods are reported.
2.4.1 Extraction yield

The extraction yield of the protein from the wool sample was evaluated using the following equation:

$$Y(\%) = \frac{W_s}{W_{CW}} \times 100$$

Equation 2.1 - Evaluation of extraction yield

where Y is the yield, $W_s$ is the dry weight of extracted samples obtained after freeze-drying, and $W_{CW}$ is the initial dry weight of the wool sample extracted.

2.4.2 Molecular weights determination:

The molecular weights of samples were determined by SDS-PAGE analysis. Keratin freeze-dried samples were dissolved in a solution of dithiothreitol/urea in a buffer (pH 8.6), under a nitrogen atmosphere for 4 h. The SDS-PAGE was performed according to the Laemmli’s method [29] using XcellLock Mini-Cell (Invitrogen), on 12% polyacrylamide gels.

2.4.3 Amino acids analysis:

All the produced keratin-based material were hydrolyzed in HCl 6N for 24 h at 110 °C under nitrogen atmosphere. Free amino acid residues were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidy carbamate (AQC, Waters) and eluted on a reversed-phase column. An Alliance High Performance Liquid Chromatograph (HPLC) (Waters) was used; the eluate was detected at 254 nm. The quantitative amino acid composition, expressed as mole % for each amino acid, was determined by external standard calibration using Amino Acid Standard H (Pierce), cysteic acid and lanthionine.

2.4.4 Fourier Transform Infrared Analysis (FT-IR):

Spectroscopic evaluation of extracted samples was carried out by FT-IR, Nexus Thermo Nicolet Spectrometer 510P, in Attenuated Total Reflectance (ATR) mode. One hundred scans were taken from 4000 to 650 cm$^{-1}$ with resolutions of 4 cm$^{-1}$ and gain 8.
2.4.5 Near Infrared (NIR) analysis:

NIR spectra were acquired in the spectral region between 10,000 and 3700 cm\(^{-1}\), with an FT-NIR spectrometer (Perkin-Elmer) System, model Spectrum IdentiCheck (Monza, Italy), equipped with an IdentiCheck Reflectance Accessory (ICRA). For spectra acquisition, samples were placed on the 12 mm round window of the instrument; a mirror was used to reflect the beam back to the integrating sphere.

2.4.6 Differential Scanning Calorimetry (DSC):

DSC analysis was performed with a Mettler Toledo DSC 821 calorimeter calibrated by an indium standard. The calorimeter cells were flushed with 100 ml/min nitrogen. The runs were conducted on the conditioned samples (20 °C, 65% R.H.) from 30 to 400 °C, at the heating rate of 5 °C/min.

2.4.7 Thermogravimetric analysis (TGA):

TGA was performed on a Mettler Toledo TGA 850 analyzer. The temperature range was from 30 to 450 °C with a heating rate of 5 °C min\(^{-1}\) in a nitrogen atmosphere. About 3 mg of samples were used in each test using Al\(_2\)O\(_3\) crucibles. The data were collected on a computer with the Mettler Toledo STARe System.
2.5 Results and discussion

In this part result and compared discussion are discussed.

2.5.1 Extraction yield

The extraction yields (% w/w) were reported in Table 2-1.

<table>
<thead>
<tr>
<th>Extracted keratin</th>
<th>Extraction Methods</th>
<th>Extraction yield (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratones</td>
<td>Oxidation</td>
<td>31±2</td>
</tr>
<tr>
<td>Kerateine</td>
<td>Reduction</td>
<td>29±3</td>
</tr>
<tr>
<td>Sulfo-kerateine</td>
<td>Sulfitolysis</td>
<td>32±2</td>
</tr>
<tr>
<td>Hydrolyzed keratin</td>
<td>Superheated water hydrolysis</td>
<td>31±3</td>
</tr>
</tbody>
</table>

In general, there are no significant differences in the extraction yield between different extraction methods.

Similar yields (around 30% w/w) were obtained for sulfitolysis, [58] while extraction yield using superheated water hydrolysis is related to temperature, reaction time and material to liquor ratio [27].

2.5.2 Molecular weight (SDS-Page)

Figure 2-1 shows the electrophoresis pattern of all keratin extracted samples compared with original wool as a reference. The wool sample (lane 2) shows two important protein fractions at 67 and 43 kDa which corresponds to low-sulfur (LS) proteins of intermediate filaments present in the wool cortex and the protein fraction of high-sulfur (HS) proteins in the cuticle and different bands at molecular weight lower than 10 kDa, corresponding to the high glycine, tyrosine protein of the matrix between cuticular and cortical cells [63].
In the keratoses sample (Lane 3), the most notable bands visible are at about 60 and 54 kDa, and they are originating from the LS protein of the cortex as already mentioned by other authors [21]. In fact, due to the specifically attach of organic peracidic acid to the −S−S− bond, the main chains of peptide bonds remain with low damage and the HS protein in keratoses sample is paled.

The electrophoresis patterns of kerateine and Sulfo-kerateine (Lane 4 and 5 respectively) show not significant degradation; in fact, the two extracted keratins via reduction and sulfitolysis ways showed the same molecular weight distribution of the original wool [58].

When hydrogen peroxide in alkali media is used as oxidation agent, 90% of the keratin solubilizate contain protein fragments with MW between 3.5 and 12 kDa, which proves the low specificity and higher severity of the H$_2$O$_2$ attack [66].

The electrophoresis patterns of superheated water hydrolyzed sample show that the strong bands of LS proteins disappeared and bands of high protein density appeared in the low fraction range at around 14–3 kDa. The loss of high molecular weight keratins indicates that the chemical structure of wool was significantly affected by the high temperature of the hydrolysis treatments with the cleavage of peptide bonds [63].
2.5.3 Amino acid Analysis

The amino acid amount of all extracted samples compared with original wool is presented in Table II. Wool is essentially pure protein (keratin) and hydrolysis yields the amino acids commonly present in the hydrolysates of most proteins. However, the relative amounts of these amino acids may vary from one sample to another, in relation to sheep breed, diet, health, etc.

During HPLC preparative hydrolysis of keratins with HCl, several amino acid residues undergo various degrees of degradation and conversion. Tryptophan residues are completely destroyed by the acidic conditions, methionine, cystine, cysteine and tyrosine undergo partial degradation, while asparagine and glutamine residues are completely converted to aspartic and glutamic acid, respectively; cystine and cysteine are detected as 1/2 cystine.

During different extraction processes, cystine disulfide bonds are cleaved, and new residues are formed according to the extraction process.
Table 2-2: Amino acid analysis: keratoses, kerateine, sulfo-kerateine and hydrolyzed keratin compared to amino acid analysis of original wool.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Wool fiber</th>
<th>Keratoses</th>
<th>Kerateine</th>
<th>Sulfo-kerateine</th>
<th>Hydrolyzed keratin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteic acid</td>
<td>0.2</td>
<td>7.8</td>
<td>0.3</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.3</td>
<td>10.5</td>
<td>9.6</td>
<td>8.4</td>
<td>8.8</td>
</tr>
<tr>
<td>Serine</td>
<td>11.7</td>
<td>11.6</td>
<td>11.0</td>
<td>11.9</td>
<td>10.7</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>15.6</td>
<td>17.7</td>
<td>15.6</td>
<td>14.6</td>
<td>19.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.3</td>
<td>6.7</td>
<td>8.1</td>
<td>8.1</td>
<td>9.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.9</td>
<td>6.0</td>
<td>6.4</td>
<td>6.1</td>
<td>5.9</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.8</td>
<td>7.0</td>
<td>6.6</td>
<td>7.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.7</td>
<td>5.8</td>
<td>5.6</td>
<td>5.2</td>
<td>8.3</td>
</tr>
<tr>
<td>Proline</td>
<td>3.1</td>
<td>3.2</td>
<td>3.2</td>
<td>4.2</td>
<td>3.7</td>
</tr>
<tr>
<td>Lantionine</td>
<td>0.4</td>
<td>0.6</td>
<td>0.6</td>
<td>0.7</td>
<td>2.3</td>
</tr>
<tr>
<td>1/2Cystine</td>
<td>9.5</td>
<td>0.0</td>
<td>8.1</td>
<td>10.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.5</td>
<td>2.2</td>
<td>3.0</td>
<td>2.7</td>
<td>2.4</td>
</tr>
<tr>
<td>Valine</td>
<td>5.5</td>
<td>5.3</td>
<td>5.3</td>
<td>5.1</td>
<td>5.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.4</td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.0</td>
<td>3.5</td>
<td>3.5</td>
<td>2.8</td>
<td>3.4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.9</td>
<td>2.7</td>
<td>2.8</td>
<td>2.6</td>
<td>2.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.1</td>
<td>7.3</td>
<td>7.6</td>
<td>6.3</td>
<td>7.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.8</td>
<td>1.6</td>
<td>2.0</td>
<td>1.7</td>
<td>1.6</td>
</tr>
</tbody>
</table>

One of the main changes in amino acid composition among the samples is related to the conversion of cystine (and cysteine) to cysteic acid from original wool to keratoses. Cysteic acid is found in very small amount in original wool where it is formed by the oxidizing effect of solar light on the fleece. Due to the oxidation process for the cleavage of disulfur bonds, the ½ cystine residues are completely converted to the cysteic acid, and there is no present ½ cystine in keratoses. In keratoses, the amino acid composition can also be highlighted a reduction of those amino acids which present easily oxidizable sites (such as tyrosine from 2.5 to 2.2 mole%, lysine from 4.0 to 3.5 mole %, and methionine from 0.4 to 0.1 mole %).
Moreover glutamic acid, aspartic acid, leucine, arginine, which are the amino acids contributing to the α-helix assembling of the LS proteins, were more abundant in keratoses than in original wool, as confirmed by molecular weight distribution where especially low sulfur proteins of intermediate filament are present (see Figure 2-1). Only the lysine amount is not in agreement with this behavior. In keratoses, cysteic acid is present in a lower amount that ½ cystine in original wool probably due to the lower amount of cystine in low sulfur proteins extracted by oxidation from cortical cells [21]. Moreover, glycine and tyrosine, mainly found in low molecular weight proteins of the matrix between cuticular and cortical cells, are present in keratoses in a lower amount than in original wool.

Applications of this reaction for the regeneration in useful biomaterials must be related to an effective way to promote re-crosslinking and restore the disulfide bridge.

From a practical point of view, the control of the cleavage degree and the final reaction products may be conveniently achieved by oxidative sulfitolysis, which basically proceeds according to Error! Reference source not found., but in the presence of appropriated oxidants and oxidant: sulfite ratios that provide the reiteration of the reduction-oxidation cycle, until almost all sulfhydryl groups are converted to S-sulfocysteine. The process is usually conducted in the presence of thionates or alkaline cupric ions, as oxidizing agents.

The process rate is prolonged, and the copper ion, which is tightly bound to the protein substrate [67] must be entirely removed prior to the use of keratin product for any subsequent application. Kelly et al. (2006) [68] patented a two-stage process for the obtaining of high molecular weight soluble S-sulfonated keratin forms, by oxidative sulfitolysis with sodium sulfite and cuprammonium hydroxide, without the use of chaotropic agents. Two intact protein fractions were isolated: the S-sulfonated keratin intermediate filament protein, having a MW distribution predominantly in the range 30-60 kDa, and the S-sulfonated keratin high sulfur protein having a MW predominantly in the range of 15-25 kDa. The process is economically and environmentally acceptable but is intricate and time-consuming.

Oxidative sulfitolysis introduces a S-sulfo functional group with solubilizing properties comparable to those of the sulfonic group, without the destruction of certain amino acid side chains that accompanies oxidation by peracids. The hydrophilic and ionizable S-sulfo groups preserve functionality and amenability to re-crosslinking, and the hydrophobic/hydrophilic
character of the S-sulfokerateine can be easily controlled by the disulfide/S-sulfo group ratio. The SDS-PAGE electrophoretic pattern of S-sulfokerateine powder shows two higher MW bands at 45-60 kDa related to the low sulfur keratin from microfibrils, and a low MW bands between 20-9 kDa, related to the high sulfur keratin from the matrix. FTIR spectra of sulfitolyzed keratin from wool showed the prevalence of α-helix secondary structures and random coil disordered portions, which explain the solubility of the regenerated keratin, while the reduced keratin showed the prevalence of β-sheet structures [112].

Other differences in amino acid composition are found between original wool and hydrolyzed sample. In the hydrolyzed sample, the amount of ½ cystine was very low compared to the original wool. Most of the cystine was destroyed because of the temperature of the process [27] and only a very small amount of cysteic acid, characteristic of undamaged wool, was found. The loss of cystine associates with the thermal treatment and the cleavage of -S-S- bonds bring to the formation of lanthionine (2.3 mole%) through the formation of dehydroalanine and free sulfidryl groups. Dehydroalanine and the free sulfidryl group of cysteine can further react to form the irreversible cross-link -S- of lanthionine. H₂S is also produced from the degradation of cystine. In addition, small changes detected in the amount of the other amino acids in the hydrolyzed sample can be correlated with their thermal stability and secondary with their solubility in water. Moreover, breaking of disulfide bonds as a result of cystine destruction combined with the breaking of peptide bonds shown by electrophoresis (see Figure SDS Page) caused a considerable amount of peptides and free amino acids to be dissolved in water.

Keratin hydrolysis determines the disaggregation of the protein structures at all hierarchical levels, which implies splitting of both amide (–CO–NH–) and disulfide (–S–S–) bonds. Molecular weights of the hydrolysis products cover a wide range, depending on the specific hydrolytic conditions; in severe conditions, the peptide backbone can be entirely broken down into the constitutive amino acids.

2.5.4 FT-IR Spectroscopy

Figure 2-2 shows the infrared spectra of all extracted samples compared with the original wool spectrum.
The infrared absorption spectra of all samples show characteristic absorption bands attributed to the peptide bonds (−CONH−). The vibration in the peptide bonds originate bands known as amide A, amide I, amide II, and amide III. The amide A band at 3282 cm\(^{-1}\) is connected with the stretching vibration of the N–H bond. Amide I, which is related mainly to the (C=O) stretching vibration, has an intense peak at 1630 cm\(^{-1}\). Amide II, which is related to the N–H band, has a peak at 1515 cm\(^{-1}\), while the amide III band at 1230 cm\(^{-1}\) is referred to the in-phase combination of C–N stretching and N–H in-plane bending, with some attributed of C–C stretching and C=O bending vibrations [69].

Peaks that appear between 1200 and 1000 cm\(^{-1}\) are attributed to the S–O vibration (see Figure 2-2). In particular, these peaks are present in the spectra of keratoses and sulfo-kerateine in comparison with original wool. The absorption bands at 1174 cm\(^{-1}\) and 1040 cm\(^{-1}\) in the keratoses spectra are related to the asymmetrical and symmetrical S–O vibrations of sulfonate (SO\(_3^−\)) in cysteic acid [70]. Because of the complete oxidation of cystine to cysteic acid during the oxidation process to obtain keratoses, these two vibration bands appeared in the keratoses sample; while the absorptions bands of cystine monoxide (−S-SO−) and cystine dioxide (−S–SO\(_2^−\)) at 1078 cm\(^{-1}\) and 1120 cm\(^{-1}\) respectively, which are intermediate products in oxidation of cystine to cysteic acid, are nearly not present in keratoses.

In the sulfo-kerateine spectrum, two absorption bands are clearly visible at 1022 cm\(^{-1}\) and 1095 cm\(^{-1}\) assigned to the sulfur-oxygen vibration of sulfonate in the cysteine sulfonic acid (−S-SO\(_3^−\) Bunte salts).
In previous studies, FTIR spectroscopy applied to examine secondary structure of keratin extracted from wool by severe alkaline hydrolysis (2% NaOH, for 3h, at 62 - 65°C) confirmed the macromolecule reorganization with alteration of the α-helix / β-sheet / disordered regions ratio (25.7% / 51.8% / 22.5%).

In the spectra of kerateine and hydrolyzed keratin, no evident differences were found in comparison with original wool spectrum.

2.5.5 NIR Spectroscopy

Figure 2-3 shows the FT-NIR spectra of extracted samples compared with the original wool spectrum.

The absorption bands in the near infrared region (between 10,000 and 3700 cm⁻¹) are the result of overtones and combinations originating in the fundamental mid-range infrared region of the spectrum. The bonds involved are generally C–H, N–H, O–H, etc., Before NIR analysis, all the samples were previously stored for 24 h in a standard atmosphere (20 °C, 65% RH) in order to normalize the water amount.

The shoulder at 7000 cm⁻¹ is related to the first overtone of O–H stretching vibration of water and the band at 5200 cm⁻¹ is assigned to a combination of the O–H stretch and H–O–H bending vibration of the hydroxyl group from the water. The doublet at 5800 cm⁻¹ is an overtone of the C–H stretch of protein side chains and lipids. The bands between 5000 cm⁻¹ and 4000 cm⁻¹ are attributed to the characteristic molecular conformation and amino acid composition of the...
keratin samples. In details, the bands between 5000 and 4500 cm\(^{-1}\) are related to the secondary structure of the proteins, and the bands from 4500 to 4000 cm\(^{-1}\) are mainly attributed to the nature of the protein side chains [3].

Figure 2-3-Near infrared spectra of (a) wool, (b) keratoses, (c) kerateine, (d) sulfo-kerateine, (e) hydrolyzed keratin.

By comparing spectral absorption of all samples, as can be seen, there are small differences in the achieved spectra, which are related to the hydration state of the samples (shoulder at 7000 cm\(^{-1}\) and bond at 5200 cm\(^{-1}\)) [71].

Moreover, the second derivate spectra of wool and extracted keratin were compared (see Figure 5). The band at 4525 cm\(^{-1}\), which seems to be associated with the β-sheet structure, is similar and very low in all samples. Significant differences are visible in the region between 4500 to 4000 cm\(^{-1}\) that is considered to reflect the amino acid composition of samples [72].
2.5.6 Thermal behavior

In Figure 2-5, the DSC thermograms of wool extracted or hydrolyzed in different ways are shown and compared with original wool. In each thermogram, the first endotherm peak below 100 °C due to water evaporation is followed by peaks related to protein denaturation.
In original wool, the α-helix denaturation of cortical cells takes place at about 230 °C with a peak that includes the denaturation of α-helical crystalline material in the domain of both ortho and para cortical cells [73].

The analytical traces of extracted keratin are very different, both for the temperature and for the form of denaturation peaks. The thermogram of hydrolyzed wool does not show typical denaturation or degradation temperatures, such as keratoses or sulfo-keratine traces, but broad peaks in a wide range at lower temperatures indicating that the thermal stability of hydrolyzed keratin is reduced with respect to keratoses or sulfo-keratine. Similar results were obtained by Fortunati et al. comparing enzyme hydrolyzed keratin with sulfo-keratine extracted from merino wool and brown alpaca fibers [45]. Also, keratine, characterized by the re-oxidation of disulfur bonds from thiols, shows that peaks not well defined peaks in a broad temperature range.

The hydrogen and ionic bonds in sulfo-keratine and keratoses proteins make them degrade at the precise temperatures with well-defined melting peaks, at about 240 °C for sulfo-keratine and more than 300 °C for keratoses.

The higher thermal stability of keratoses and the lower thermal stability of hydrolyzed wool are confirmed by thermogravimetric analysis (see Figure 2-6).

*Figure 2-5-Differential scanning calorimetry curves of (a) wool, (b) keratoses, (c) keratine, (d) sulfo-keratine, (e) hydrolyzed keratin.*
Figure 2-6-Thermogravimetric analysis of extracted keratins.
2.6 Conclusions

Keratin from wool fibers was extracted by oxidation, reduction, sulfitolysis, and superheated water hydrolysis, and the different extracts obtained were compared. Although extraction yields are quite similar, evident differences can be noticed in the time, cost, and environmental sustainability of the extraction methods. Among the selected extraction agents, sodium metabisulfite, used for sulfitolysis, seems to be the cheapest and least harmful agent in comparison with peracetic acid and dithiothreitol, utilized for oxidative and reductive extraction, respectively, being similar to the molecular weights of the products obtained. Superheated water hydrolysis highly decreases the molecular weight of the peptides obtained, with loss of the most temperature sensitive amino acids, but it is a cheap and environmentally friendly method, easy to implement on a large scale.

Although extracted keratins via oxidation, reduction, and sulfitolysis can be used in highly technological fields, such as the biomedical sector for scaffold production or the filtration sector, where the mechanical characteristics of the proposed materials are also to be tailored, the hydrolysis of keratin represents one of the most promising ways to extend the practical application of keratin in the agricultural, animal feed, or cosmetic fields. Other characteristics, such as solubility, the presence of polar groups (e.g., the cysteic acid in keratoses), reformation of the disulfur bond capacity, can be considered to use keratin itself extracted in different ways or extracted keratin in a blend with other polymers, for tailoring specific material characteristics.
Chapter 3

3 Film and microcapsule

3.1 The aim of this part

The purpose of this chapter is the production of keratin-based microcapsules and film, following by physicochemical characteristic of produced microcapsule and film with perspective bio-application.

3.2 Preliminary discussion

Numerous methods of microcapsule preparation have been investigated, which include solvent extraction, spray-drying, interfacial polymerization with emulsions and microemulsions, coacervation/precipitation, organic phase separation, emulsion cross-linking, ionic gelation, etc. [41], [74]–[79]. Among these methods, the sonication technique is considered as a proper approach to producing biocompatible protein microcapsules for those materials having the potential for employing this approach for fabricating microcapsules. The advantage of this method is that desired targeting material can be readily loaded into protein [80].

The shell part of the microcapsules is an essential compound for final products properties. For each application, the material utilized as the shell part functioning differently. Keratin, one of
the type of proteins utilized, is a proper material for employing in bio-fields. Keratin can be extracted via different methods, and the final products of each keratin extraction process will have different characteristics. In the current study, water-soluble keratins obtained via oxidizing extraction of wool, known as keratoses, was successfully used for the encapsulation of hydrophilic molecules, using an easy detectable blue dye. The encapsulation process was carried out via sonication [21], [41], [81]–[85].

The previous study about keratin microcapsules indicates that keratin capsules were stable in boiling water and different organic solvent and less leaky than myoglobin and BSA capsules. Although, subjected fragmentation might lose their shape under heating in a dilute aqueous solution of mercaptanol [86].

On the other part of this section blend films were prepared at different PVA/KOx ratios and investigated with Scanning Electron Microscopy, Fourier Transform Infrared Spectroscopy, Differential Scanning Calorimetry and tensile measurement for collecting knowledge for further processing and applications.
3.3 Materials and methods for producing microcapsules

Keratoses were obtained via oxidizing extraction from pristine wool, 21 µm mean fiber diameter, in the form of top (the fiber sliver submitted to industrial scouring, carding, and combing) supplied by The Woolmark Co., Italy. The hydrophilic dye used to fill the microcapsules was Telon Blue BRL (N-aminophenylacetamide) from Dystar. Peracetic acid (36-40% wt. in acetic acid) and Tris(hydroxymethyl)aminomethane (≥ 99.9 %) used for the oxidizing extraction, and toluene (anhydrous 99.8%), utilized for the microencapsulation process, were purchased from Sigma-Aldrich®.

3.3.1 Keratoses oxidizing extractions

Keratin was extracted via oxidizing extraction from pristine wool described in the previous chapter. Briefly, Extraction was carried out with peracetic acid (36-40% wt. in acetic acid) and Tris (hydroxymethyl)aminomethane (≥ 99.9 %) following the method used by L. Burnett and S.A. Boyd [87]. Wool fibers, previously cleaned by Soxhlet extraction with petroleum ether, were immersed in peracetic acid at 2% w/v and left in a thermostatic water bath for 24 hours at 25 ºC. Further, the fibers were washed to remove unreacted peracetic acid, dried at 90 ºC and vigorously shaken in TRIS 1 M (121.14 g/l) at 37 ºC for 2 h. The resulting solution of keratoses was filtered with a 120 mesh sieve, neutralized with hydrochloric acid and dialyzed for 48 h against fresh water, fed by a peristaltic pump to accelerate the osmosis process. At the end of the dialysis, the solution was centrifuged to remove insoluble keratin particles, frozen and lyophilized.

The hydrophilic dye used to fill the microcapsules was Telon Blue BRL (N-aminophenylacetamide) from Dystar.

Toluene (anhydrous 99.8%) from Sigma-Aldrich was used as the non-polar solvent to promote formation and emulsification of the keratoses microcapsules.
3.3.2 Preparation of microcapsules

The procedure of encapsulation was performed following the method used by K. Yamauchi, A. Khoda [86]. An aqueous solution of keratoses 1.8% wt. concentration was added with 0.09 mg/ml hydrophilic dye. After complete dissolution, toluene was added in the proportion 1:0.5 v/v. Toluene (anhydrous 99.8%) was used as the non-polar solvent to promote formation and emulsification of the keratoses microcapsules. The solution (25 ml) was sonicated for 3 min with a Sonics Vibracell 750 (Cole Parmer) sonicator equipped with a stainless steel ½ inch “solid” probe. Power was adjusted to 150 W at the frequency 20 kHz at room temperature, leaving the solution under constant stirring with a magnetic plate. The resulting foamy emulsion was centrifuged for 15 min at 12000 rpm, in order to separate the foamy phase from the solvents that didn’t take part in the formation of microcapsules. Centrifugation originated a triple stratification with toluene on the top, the foam containing the microcapsules in the middle and the aqueous solution containing the residual keratoses and the dye not embedded in the microcapsules on the bottom.

3.3.3 Measurement and characterization methods

The microscopic investigation was carried on microcapsules out with a DM-L Light Microscope (Leica) in the Transmitted Light mode. The specimens were prepared to mount some drops of emulsion on glass microscope slides; in some cases, the microcapsule emulsion was diluted with water for a better investigation.

Scanning Electron Microscopic (SEM) analysis was carried out on microcapsules with a LEO 435 VP SEM (Leica Electron Optics) at 15 kV, 30 mm working distance. Aluminum specimen stubs were used to mount the samples using a double-sided adhesive tape. Samples were sputter-coated with 20 nm thick gold layer in rarefied argon, with a K 550 Sputter Coater (Emitech) at 20 mA for 180 s.

Morphological and surface investigations were carried out on microcapsules with a Nano-R2 Atomic Force Microscope (Pacific Nanotechnology) and were evaluated from 4 µm images. The AFM imaging technique was the “Close Contact” mode with highly doped single crystal silicon probes (APPNANO) of 125 µm nominal length. Data were acquired by means of the
SPM Cockpit Software, processed and analyzed by the Nanorule+ software, both equipped with the Nano-R2 system.

The microcapsules production yield was determined by difference with the amount of keratoses still remaining in the water solution (not belonging to the microcapsule) by an UV-vis spectrophotometer Lambda 40 (Perkin Elmer).

Thermal properties of keratoses and microcapsules were investigated by a Differential Scanning Calorimeter DSC 821 (Mettler Toledo), calibrated by an indium standard. A small amount of dried microcapsules were introduced inside an Al crucible and the analyses were performed at 10 °C/min heating rate, in the range 25-500 °C, flushing the calorimeter cell with 100 ml/min nitrogen.
3.4 Result and discussion

When the biphasic solution of keratoses and toluene is vigorously agitated by sonication, the long and hydrophilic chains of keratoses came into contact with the toluene repulsing it and rolling up, resulting in spheres. During the process, the dye is entrapped in.

In order to detect the presence of hydrophilic dye inside the keratoses microcapsules, the foamy emulsion isolated from the solvents was analyzed by Transmitted Light Microscopy.

Figure 3-1 shows the images of the keratoses microcapsules at 200 (a) and 400 (b) magnification, after dilution in water. Microcapsules presented a varied range of size distribution, with diameters of few micrometers, influenced by many parameters including sonication power, time, and agitation speed [88]–[91].

![Image of keratoses microcapsules](image1.png)

(a)  
(b)

*Figure 3-1: Transmitted Light Microscope images of keratoses microcapsules 200x (a) and 400x (b) after dilution with water.*

At this observation, microcapsules have no sign of collapses or rupture due to no preparation of inspection process. Agitation during the development processes had a significant effect on the average microsphere diameter.

A smaller size of microsphere diameter, on average, was detected while the agitation power was increased—which can be considered as the most essential factor on microcapsules sizes.
SEM observation evidenced the ultrastructure, morphology and size distribution of the keratoses microcapsules.

Drops of the microcapsules emulsion were mounted directly on the specimen stubs surface and introduced in the sputter coater without previous drying to avoid the fracture of the shells. In this way, evaporation of the solvents occurred under high vacuum. Those deformations in the structure, as shown in Figure 3-2 (a), can be explained by that high vacuum preparation which leads to destroying a major proportion of the microcapsules.

SEM images presented in Figure 3-2 evidenced the presence of round-shaped vesicles (the keratoses microcapsules) with a wide range of size distribution, which also confirms the findings of the light microscopy investigations as stated previously. The diameters of the microcapsules were found to be in between 0.5 and 4 µm.
Figure 3-2: SEM images of keratoses microcapsules 1500x (a) and 3000x (b).

Figure 3-3 reports AFM images of the keratoses microcapsules after treatment under high vacuum to remove the solvents. The diameters of microcapsules were obtained in the range 0.3 - 1 µm with a rough surface. Furthermore, the SEM results reveal a wide size distribution of the microcapsules, which is similar to that of the results obtained from transmitted light microscopy tests.

Figure 3-3: AFM images of microcapsules

The microcapsule yield was determined by UV-vis spectrometry measuring the residual amount of keratoses and dye still found in the aqueous solution separated by centrifugation.

An aliquot of the balk solution was taken from the suspension to measure the UV absorbency. Absorbance was measured at the maximum peak corresponding to 215.1 nm for keratoses (UV field) and 607.8 nm for Telon Blue (visible field).
The yield of microcapsules corresponded to 28.87 ± 3%.

The UV analysis was used to calculate the encapsulation yield of the dye, using the following equation:

\[
Dye\text{\hspace{1mm}encapsulation\hspace{1mm}yield}(\%) = \frac{dye\hspace{1mm}encapsulated\hspace{1mm}(g)}{initial\hspace{1mm}amount\hspace{1mm}of\hspace{1mm}dye\hspace{1mm}(g)} \times 100
\]

where the initial amount of dye was the quantity of dye used in the encapsulation process, and the dye encapsulated was obtained by subtracting the quantity of dye remained in the solution from the initial amount of dye. The dye encapsulation yield was found to be 83.62±5 %.

The DSC thermograms, reported in Figure 3-4, show three main endothermic events in the thermal behavior of both, i.e., the keratoses and the microcapsules dried in vacuum. The peaks corresponding to water evaporation fell in the range 65-70°C for both samples. The denaturation peaks were found to be very close (225°C for the keratoses and 233°C for microcapsules), while the degradation peak of microcapsules broadened and shifted to lower temperatures (297°C against 314 °C), probably because of interactions between keratoses and the dye inside the capsules.

*Figure 3-4-DSC thermograms of keratoses and keratoses microcapsules*
3.5 Materials and methods for producing blend film

KOx were obtained via oxidizing extraction from pristine wool, 21 μm mean fiber diameter, in the form of top (the fiber sliver submitted to industrial scouring, carding and combing), that was supplied by The Woolmark Co., Italy.

PVA fibers were supplied from Citterio S.P.A. - Monza, Italy.

All the other reagents were purchased from Sigma-Aldrich®, unless otherwise specified.

3.5.1 Preparation of PVA/KOx blend films

PVA fibers were cleaned by Soxhlet extraction using petroleum ether, washed with distilled water and conditioned at 20 °C, 65% r.h. for 24 h. PVA/KOx solutions were obtained by dissolving KOx and PVA (5% w/w) in water as a common solvent. The pure solutions of PVA and KOx were mixed in different proportions (0/100, 10/90, 30/70, 50/50, 70/30, 90/10, 100/0 w/w PVA/KOx) under stirring at 65°C for 1h and cast on polyester plates at room temperature overnight to obtain regenerated PVA/KOx blend films.

3.6 Blend film characterization

Blend film characterization has been carried out with different assays.

3.6.1 Morphological structure

Blend films were successfully obtained at all PVA/KOx ratio except for films with a KOx amount of 100% and 90% because at this KOx amount, films became fragile and hard to peel from the cast. The films are visually transparent especially those with higher PVA amount and as the KOx content increased the film's color tend to brownish (see Figure 3-5). The surface of the films of 100/0, 90/10, 70/30, 50/50, and 70/30 PVA/KOx look quite smooth, but with 100/0 PVA/KOx, the surface appearance becomes rigid. The morphology of the films are more irregular with increasing of the keratoses amount. As it shown in figure 2 the structure of the film
becomes fragile with decreasing the PVA amount that in 0% and 10% PVA, producing the film is not possible and also film with this amount of PVA hardly peeled from the casts.

![Image](image_url)  
Figure 3-5-Visual appearance of PVA/KOx films. (1)100/0 PVA/KOx - (2) 90/10 PVA/KOx - (3) 70/30 PVA/KOx - (4) 50/50 PVA/KOx - (5) 30/70 PVA/KOx - (6) 10/90 PVA/KOx - (7) 0/100 PVA/KOx

Microscopic morphology was examined by SEM (see Figure 3-6). The SEM images show that the films have a dense and continuous structure, and in 100/0, 90/10, and 70/30 PVA/KOx void were not found in entire structures and the films show good structural integrity in total shape, and the phase separation does not appear in any blended film among these three samples. As the amount of keratoses increased (50/50, 30/70, and 10/90 PVA/KOx), the smoothness of the blend films become rough and fluctuating, with some fracture structure revealed with a higher amount of keratoses ratio. This suggests that the presence of keratoses probably effect on the crystallization of blended film and higher amount of keratoses causes phase separation and aggregation of each polymer in matrix. This phase separation also occurs in other study with similar two polymers without using cross-linker [57].
Figure 3-6-SEM pictures of PVA/KOx films. (1) 100/0 PVA/KOx - (2) 90/10 PVA/KOx - (3) 70/30 PVA/KOx - (4) 50/50 PVA/KOx - (5) 30/70 PVA/KOx - (6) 10/90 PVA/KOx - (7) 0/100 PVA/KOx
The film thickness decreases by increasing the amount of keratoses in the blends from 0% to 30%. As the amount of keratoses increased, the thickness of the films increase.

Table 3.1: Thickness of blended films

<table>
<thead>
<tr>
<th>Film</th>
<th>Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0 PVA/KOx</td>
<td>97.54</td>
</tr>
<tr>
<td>90/10 PVA/KOx</td>
<td>64.99</td>
</tr>
<tr>
<td>70/30 PVA/KOx</td>
<td>46.90</td>
</tr>
<tr>
<td>50/50 PVA/KOx</td>
<td>53.97</td>
</tr>
<tr>
<td>30/70 PVA/KOx</td>
<td>55.71</td>
</tr>
</tbody>
</table>

3.6.2 Thermal analysis

Figure 4 shows the DSC traces recorded for the heating scan in the range 50 to 400 °C. In the blend films containing PVA/KOx, the curves display two main endothermic peaks. The first peak around 80 °C is related to the moisture evaporation and may be glass transition [92]. The endothermic peaks observed at 212 °C are related to the melting point of the PVA crystalline phase in the blend films which the peak become fading with decreasing of PVA amount and with increasing content of keratoses this peak shows the same temperature with all keratoses amount indicating there is no effect of keratoses on PVA crystallite structure.

From DSC with curves of 0/100 PVA/KOx, the peak at 295 °C was observed, indicating decomposition of keratoses and this decomposition did not appear in 100% PVA/0% keratoses. As the content of PVA increase, the decomposing and depolymerization peak was shifted to 300 °C, suggesting slightly higher stability of blend film than pure keratoses. As the keratoses contain different amino acids with hydrogen bonds, the melting energy in blend film causes increasing of the Tm in blend films. Basically, bending two mentioned macromolecule has no significant consequence on thermal properties.
3.6.3 FTIR analysis

Figure 3-8 shows the infrared spectra of all blend samples. The investigation of the keratoses films illustrates the typical bending and stretching vibration keratoses containing amino acid. In detail, the blend samples containing keratoses, there is a characteristic absorption which is related to the peptide bonds (−CONH−). The amide A has vibration at 3282 cm⁻¹ in the peptide bonds connected with the stretching vibration of N–H bond. The amide I is related mainly to the (C=O) stretching vibration having an intense peak at 1630 cm⁻¹. The amide II which is attributed to the N–H band has a peak at 1515 cm⁻¹, and the amide III band at 1230 cm⁻¹ is related to the in phase combination of C–N stretching and N–H in plane bending, with some attribution of C–C stretching and C=O bending vibration. The absorption bands at 1174 cm⁻¹ and 1040 cm⁻¹ in the keratoses spectra are related to the asymmetrical and symmetrical S–O vibrations of sulfonate (SO₃⁻) in cysteic acid [70].

As it can seen, the location of the main characteristic absorption peaks in keratoses remains in blend films. With adding and increasing of PVA in keratoses film, the absorption peak at 2940 cm⁻¹ appeared and broanded, this because this peak is related –H stretching and bending vibration of PVA [93].

The peaks present in blend film containing PVA are mainly related to the hydroxyl and acetate groups. The bands in 3270 cm⁻¹ are attributed to the stretching O–H from the
intermolecular and intramolecular hydrogen bonds. The other close vibrational bands are at 2840 and 2940 cm$^{-1}$ and are related to the stretching C−H form alkyl groups. Moreover, O–H plane bending motion is coupled with the other molecular motions that involve frequencies in the range 600-1500 cm$^{-1}$.

![FTIR spectra of blended films of PVA/KOx with different ratios.](image)

The bands at 1700 cm$^{-1}$ are related to absorption of the C=O and C−O from residual acetate groups attributed to the producing PVA from hydrolysis of polyvinyl acetate. The intensity of the absorption band at 1144 cm$^{-1}$ is related to the crystallinity degree of PVA. The presence of this band indicates that in blend films PVA maintains a certain degree of crystallinity as confirmed by the presence of melting peak in DSC curves. With increasing PVA amount, new absorption bonds emerged at about 1330 cm$^{-1}$ attributed to the CH−OH bending vibration of PVA. With increasing PVA, in PVA/KOx blend films, the absorption peak at 2940 cm$^{-1}$ appeared and broaded, because this peak is related to H stretching and bending vibration of PVA [93]. In blend PVA/KOx films it seems that both PVA and KOx peaks are present or overlapped indicating no covalent interaction between the two components in the blend.

### 3.6.4 Mechanical properties

The tensile strength and the modulus of cast films increase as increasing the amount of KOx in the blend until respectively 30% and 50% w/w. In blend film containing 70% of KOx these
parameters decrease sharply. On the other hand PVA cast film elongation at break decreases by adding KOx in the blend films. The similar result had been reported by other studies. Dou et al. indicated that, without a crosslinking agent, the increase of PVA content causes both tensile strength and elongation to increase, proposing high probably the hydrogen bonds between keratin and PVA were generated in blend film structure. Also, it suggested that the improved mechanical properties of keratin and PVA blend film possibly caused by the increase of crystallinity of blend film because of increased crystallizable PVA[93]. However, in the gelatin, the other protein-based material, gelatin/PVA blend film revealed that increasing PVA concentration causes decrease of the tensile strength of blend film [94].

The mechanical properties of the obtained film are summarized in Table 3-2-Tensile strength, elongation at break and modulus of PVA/KOx blend films. The blend films of 10/90 and 0/100 PVA/KOx are too fragile for film fabrication.

<table>
<thead>
<tr>
<th>PVA/KOx</th>
<th>Tensile strength (MPa)</th>
<th>Elongation at break (%)</th>
<th>Modulus at 0.5 mm elongation (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0</td>
<td>18.1</td>
<td>148</td>
<td>2.1</td>
</tr>
<tr>
<td>90/10</td>
<td>21.4</td>
<td>80</td>
<td>3.7</td>
</tr>
<tr>
<td>70/30</td>
<td>24.7</td>
<td>37</td>
<td>10.7</td>
</tr>
<tr>
<td>50/50</td>
<td>23.1</td>
<td>6</td>
<td>16.4</td>
</tr>
<tr>
<td>30/70</td>
<td>5</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3-2-Tensile strength, elongation at break and modulus of PVA/KOx blend films.
3.7 Conclusion

In this study, we have demonstrated the encapsulations of hydrophilic molecules into keratoses microcapsules using a sonication process. The keratoses were prepared via oxidizing an extraction of wool keratin and then analyzed by FTIR, HPLC, and SDS-PAGE. The results from SDS-PAGE shows that the molecular weights of the keratoses lie in the range of 38-62 kDa, and FTIR and HPLC results confirm the suitability of extracted keratoses to use via sonication method for the fabrication of microcapsule shells. Resultant microcapsules validate that Telon Blue BRL dye (N-aminophenylacetamide) has been entrapped as a core part and that the keratoses have a suitable structure to be a shell part of the microcapsules. The size of fabricated microcapsules was determined between 0.5 and 4 µm, and the dye encapsulation yield shows that the majority of the dye became entrapped inside keratoses microcapsules. Consequently, keratoses microcapsules can be a valuable and promising carrier for different applications, due to their level of biodegradability and high encapsulation rate.
Chapter 4

4 Conclusions

With unique properties of biodegradability, biocompatibility, non-toxicity, cross-linking and self-assembling ability, keratin is a versatile biopolymer that can be modified and developed in various forms, which can find applications in different fields, from agriculture to cosmetics and regenerative medicine. In this study, keratins have been extracted from keratinous sources with different methods. Chemical methods can obtain different kinds of solubilizates with distinctive characteristics. The properties of obtained keratins significantly are related to the extraction procedure. Choosing the appropriate process for obtaining keratin based on final requirements needs to be considered. The oxidation, reduction, and sulfitolysis are the pathways which consider it as a benchmark with least damaging of disulfide bonds. In particular, in oxidation method, the amount of cystiene has been increased and different keratin fraction can be obtained. The production yield of each method shows almost the same amount.

Chemical pathways for keratin solubilization are not always convenient from a technological, economical or environmental point of view. Within the contemporary ecological development context, emerging biotechnological methods employing keratinolytic microorganisms or chemically-free alternatives start to play a key role in processing keratinous waste and resources.
Development of feasible pathways to create value-added materials and applications starting from materials with high potentials, such as keratin wastes and by-product, is a contribution to environmental sustainability and progress of different economical and social fields. Moreover, keratins are biodegradable and biocompatible and have potential to be used in control release process. Further research is needed to take advantage of the keratin biopolymer potential fully.

This thesis reported the variety of keratin biomaterial and emphasized the properties of each keratin material. Particularly, keratin materials have different specific structure that makes it possible to apply in various particular tailored biomaterial by designing and modification of the structure and choosing proper extraction way.
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List of publications

Hossein Rajabinejad

PEER-REVIEWED JOURNAL PUBLICATIONS


- **Hossein Rajabinejad,** Alessia Patrucco, Rosalinda Caringella, Alessio Montarsolo, Marina Zoccola, Pier Davide Pozzo, *Fabrication and Properties of Keratoses/ Polyvinyl alcohol (PVA) Blend Film, submitted in Polymers MDPI, Oct 2017*


Project and Paper in Progress

- **Producing silk microsphere for drug delivery,** Soochow University, National Engineering Laboratory For Modern Silk, From Dec 2016- Nov 2017, *The paper will be submitted by October 2017*

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