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XXXVIII CYCLE

**From physical blending to polymerization-assisted
integration: degradable polyester/keratin biocomposites
for functional applications**

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ABBREVIATIONS AND ACRONYMS

PLA	Poly(lactic acid)
PHA	Poly(hydroxyalkanoates)
PBS	Poly(butylene succinate)
PCL	Poly(ϵ -caprolactone)
PBAT	Poly(butylene adipate- <i>co</i> -terephthalate)
PGA	Poly(glycolic acid)
PLGA	Poly(lactic- <i>co</i> -glycolic acid)
PBA	Poly(butylene adipate)
PHB	Poly(3-hydroxybutyrate)
PBSA	Poly(butylene succinate- <i>co</i> -adipate)
T_g	Glass transition temperature
T_{max}	Temperature of maximum degradation rate
$T_{5\%}$	Temperature at 5% weight loss
T_m	Melting temperature
ϵ -CL	ϵ -caprolactone
wt%	Weight percent
ECM	Extracellular matrix
RGD	Arginyl-glycyl-aspartic acid
LDV	Leucine-aspartic acid-valine
EDS	Glutamic acid-aspartic acid-serine
HaCaT	Human cultured keratinocytes
MC3T3-E1	Mouse calvaria 3-day transfer
3D	Three-dimensional
2D	Two-dimensional
ROP	Ring-Opening Polymerization
TEO	Thyme Essential Oil
DPPH	(2,2-difenil-1-picrilidrazile)
TBARS	Thiobarbituric acid reactive substances
MDA	Malonic dialdehyde
SA	Succinic acid
BD	1,4-butanediol
u-PBS	unsaturated-poly(butylene succinate)
Sn(Oct) ₂	Tin(II) 2-ethylhexanoate
AlL ₂ Me	Aluminum phenoxyimine methyl
TOF	Turnover frequency
Ti(OBu) ₄	Titanium(IV) butoxide
FDA	Food and Drug Administration
CDCl ₃	Deuterated chloroform
UV	Ultraviolet
GPC	Gel Permeation Chromatography
THF	Tetrahydrofuran
M_n	Number-average molecular weight
$\bar{D}$	Dispersity (M_w/M_n)

FT-IR	Fourier Transform Infrared Spectroscopy
DSC	Differential Scanning Calorimetry
SEM	Scanning Electron Microscopy
NMR	Nuclear Magnetic Resonance
TGA	Thermogravimetric analysis
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight
FWHM	Full Width at Half Maximum
K	Keratin
KE	Keratin Extract
FK	Feathers Keratin
NK	Native Keratin
DK	Denatured Keratin
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	Size Exclusion Chromatography
TMS	Tetramethylsilane
ATR	Attenuated Total Reflectance
CHCl ₃	Chloroform
WCA	Water Contact Angle
HSD	Honestly significant difference
ΔH_m	Melting enthalpy
χ_c	Crystallinity degree
ΔW	Weight loss
T.W.L.	Total weight loss
R.M.	Residual mass
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
SD	Standard deviation
MWCO	Molecular weight cut-off
LDH	Lactate dehydrogenase
OCN	Osteocalcin
FAK	Focal adhesion kinase
ROCK	Rho-associated coiled-coil kinase
Runx2	Runt-related transcription factor 2
DPBS	Dulbecco phosphate-buffered saline
DMSO	Dimethyl sulfoxide

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Chapter I

INTRODUCTION

Traditional fossil-based plastics are specifically engineered to exhibit high durability, chemical resistance, and mechanical stability, which are undoubtedly beneficial during their useful life but become critically problematic once these materials reach their end-of-life.¹ The extensive and indiscriminate use of synthetic polymer materials derived from fossil resources has raised serious environmental concerns, primarily related to their persistence in natural ecosystems and the continued accumulation of plastic waste in landfills, soils, and marine environments.^{2,3} Consequently, plastic pollution has emerged as one of the most pressing global environmental challenges, attracting growing attention from the scientific community, industry stakeholders, and policymakers. This phenomenon is the direct consequence of a production paradigm that, for decades, has prioritized material performance, durability, and low cost. The limited recyclability of many polymeric materials, combined with inefficient waste management strategies and the steadily increasing global plastic production, has further exacerbated this issue. Current estimates indicate that global plastic production has exceeded 400 million tonnes per year, while less than 10% of post-consumer plastic waste is effectively recycled on a global scale.^{4,5}

A further critical aspect associated with the persistence of conventional plastics is their progressive fragmentation into microplastics and nanoplastics, which are now recognized as ubiquitous contaminants in terrestrial, aquatic, and atmospheric environments.⁶ It has been estimated that microplastics, typically defined as plastic particles smaller than 5 mm, originate both from the direct release of primary microplastics and from the long-term degradation and mechanical fragmentation of larger plastic objects. Recent studies report that several million tonnes of microplastics are generated annually worldwide, with significant accumulation observed in marine sediments, freshwater systems, soils, and even in remote environments.^{7,8} Due to their small size and high surface-area-to-volume ratio, microplastics can persist for decades, act as carriers for persistent organic pollutants and heavy metals, and interact with biological systems across multiple trophic levels.⁹ Increasing evidence of microplastic ingestion by microorganisms, invertebrates, fish, and higher organisms has raised serious concerns regarding their potential impact on ecosystems and human health.^{10–12}

In this scenario, the development of degradable polymeric materials, capable of complete and predictable degradation, without generating persistent residues, represents a fundamental objective for a sustainable solution. These materials must therefore not only maintain adequate performance

during use but also exhibit controlled degradation or complete assimilation into natural cycles after disposal.^{13,14} In this direction, degradable polymers have emerged as one of the most promising solutions to address these challenges, representing a conceptual shift from materials designed for permanence to materials intentionally engineered for a finite and controlled lifespan. In particular, biodegradable polymers have been engineered to degrade through hydrolytic and/or enzymatic processes, resulting in the formation of non-toxic, low-molecular-weight byproducts that can be metabolized by microorganisms or integrated into natural biochemical pathways.¹⁵

Furthermore, the biobased polymer materials have gained considerable attention as a complementary strategy to biodegradability. These polymers are derived, partially or entirely, from renewable biological resources such as biomass, agricultural feedstocks, or biorefinery streams, enabling a reduction in fossil carbon consumption and related greenhouse gas emissions. The development of biodegradable and biobased polymers is increasingly considered a synergistic approach, capable of addressing both resource depletion and waste accumulation.¹⁶

Beyond waste reduction, biobased and biodegradable polymers also offer additional benefits, including reduced dependence on fossil resources and the ability to tailor their degradation behaviour to specific application requirements. In line with this growing interest, global production capacity for biodegradable and bio-based polymers has increased steadily over the past decade. Recent market and industry analysis estimate that production has exceeded 4-5 million tons annually, with projected annual growth rates exceeding 10%, driven primarily by applications in the packaging, agriculture, and biomedicine sectors.^{17,18} Although these materials still represent a relatively small fraction of the total plastics market, their rapid growth reflects growing regulatory pressure, technological maturation, and social demand for more sustainable material solutions.

Sustainable materials must be conceived as part of an integrated system in which raw materials, production processes, use phases, and end-of-life scenarios are all interconnected. Unlike the traditional linear model based on production, consumption, and disposal, the circular economy aims to maintain the value of materials and resources within the economic system for as long as possible.¹⁹ In this regard, aliphatic polyesters represent a widely studied class of materials, already used in industrial applications and available on a commercial scale, as in the case of poly(lactic acid) (PLA), as well as a consolidated platform for the development of sustainable alternatives to conventional plastics.²⁰ Their widespread use is mainly attributed to the combination of relatively simple synthesis methods, process versatility, and a wide range of achievable physicochemical properties, which can be modulated through molecular design and formulation strategies.. Aliphatic polyesters are particularly attractive for biodegradable material applications because their ester bonds are

susceptible to hydrolytic and enzymatic cleavage, allowing for degradation under environmental or physiological conditions.²¹ At the same time, the diversity of monomer structures and polymer architectures allows for materials ranging from rigid thermoplastics to flexible elastomeric systems, covering application areas ranging from packaging to agriculture to biomedical devices.²²

From a sustainability perspective, polyesters can be broadly classified based on the origin of their constituent elements. Some polyesters are completely or partially of biological origin, derived from renewable resources such as biomass or fermentation products, such as poly (lactic acid) (PLA), poly(hydroxyalkanoates) (PHA), and, to some extent, poly(butylene succinate) (PBS). Others, however, are synthesized from fossil-based monomers but still exhibit biodegradability due to the presence of ester bonds in their chemical structure, such as poly(ϵ -caprolactone) (PCL) and poly(butylene adipate-*co*-terephthalate) (PBAT).²³

In addition to their origin, polyesters can be differentiated based on their degradation behaviour and functional performance. Some polymers exhibit relatively high degradation rates and limited long-term mechanical stability, making them unsuitable for short-term applications, such as disposable packaging or temporary biomedical devices. These include, for example, PLA and PHA for packaging, and poly(glycolic acid) PGA or poly(lactic-*co*-glycolic acid) PLGA copolymers for biomedical applications..^{13,14} Conversely, other polyesters exhibit slower degradation kinetics and greater mechanical strength, allowing them to be used in applications requiring long-term structural integrity and predictable performance; representative examples include PCL, PBS, and PBAT.^{24,25} Selecting a specific macromolecular composition therefore involves a trade-off between sustainability parameters, degradation profile, and functional performance. This balance is a central aspect of sustainable materials design and represents one of the main challenges in the transition from conventional plastics to more sustainable polymeric systems.²⁶

Within the broad family of sustainable polyesters, several representative systems have been developed and extensively studied, each occupying a distinct position in terms of origin, degradation behaviour, and application potential. Polyesters derived from α -hydroxy acids, such as PLA and PGA, represent some of the most established materials in this field.^{27,28} These polymers generally exhibit relatively high rigidity, while their hydrolytic degradation kinetics can vary significantly depending on their composition and microstructure: PLA can exhibit even prolonged degradation times, particularly in semi-crystalline forms,²⁹ while copolymers such as PLGA are characterized by faster and more tunable degradation. These properties have led to their widespread use in short- and medium-term applications, including compostable packaging, absorbable medical devices, sutures, and drug delivery systems.^{30,31} PLA, in particular, has attracted considerable industrial interest due to

the possibility of producing its monomer, lactic acid, from renewable resources through microbial fermentation processes starting from various matrices of biological origin, including sugars derived from starchy crops, sucrose from sugar cane or beet, as well as by-products of the dairy chain, such as whey and whey permeate, as well as carbohydrates obtained from lignocellulosic biomass.^{32,33} However, despite its biobased origin and biodegradability, PLA exhibits intrinsic limitations related to brittleness, low impact resistance, and limited thermal stability, which often require blending or modification to extend its application range.^{34–36}

A second important category is represented by aliphatic polyesters obtained from the polycondensation of diacids and diols, such as PBS, poly(butylene adipate) (PBA), and related copolymers, including PBAT.^{37,38} These materials are generally characterized by improved flexibility and melt processability compared to PLA-based systems, making them suitable for applications such as agricultural films, flexible packaging, and disposable products.^{39,40}

PHAs constitute a further relevant class of biodegradable polyesters, distinguished by their biological origin. PHAs are biosynthesized by microorganisms as intracellular energy storage materials and include poly(3-hydroxybutyrate) (PHB) and a wide range of copolymers.^{41,42} Their fully biobased nature and inherent biodegradability have generated considerable interest for both biomedical and packaging applications.⁴³ Nonetheless, PHAs often suffer from processing challenges, narrow thermal processing windows, and brittleness, which have limited their large-scale adoption despite their favourable sustainability profile.^{41,44}

In this heterogeneous landscape, lactone-based polyesters such as PCL occupy a distinct position. PCL is a semicrystalline polymer with a variable degree of crystallinity (up to 69%), a low glass transition temperature (T_g), which, depending on the polymer molecular weight, ranges from -65°C to -60°C , and a low melting temperature ($T_m = 56\text{--}65^{\circ}\text{C}$) with slow degradation kinetics under physiological and environmental conditions. Its decomposition temperature is 350°C .^{25,45} These characteristics confer high ductility and long-term structural stability, making PCL particularly attractive for applications requiring prolonged functional performance, including long-term implantable devices and controlled-release systems.⁴⁶ PCL can be completely degraded by many natural microbes,⁴⁷ its degradation is strongly affected by the polymer features, such as molecular weight and crystallinity, and by the external conditions of degradation (pH, temperature, solvents); it could take from several months to several years.⁴⁸ It is a highly versatile polymer from an applicative standpoint, with uses ranging from sutures, wound dressings, bone or nerve tissue regeneration, to artificial blood vessels and food packaging systems.^{49–54} Although PCL is typically synthesized from fossil-based monomers through the polymerization of ϵ -caprolactone (ϵ -CL), its biodegradability and

excellent processability have made it a reference material in polymer research; in parallel, more sustainable synthetic strategies have been explored and proposed to obtain the monomer itself from renewable sources (*e.g.*, derived from sugars, agricultural residues or lignin), which aim to reduce the fossil footprint of the production cycle.^{55,56}

Overall, these examples illustrate how biodegradable polyesters encompass a wide spectrum of material behaviours, ranging from rigid and fast-degrading systems to flexible and slowly degradable materials.^{24,25} This diversity provides a versatile toolbox for the design of biodegradable materials but also highlights the necessity of carefully selecting or modifying polymer matrices to meet specific functional and sustainability requirements.²⁶

Despite the remarkable versatility of biodegradable polyesters and their successful applications across a variety of sectors, the use of these materials in their pure or unmodified form is often associated with inherent limitations that limit their functional performance.^{13,57} These limitations become particularly evident when biodegradable polyesters must operate in harsh environments or provide additional functionality beyond structural support.^{58,59} One of the most common limitations of aliphatic polyesters is their inherent hydrophobicity and chemical simplicity, which translates into limited surface reactivity and poor interaction with biological systems. In biomedical applications, this often results in suboptimal cell adhesion, limited bioactivity, and a lack of specific biological signaling, especially for polymers such as PCL and PLA, which have relatively inert surfaces.^{60,61} From a mechanical standpoint, many biodegradable polyesters exhibit a narrow window of usable properties. Rigid systems, such as PLA and PGA, tend to exhibit brittleness and poor impact resistance, while more flexible polyesters may lack sufficient stiffness or long-term mechanical stability.^{31,35} Achieving a balanced combination of strength, ductility, and toughness often requires modification strategies that go beyond simple polymer selection. Thermal and degradation limitations further complicate the use of pure biodegradable polyesters. Materials characterized by rapid degradation kinetics may prematurely lose mechanical integrity, limiting their applicability in long-term applications.⁶² Conversely, slowly degrading polymers may persist longer than desired, particularly in environmental settings, raising concerns about incomplete degradation and the formation of lingering residues under certain conditions.^{6,62}

In addition to these issues, biodegradable polyesters typically offer limited functional versatility. In their pure form, they are primarily structural materials and do not inherently possess properties such as antimicrobial activity, bioactivity, controlled release capacity, or responsiveness to external *stimuli*.^{63,64} As a result, the development of advanced biodegradable materials increasingly relies on strategies to introduce additional functionalities into the polymer matrix. Overall, these inherent

limitations highlight that replacing conventional plastics with biodegradable polyesters cannot rely solely on polymer chemistry. Instead, a materials design approach that integrates complementary components and functional modifiers is needed to overcome these constraints and fully exploit the potential of biodegradable polyesters in advanced applications.⁶⁵

In response to the inherent limitations of pure biodegradable polyesters, a wide range of material design strategies has been developed to improve their functional performance and expand their application potential. Among these approaches, the integration of additional components into polyester matrices has proven to be one of the most effective, simple and versatile solutions, allowing for the modulation of physicochemical, mechanical, and biological properties without radically altering the polymer structure.^{66–68} Traditionally, inorganic fillers and synthetic additives have been used to modify polymer properties; a growing emphasis has been placed on the use of bioderived and waste-derived components as functional modifiers.^{69,70} This shift reflects not only the need to improve material performance but also the growing demand for sustainability-oriented design strategies aligned with the principles of the circular economy.^{58,71}

Agri-food waste streams represent a particularly valuable source of bioderived components, as they are generated in large quantities, are constantly available, and often contain structurally complex biomolecules.^{67,72} Incorporated into biodegradable polyester matrices, these components can perform multiple functions, acting as reinforcing agents, surface modifiers, or functional additives capable of introducing new properties such as increased hydrophilicity, bioactivity, or controlled interaction with biological environments.⁷³ The integration of bioderived components into polymer matrices can be achieved through various approaches, ranging from simple physical mixing to more advanced strategies involving surface functionalization or chemical bonding.⁷⁴ While physical incorporation offers simplicity and scalability, chemically assisted approaches provide better interfacial compatibility and more stable property enhancement, highlighting the importance of selecting appropriate integration strategies based on the desired material performance.⁷⁵

It is important to emphasize that the use of waste-derived components extends beyond the concept of passive fillers. In many cases, these biomolecules actively contribute to the functionality of the final material, enabling the development of multifunctional systems that combine structural integrity with biological or chemical activity.⁷⁶ This paradigm shift from additive-based modification to functionality-oriented material design is particularly relevant in the context of biodegradable polymers, where added functionality must be balanced with degradation behaviour and environmental compatibility. In this regard, the integration of protein components offers unique opportunities. Proteins offer a high density of functional groups, intrinsic biocompatibility, and the potential to

interact with both polymer matrices and biological systems.⁷⁶ Consequently, protein-polyester composites have attracted increasing attention as advanced biodegradable materials capable of meeting both performance constraints and sustainability objectives. Within the broader class of bio-derived components, proteins recovered from agro-food waste streams have emerged as particularly attractive candidates for the development of advanced biodegradable materials.^{77,78} The interest in protein-based systems stems from their intrinsic biodegradability, chemical versatility, and biological relevance, as well as from the large volumes of protein-rich residues generated by agricultural practices and food processing industries. Several waste-derived proteins have been investigated for material applications, including collagen and gelatin obtained from slaughterhouse by-products, casein from dairy industry residues, plant proteins such as soy and gluten, and proteins recovered from seafood processing waste. Many of these biomolecules are already efficiently integrated into consolidated industrial value chains, finding application in food, feed, cosmetic, pharmaceutical, and biomedical sectors.⁷⁹

In this context, keratin represents a particularly advantageous protein-based resource. Keratin is a fibrous structural protein widely distributed in nature and constitutes the main component of hair, wool, feathers, horns, nails, and other corneous tissues.⁸⁰ Unlike several other protein sources, keratin, especially when derived from poultry feathers, combines high availability with limited current exploitation in high-value applications, making it an attractive candidate within a circular economy framework.⁸¹ At the molecular level, keratin is characterized by a complex hierarchical organization, spanning from amino acid sequences to supramolecular assemblies. Keratins are rich in cysteine residues, which form extensive disulfide crosslinks that confer remarkable chemical, thermal, and mechanical stability. Based on their secondary structure, keratins can be broadly classified into α -keratins and β -keratins. Mammalian α -keratins, such as those found in wool and hair, adopt α -helical coiled-coil conformations that assemble into intermediate filaments, providing elasticity and toughness. These properties have enabled the long-standing use of wool in textile and insulation applications, as well as its more recent exploitation in selected biomedical and cosmetic products.⁸² In contrast, avian β -keratins, which constitute the predominant component of chicken feathers, are characterized by a β -sheet-rich structure leading to a more compact, rigid, and highly ordered molecular arrangement. This structural organization results in increased stiffness and mechanical robustness compared to α -keratins. Despite these advantageous characteristics, feather-derived β -keratin has not yet found widespread high-value material applications, representing a largely untapped resource when compared to wool-derived keratin.⁸³ From a quantitative perspective, the relevance of chicken feathers as a keratin source is significant. The global poultry industry produces on the order of 100 million tonnes of chicken annually, and feathers account for approximately 3–7%

of the total body weight. This corresponds to several million tonnes of feather waste generated every year worldwide. Despite this continuous and abundant supply, feather waste is still largely disposed of through landfilling, incineration, or low value uses, resulting in both environmental burden and loss of a highly concentrated biomaterial resource. Notably, chicken feathers are composed of more than 90 wt% keratin, making them one of the richest natural sources of this protein.⁸¹ Figure 1 shows a schematic representation of the recovery and characterization of feather keratin. Beyond its structural role, keratin also exhibits intrinsic biological functionality. The protein rich in short amino acid sequences present in several proteins of the extracellular matrix (ECM) that promote cell adhesion, such as arginyl-glycyl-aspartic acid (RGD), leucine-aspartic acid-valine (LDV) and glutamic acid-aspartic acid-serine (EDS) peptide domains.⁸⁴ These features enable interactions with cell surface receptors and contribute to the promotion of cell adhesion, proliferation, and differentiation. As a result, keratin is not merely a passive structural component, but an active biomolecule capable of influencing biological responses at the material–cell interface.^{85,86} The combination of high availability, limited competition with existing industrial uses, structural robustness, chemical functionality, and inherent bioactivity positions feather-derived keratin as a unique protein-based resource among agro-food waste materials. When integrated into biodegradable polyester matrices, keratin offers the opportunity to introduce functional groups, biological cues, and enhanced interfacial properties, thereby contributing to the development of multifunctional materials that address both performance requirements and sustainability objectives.⁸⁰

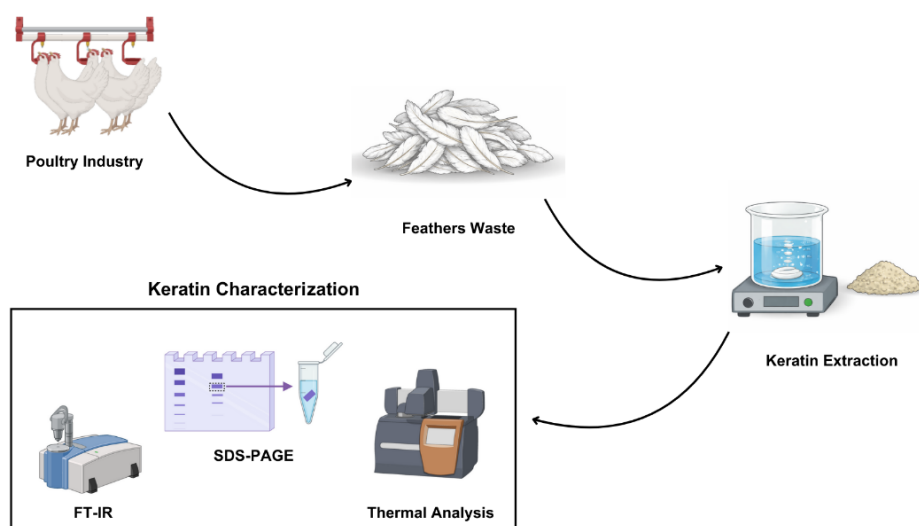


Figure 1. Schematic representation of the recovery and characterization of keratin from poultry industry waste.

The integration of keratin into biodegradable polyester matrices has been widely studied as a strategy to combine the favourable mechanical and processing properties of synthetic polymers with the

biological functionality of protein-based materials. From a materials science perspective, keratin offers a unique set of advantages, including a high density of polar functional groups, intrinsic bioactivity, and the ability to modulate surface properties, which are particularly useful when combined with chemically simple and relatively bioinert polyesters.^{87–89} Most keratin/polyester systems reported in the literature are based on physical blending approaches, in which keratin is incorporated as a bioactive phase dispersed within the polymer matrix. This strategy has been widely adopted due to its conceptual simplicity, versatility, and compatibility with conventional polymer processing techniques. Physical mixing has proven effective in improving surface wettability, promoting protein adsorption, and enhancing cell-material interactions, especially in applications such as films, fibrous membranes, and porous scaffolds for biomedical use. In these systems, the polyester matrix provides structural integrity and processability, while keratin acts primarily as a functional modifier, compensating for the inherent bioinertness of many biodegradable polyesters.^{87–89}

⁸⁹ The ability to fabricate keratin-containing materials in diverse architectures, including solvent-cast films, electrospun fibrous mats, and three-dimensional porous scaffolds, has further contributed to the widespread adoption of physical mixing as the first and most robust strategy for keratin integration. At the same time, it is important to recognize that the vast majority of blend-based keratin–polyester systems described to date employ keratins of mammalian origin, such as those extracted from wool or human hair. These α -keratins have been extensively investigated due to their well-established availability and well-characterized physicochemical properties. In such systems, physical blending has consistently produced reproducible improvements in surface properties and biological performance, confirming its validity as a robust and versatile integration strategy. In contrast, avian keratins, composed of β -keratin and derived from chicken feathers, have received comparatively limited attention in polymer composite systems. The structural differences between α -keratins and β -keratins, particularly in terms of secondary structural organization and crosslink density, suggest that their interaction mechanisms with polyester matrices, as well as the resulting material properties, may differ significantly from those observed in mammalian keratin-based systems. Addressing this aspect is essential to assess the true potential of feather-derived keratin as a functional component in biodegradable polyester composites. Beyond physical blending approaches, increasing attention has been directed toward the development of chemically assisted integration strategies aimed at improving interfacial stability and enabling a more intimate association between proteins and polymer matrices. Chemical modification routes, including coupling reactions, surface functionalization, and grafting strategies (*e.g.*, thiol–ene reactions or other reactive approaches), have been proposed as effective tools to promote covalent bonding between bio-derived components and synthetic polymers. Such approaches are particularly relevant for overcoming limitations associated

with phase separation, component migration, or limited long-term stability, while preserving the functional contribution of the protein component. Within the broader landscape of biodegradable polyesters, PCL has emerged as one of the most promising matrices for keratin integration.^{80,90,91} A recent comparative study in which PCL was blended with different proteins, including gelatin, zein, and keratin, to produce electrospun fibrous scaffolds demonstrated that keratin–PCL systems exhibited the most favourable biological performance.⁹² This relevance can be attributed to PCL's advantageous combination of properties, including low glass transition temperature, high chain flexibility, slow and predictable degradation kinetics, and excellent processability, which make it particularly suitable for blending with protein-based materials and for fabricating complex architectures relevant to biomedical applications. At the same time, PCL is intrinsically hydrophobic and chemically simple, lacking specific functional groups required for direct biological signaling. While physical blending with keratin represents an effective strategy to enhance surface bioactivity, the establishment of covalent interactions between PCL chains and keratin remains largely unexplored. The development of chemical strategies capable of linking keratin to PCL at the molecular level therefore represents an important opportunity to further improve interfacial compatibility, stability, and functional control in keratin–PCL systems.⁵² In this context, keratin–PCL materials constitute an effective and versatile platform for investigating the interplay between polymer structure, processing, and biological response.⁸⁵ Overall, the state of the art indicates that physical blending is a validated and highly effective strategy for integrating keratin into biodegradable polyesters, while also highlighting the potential added value of chemically assisted approaches aimed at achieving more stable and controlled polymer–protein integration.⁹³ Extending integration strategies to structurally distinct keratin sources and exploring covalent coupling concepts in versatile matrices such as PCL represents a logical step toward the broader valorization of agro-industrial protein waste into advanced biodegradable materials.

In light of the considerations discussed above, biodegradable polyesters emerge as versatile platforms for the development of sustainable and functional materials, while agro-industrial protein waste offers unique opportunities to introduce biological functionality and added value within polymeric systems. The demonstrated effectiveness of physical blending strategies, together with the limited exploration of structurally distinct keratin sources and chemically integrated keratin–polyester systems, highlights the need for systematic investigations aimed at assessing material performance, biological response, and integration strategies in carefully selected polyester matrices. Within this framework, the present thesis is directed toward the development and characterization of keratin-based biodegradable materials, with particular emphasis on PCL as a model platform, as outlined in the following section.

Chapter II

DESIGN OF KERATIN-ENRICHED POLYMERIC MATERIALS

II.1 Purpose of the study

The aim of this doctoral thesis is to develop biodegradable polymeric materials incorporating keratin recovered from agro-industrial waste, with the aim of exploiting its bioactivity and structural functionality within sustainable polymer systems.

Specifically, the work focuses on the valorization of keratin extracted from chicken feathers as an abundant and underutilized protein source and its integration into biodegradable polyester matrices for advanced materials applications. The research focuses primarily on PCL as a model platform for biodegradable polyester. PCL was selected for its favourable combination of processability, mechanical stability, and controlled degradation behaviour, as well as its widespread use in biomedical materials research. In this context, keratin is employed as a functional component capable of modulating the surface properties, biological response, and functionality of the material, rather than simply serving as an inert filler. The first part of the thesis focuses on the development and characterization of keratin/PCL materials obtained through physical mixing approaches. In this phase, feather-derived β -keratin is incorporated into PCL matrices and transformed into two-dimensional systems, such as solvent-based films, with the aim of improving surface properties and biological response for skin applications. In particular, cell viability was assessed using HaCaT keratinocytes, an immortalized cell line of human epidermal origin, commonly used as an *in vitro* model for preliminary screening of biomaterials for skin applications, due to its high reproducibility and ability to respond to the chemical and physical properties of the substrate surface.

Subsequently, the physical mixing strategy is extended to the fabrication of three-dimensional porous scaffolds by salt leaching, allowing the evaluation of keratin–PCL systems as scaffolds for bone tissue engineering, a field in which cell–material interactions and structural characteristics play a crucial role. In this context, the biological response was investigated using MC3T3-E1 pre-osteoblasts, a cell line widely used as an *in vitro* model for studying adhesion, proliferation, and osteogenic differentiation on biomaterials intended for bone regeneration. Building on the knowledge gained with mixing-based systems, the research then moves towards a more advanced materials design strategy in which keratin is directly involved during polymer synthesis. In this approach, keratin is employed *in situ* during the ring-opening polymerization (ROP) of ϵ -CL, with the aim of promoting a tighter association between the protein and the growing PCL chains. This step represents a

conceptual transition from keratin as a dispersed functional modifier to its use as an active macroinitiator within the polymerization process, opening new perspectives for the design of multifunctional biodegradable materials.

Based on the results and insights derived from the *in situ* polymerization approach, the thesis further explores the possibility of extending keratin integration strategies to alternative polymerization pathways. Specifically, keratin incorporation is studied in the context of polycondensation reactions leading to the synthesis of PBS-based materials. Figure 2 shows a conceptual representation of the strategies adopted in this thesis. This part of the work aims to evaluate the compatibility of keratin with condensation polymerization mechanisms and assess the feasibility of transferring protein integration concepts beyond ring-opening polymerization systems. Overall, the thesis follows a progressive and exploratory research path, moving from established physical mixing approaches to more advanced polymerization-based strategies, and finally assessing the transferability of these concepts to different classes of biodegradable polyesters. This systematic approach allows exploring the potential of feather-derived keratin as a functional component in biodegradable polymeric materials, providing preliminary insights for the rational design of application-oriented sustainable biocomposites.

In the following paragraphs, a brief overview of the main sections of the thesis is provided, outlining the rationale, scope, and key objectives of each research stage, from physically blended systems to chemically integrated keratin–polyester materials.

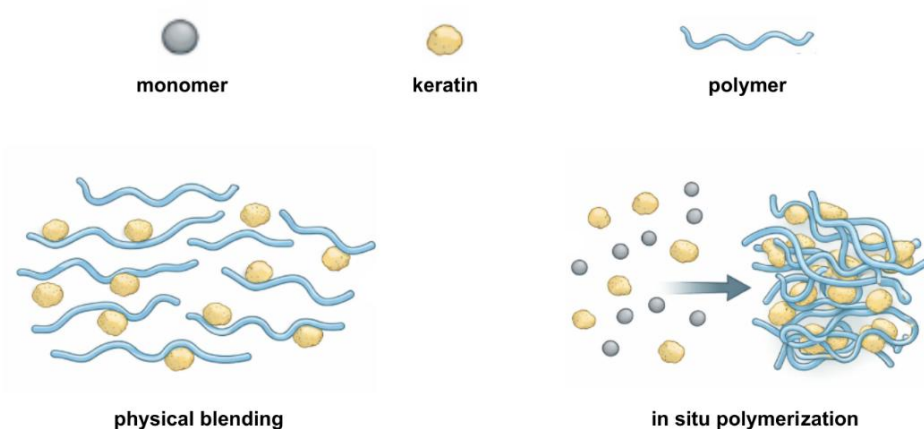


Figure 2. Schematic comparison of different conceptual strategies for keratin integration into polymeric systems

II.2 Two-dimensional keratin/PCL biocomposites for skin-related biomedical applications

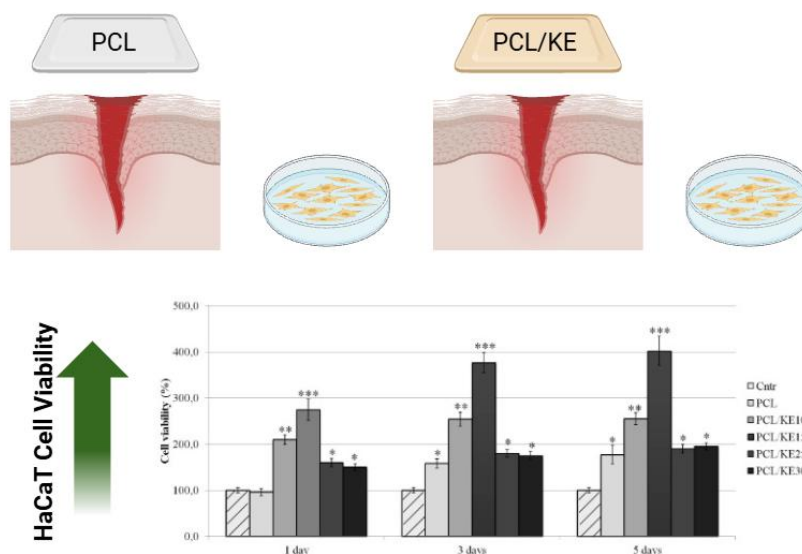


Figure 3. Schematic representation of two-dimensional PCL and PCL/keratin (PCL/KE) films evaluated for skin-related applications.

Two-dimensional (2D) polymeric systems, such as films and membranes, represent a straight forward platform for investigating the functional integration of bio-derived components into biodegradable polymers. In these systems, material performance is predominantly governed by surface-related properties, including surface chemistry, wettability, and interfacial interactions with the surrounding environment, rather than by bulk mechanical behaviour alone. As a result, two-dimensional architectures provide a controlled and accessible framework for evaluating the impact of functional modifiers on polymer surface properties and surface-mediated biological responses (Figure 3).

In biomedical applications involving skin contact, wound protection, or temporary barrier functions, materials must combine flexibility, conformability, and biocompatibility, while promoting favourable interactions with cells at the material interface. For these applications, the surface-mediated biological response plays a dominant role, providing an ideal context for evaluating the contribution of bioactive components introduced into otherwise bioinert polymer matrices.

Among biodegradable polyesters, PCL has been widely employed in surface-based biomedical systems due to its excellent processability, low glass transition temperature, and mechanical flexibility. These properties enable the fabrication of thin, flexible films capable of conforming to irregular surfaces without mechanical failure. At the same time, PCL is characterized by intrinsic hydrophobicity and limited chemical functionality, which restrict its ability to directly interact with

biological environments. When used in its pristine form, PCL often exhibits suboptimal cell adhesion and limited bioactivity, particularly in applications where surface interactions are critical. These limitations make PCL an ideal model system to investigate functional modification strategies aimed at enhancing biological performance without altering the polymer backbone.

Protein-based materials offer unique advantages as functional modifiers of biodegradable polymers, owing to their high density of polar functional groups and intrinsic biological relevance. Among them, keratin represents a particularly attractive candidate, combining structural stability with the presence of motifs associated with cell adhesion and tissue interaction. While keratins of mammalian origin have been extensively investigated in polymeric films and membranes, keratin derived from chicken feathers- predominantly composed of β -keratin -remains comparatively underexplored in surface-based composite systems. The distinct molecular organization of β -keratin suggests that its interaction with polymer matrices and biological environments may differ from that of α -keratin-based systems, warranting targeted investigation., Feather-derived keratin also offers clear advantages, as from a sustainability perspective it originates from an abundant agro-industrial waste stream, and moreover it is largely unexploited in high-value material applications. Its use as a functional modifier in biodegradable polymer films therefore represents both a scientific and environmental opportunity. For surface-based applications, physical blending provides a simple and effective strategy to incorporate keratin into PCL matrices. This approach allows the introduction of bioactive components without modifying the polymer backbone or relying on complex chemical coupling reactions, while maintaining processability with scalable processing techniques such as solvent casting. In two-dimensional systems, physical blending is particularly advantageous because even limited amounts of protein can significantly influence surface properties, provided that the protein is accessible at or near the material interface. As such, keratin/PCL films obtained by physical blending constitute an ideal platform to study the relationship between composition, surface properties, and biological response in a systematic manner.

This chapter establishes the conceptual framework for the investigation of keratin/PCL systems in surface biomedical applications. By focusing on two-dimensional architectures and physical blending strategies, it defines the starting point of the research pathway developed in this thesis. The results related to the preparation, characterization, and biological evaluation of keratin/PCL films are presented and discussed in the Results and Discussion section.

The insights gained from this initial level of investigation provide the foundation for the subsequent extension to three-dimensional architectures and to more advanced keratin integration strategies explored in the following section (II.3).

II.3 Three-dimensional keratin/PCL scaffolds for bone tissue engineering: rationale and design

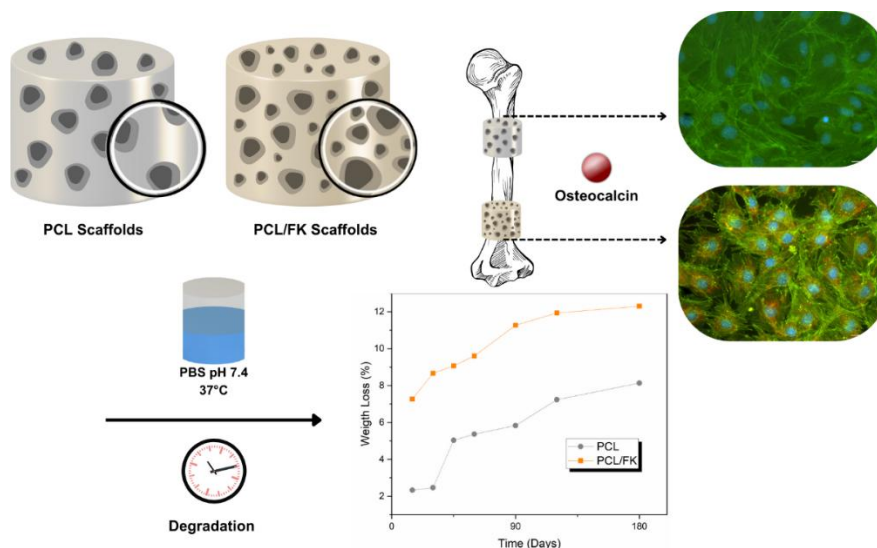


Figure 4. Schematic overview of three-dimensional PCL and PCL/keratin (PCL/FK) porous scaffolds developed for bone tissue engineering applications.

While two-dimensional polymeric systems provide an effective platform for investigating surface-mediated interactions, many biomedical applications require materials capable of supporting cell infiltration, mass transport, and tissue regeneration within a three-dimensional space. In this context, three-dimensional (3D) architectures represent a critical evolution from surface-based systems, as they introduce additional design constraints related to porosity, pore interconnectivity, and structural stability. Building upon the conceptual framework established in Chapter 1, the transition from keratin/PCL films to three-dimensional architectures enables the assessment of whether the bioactive contribution of feather-derived β -keratin can be preserved and exploited within more complex material geometries. Unlike 2D systems, where interactions are largely confined to the material surface, 3D scaffolds must support sustained cell–material interactions throughout the entire volume of the construct. Specifically, bone tissue engineering places stringent and multifactorial demands on scaffold materials. In addition to biocompatibility and biodegradability, scaffolds must feature an interconnected porous network that facilitates cell migration, vascularization, and nutrient diffusion. Mechanical stability is also necessary to maintain structural integrity during the early stages of tissue regeneration, while gradual degradation should allow the scaffold to be progressively replaced by newly formed bone tissue. Polymeric scaffolds based on biodegradable polyesters have been widely

studied for bone tissue engineering due to their tunable degradation behaviour and processability in porous architectures. However, many synthetic polymers lack intrinsic bioactivity and rely on surface modification or the incorporation of functional components to promote the osteogenic response. In this context, the integration of bioderived components such as keratin represents a promising strategy to improve the biofunctionality of the scaffold without compromising the processability of the material. Keratin has been shown to possess intrinsic biological features relevant to bone-related applications, including the presence of peptide motifs associated with cell adhesion and the ability to modulate protein adsorption at material surfaces. While these properties have been extensively investigated in surface-based systems, their role within three-dimensional environments remains less explored, particularly for feather-derived β -keratin. In 3D scaffolds, the distribution and accessibility of keratin throughout the scaffold volume become critical factors influencing cell attachment, proliferation, and differentiation. The extension of keratin/PCL systems to porous architectures therefore provides an opportunity to investigate whether the bioactive effects observed in two-dimensional configurations can be translated into volumetric constructs capable of supporting osteogenic maturation. Importantly, maintaining the same material composition while increasing architectural complexity allows the isolation of structural effects from compositional variables. This approach enables a clearer interpretation of how scaffold architecture and keratin integration jointly influence biological response. This chapter establishes the conceptual and methodological background for the investigation of three-dimensional keratin/PCL scaffolds intended for bone tissue engineering applications (Figure 4). By extending the same material system explored in Chapter 1 to porous architectures, it defines the second level of complexity within the research pathway developed in this thesis. The experimental results related to scaffold fabrication, structural characterization, degradation behaviour, and osteogenic response are presented and discussed in the integrated Results and Discussion section. The insights gained from this three-dimensional investigation provide a critical bridge between surface-based systems and the subsequent exploration of more advanced keratin integration strategies addressed in the following section.

II.4 *In situ* PCL/keratin biocomposites: synthesis, characterization, and reservoir behaviour

This section of the work focuses on the development of keratin-based polymer systems obtained via *in situ* ROP of ϵ -CL, using denatured keratin as a multifunctional macroinitiator. This approach represents a deliberate shift from the physical mixing strategies discussed in the previous chapter toward the design of chemically integrated materials, in which the protein component actively participates in polymer formation.

The shift from physical mixing to polymerization-based integration strategies is motivated by the intrinsic limitations of blend-based systems, in which the keratin is dispersed as a separate phase and its interaction with the polyester matrix is governed predominantly by weak physical forces. Such systems are inherently prone to phase separation, limited interfacial stability, and limited control over the spatial distribution of the protein component, especially under processing or long-term service conditions. Conversely, the use of keratin during ϵ -CL polymerization enables PCL chain growth to occur in close proximity to the protein phase, leading to materials in which the two components are integrated at the formation stage rather than combined post-synthetically. This molecular integration is expected to improve interfacial and thermal stability, maintain stable crystallinity, and promote a more homogeneous and stable polymer-protein architecture.

In this context, the primary objective of this section is to clarify how keratin-initiated polymerization influences polymer growth, molecular architecture, and material organization. Structural, spectroscopic, thermal, and morphological characterizations are used to evaluate the effects of *in situ* polymerization in the presence of keratin on the resulting PCL-based materials. Particular attention is paid to identifying the effects of keratin presence during polymerization on chain growth behaviour, phase organization, and thermal stability, which are key parameters for assessing the robustness and reliability of the resulting materials.

In addition to chemical and materials characterization, the systems obtained by polymerization in the presence of keratin were functionalized with thyme essential oil (TEO) to evaluate the potential functional benefit associated with keratin incorporation. Keratin is not inherently antioxidant; however, its chemical heterogeneity suggests that, when chemically bound within the polymer architecture, it could serve as a reservoir phase capable of retaining volatile bioactive compounds. TEO was chosen as a model due to its high antioxidant efficacy combined with marked volatility, which represents a rigorous test for retention within polymer matrices. Antioxidant activity tests such as (2,2-difenil-1-picrilidrazile) DPPH and (Thiobarbituric Acid Reactive Substances) TBARS were performed on a fresh chicken meat model to investigate the optimal keratin concentration that most effectively serves as a reservoir for TEO. Functionalization experiments were then designed to

evaluate whether PCL/keratin materials provide better retention and stabilization of these compounds than pure PCL, offering a functional advantage that arises directly from the presence of the protein phase rather than its intrinsic chemical activity.

Overall, this section establishes the chemical and materials basis of PCL systems obtained by polymerization in the presence of keratin, showing how moving beyond physical blending can overcome some of the limitations of composite materials based on simple phase dispersion, while enabling additional functional advantages related to controlled interactions within the polymer matrix.

Finally, this approach demonstrates that the development of keratin-based biocomposites can be effectively achieved directly during the polymerization step. The *in situ* growth of PCL chains from keratin-derived initiation sites confirms that bioderived macromolecules can be actively involved in polymerization reactions, even in solvent-free conditions, resulting in structurally integrated materials. This result provides clear evidence that biocomposite formation can be integrated into the reaction step itself, offering a chemically robust route to designing multifunctional polymer systems based on renewable components.

II.5 Keratin integration strategies in polycondensation reactions for poly(butylene succinate)

Building on the results obtained for physically blended keratin/PCL systems and for keratin-initiated ROP of ϵ -CL, the research was further extended to polycondensation-based biodegradable polyesters, with the aim of evaluating the behaviour of keratin under different polymerization mechanisms. In this context, PBS was selected as a model aliphatic polyester synthesized via step-growth polycondensation, offering a chemically and mechanistically distinct platform compared to ROP-derived systems.

Unlike the (PCL/Denatured Keratin) PCL/DK materials, for which the involvement of keratin during polymerization could be investigated through molecular-level characterization, the integration of keratin into PBS-based systems was approached in a more exploratory manner. Here, keratin was introduced during the polycondensation process as a functional additive with the aim of evaluating whether its presence during chain growth could influence the resulting material properties.

To further expand the design space, structural modification of PBS was also pursued through partial substitution (10% molar ratio) of 1,4-butanediol (BD) with *cis*-2-butene-1,4-diol, enabling the introduction of unsaturation along the polymer backbone. This modification provides additional structural flexibility and potential reactive sites, allowing the investigation of combined effects arising

from backbone modification and keratin incorporation. Both neat PBS and unsaturated PBS-based systems were synthesized in the presence of increasing keratin contents, under reaction conditions specifically tailored to preserve protein integrity during high-temperature polycondensation.

Given the complexity of these systems and the limitations associated with direct molecular-level characterization of protein/polyester interactions in polycondensation-derived materials, the focus of this section is placed on the evaluation of keratin-induced effects at the macroscopic and mesoscopic levels. Thermal, mechanical, rheological, morphological, and surface property analyses are employed to assess how the presence of keratin during polymer formation influences crystallization behaviour, viscoelastic response, mechanical performance, and wettability of PBS-based materials.

Overall, this section aims to verify whether the integration of keratin during polycondensation can represent a viable strategy for tailoring material properties, even without definitive evidence of covalent bonding. By comparing neat PBS, unsaturated PBS derivatives, and keratin-containing systems, this study explores the extent to which keratin can act as a structural modifier and functional component under step-growth polymerization conditions. This investigation represents a logical progression of the thesis, transitioning from physical blending to chemically integrated systems and finally to the assessment of keratin compatibility and effectiveness across different polymerization routes, thereby broadening the applicability of keratin-based strategies in biodegradable polyester design.

Chapter III

RESULTS AND DISCUSSION

III.1 Effect of Keratin Waste on PCL Films: Structural Characterization, Thermal Properties, and Keratinocytes Viability and Proliferation Studies

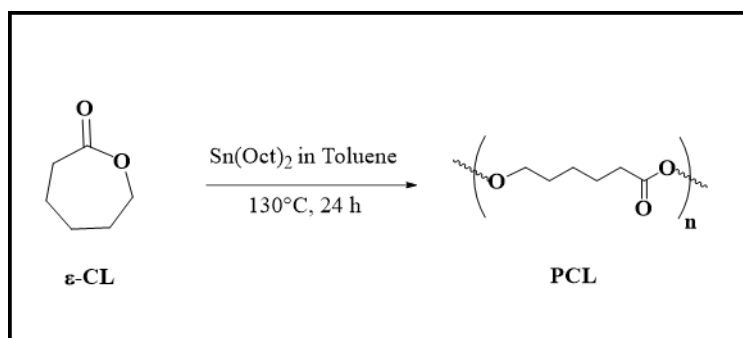
This section examines the effect of feather-derived keratin waste on PCL films obtained by physical mixing, with the aim of assessing how the incorporation of a bioderived protein influences the material's structure, thermal behaviour, and surface-mediated biological response. The analysis focuses on the relationship between keratin content and film morphology, including keratin dispersion and phase organization, as well as the impact of keratin addition on the thermal stability and crystallization behaviour of the PCL matrix. In parallel, *in vitro* biological studies are presented to assess keratinocyte viability and proliferation on keratin/PCL films, providing insight into the suitability of these two-dimensional systems for skin-related biomedical applications.

III.1.1. Synthesis and Characterization of PCL.

The synthesis of PCL has been widely reported in the literature and was achieved by ROP of ϵ -CL in the presence of tin(II) 2-ethylhexanoate ($\text{Sn}(\text{Oct})_2$) as a catalyst (Scheme 1).⁹⁴ $\text{Sn}(\text{Oct})_2$ exhibits several advantageous features, including high catalytic activity, ease of handling, and cost-effectiveness, and it has been approved by the Food and Drug Administration (FDA) for use in medical polymers, provided that the residual tin content does not exceed 20 ppm.⁹⁵

Regarding the ROP mechanisms of ϵ -CL, polymerization may proceed through cationic, anionic, or coordination–insertion pathways, depending on the nature of the catalyst or initiator employed. In particular, polymerizations mediated by well-defined single-site organometallic catalysts typically follow a coordination–insertion mechanism and are distinguished by a high degree of control over both molecular weight and molecular weight distribution.⁹⁴ This level of control enables the rational design of polymer chains, including precise control over polymer end groups. In addition, single-site catalysts make it possible to tune the polymer microstructure, either in terms of stereochemistry or, in copolymerization processes, through control of monomer sequence distribution along the chain. Consequently, this approach allows the achievement of tailor-made polymer architectures and properties.

The coordination–insertion mechanism involves an initial coordination of the monomer to the metal center, followed by insertion of the monomer into the metal–active bond, typically an alkoxide or amide species, accompanied by cleavage of the acyl–oxygen bond of the lactone. Chain propagation then proceeds through successive monomer insertions, while termination generally occurs via hydrolysis of the growing polymer chain.⁹⁶



Scheme 1. PCL synthesis reaction scheme.

The successful synthesis of the polymer was confirmed by ^1H and ^{13}C NMR. In the ^1H NMR spectrum of the product (solvent CDCl_3 , Figure 5), the characteristic chemical shifts at 1.36, 1.65–1.61, 2.29, and 4.04 ppm of the PCL were observed. Macromolecular weight and macromolecular weight distribution of the polymer were determined by GPC in THF and resulted respectively $M_n = 23,200$ g/mol and $D = 2.0$. (Figure 6). The origin of the observed dispersity value can be related to the used catalyst, Sn(Oct)_2 , which could promote transesterification reactions.⁹⁷

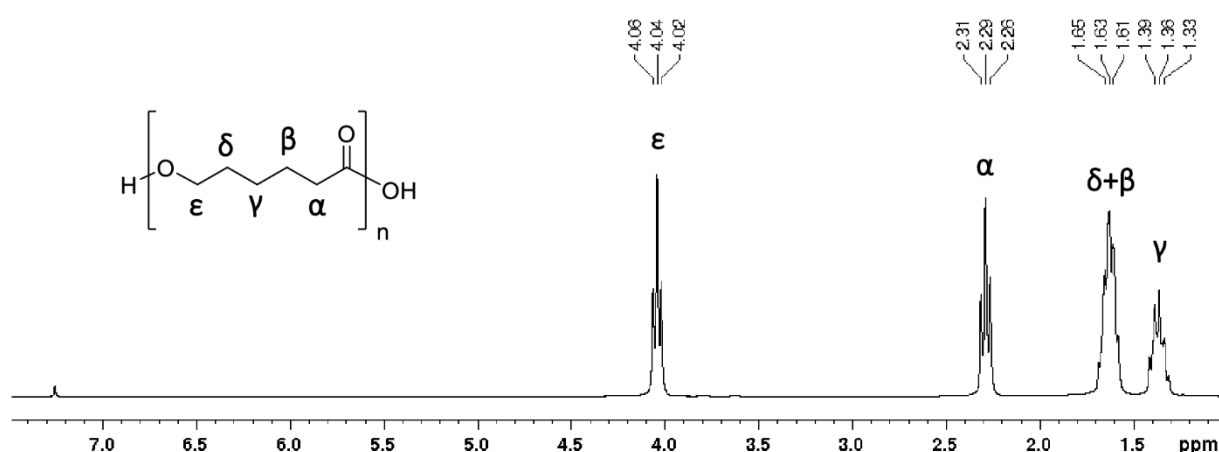


Figure 5. ^1H NMR spectrum (300 MHz, CDCl_3 , 298 K) of PCL.

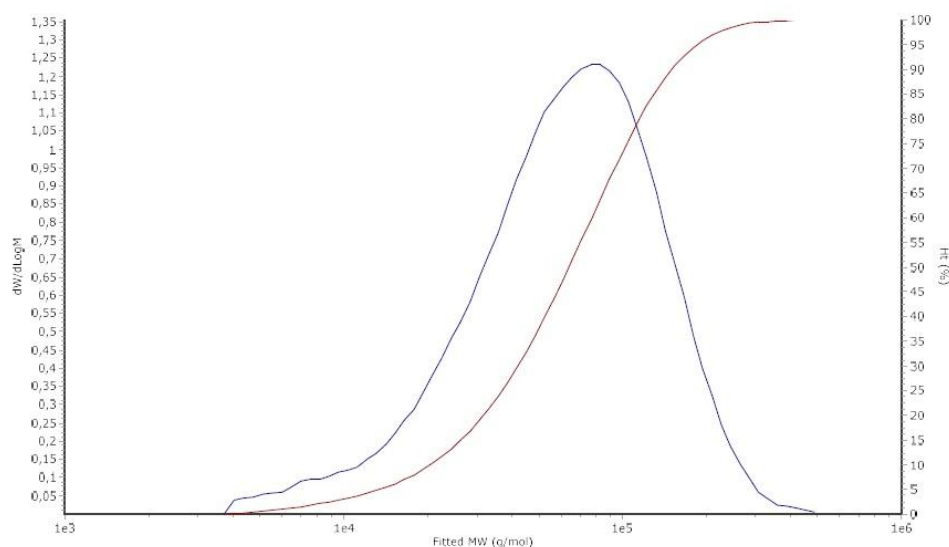


Figure 6. GPC curve of PCL.

Thermal characterizations of the polymer were performed using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). DSC analysis was used to evaluate the melting and crystallization behaviour of the material; the resulting enthalpy curve shows a well-defined melting peak at 60.7 °C during the second heating scan (Table 1).

The thermal stability of the polymer was investigated using TG analysis, the main parameters of which are summarized in Table 2. The weight loss of the synthesized PCL, measured in a controlled-flow nitrogen atmosphere, is plotted as a function of temperature in Figure 7. The onset of the degradation process was detected at approximately 300 °C. A single degradation phase was observed, characterized by an inflection point at 338.3 °C, consistent with what was reported in the literature for PCL, although with a slightly lower onset temperature in the present study. This difference can be attributed to variations in the molecular weight of the samples considered, which is known to significantly influence the degradation behaviour of PCL.^{98,99}

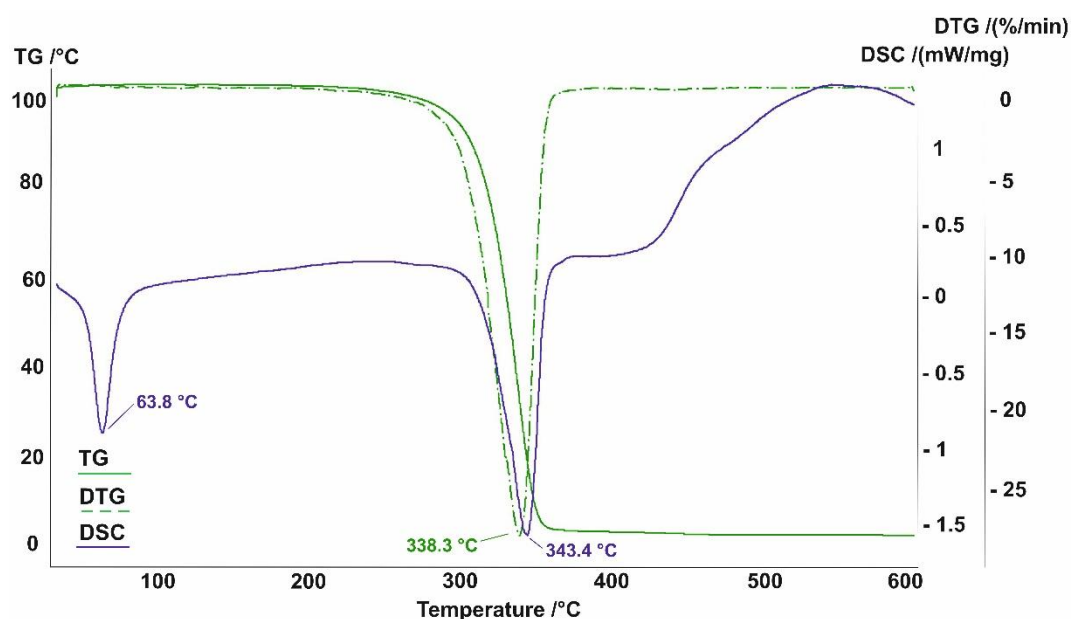


Figure 7. TG, DTG and DSC curves of PCL sample obtained by thermal analyses performed in the temperature range 40–600°C at 10°C/min.

III.1.2. Keratin extract from chicken feathers by alkaline hydrolysis: molecular and thermal characterization.

Numerous approaches for keratin extraction are reported in the literature, including acid or alkaline hydrolysis processes, strategies based on the cleavage of inter- and intramolecular disulfide bonds, as well as reduction, oxidation, or enzymatic hydrolysis methods.¹⁰⁰ Most of these techniques involve the use of organic solvents and/or chemical reagents such as sodium sulfide, metabisulfite, and 2-mercaptoethanol. However, the use of these substances is not only expensive but also has a significant environmental impact.¹⁰¹

To reduce the environmental footprint of the extraction process, alkaline hydrolysis was adopted in this work. Although this method is characterized by a relatively low extraction yield, it avoids the use of organic solvents and particularly harmful reagents, while also ensuring less alteration of keratin amino acids compared to acid hydrolysis.¹⁰¹ In this initial work, extraction was conducted using a basic aqueous solution of 0.1 M NaOH.

The molecular weight distribution of the extracted keratin (KE) was analyzed by SDS-PAGE on a 12% polyacrylamide gel, and the results are shown in Figure 8. Electrophoresis highlights the presence of two main bands, corresponding to approximately 10 and 20 kDa. These results are consistent with those reported in the literature, where the band at approximately 10 kDa is attributed

to the monomeric form, while the band at approximately 20 kDa is associated with the dimeric form, confirming the ability of keratin to self-assemble into dimers.¹⁰²

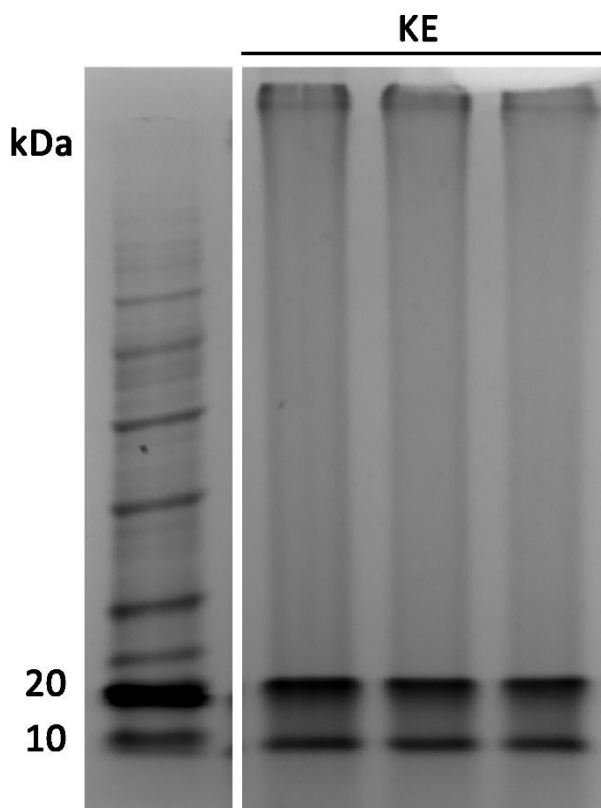


Figure 8. SDS-PAGE of keratin extract (KE). The left line is the molecular marker. kDa = kilodalton. KE = keratin extract.

The data obtained from thermogravimetric analysis, shown in Figure 9, show a progressive degradation process of the extracted keratin (KE), leading to an overall weight loss of 69.9% (Table 2). The first thermal event, observable as a peak at 73.8°C in the DTG curve, is attributable to the evaporation of water associated with the keratin through physical interactions and hydrogen bonds.¹⁰⁰

The main thermal degradation phase of keratin fibers occurs in the temperature range between 200 and 450°C, with a maximum degradation rate highlighted by a peak in the DTG curve at 330.5°C (Figure 9). This behaviour is consistent with what has been previously described in the literature for keratin-based materials.¹⁰⁰

Above 450°C, the residual mass loss, equal to 6.8% by weight, is associated with advanced decomposition processes of the keratin structure at high temperatures.¹⁰³

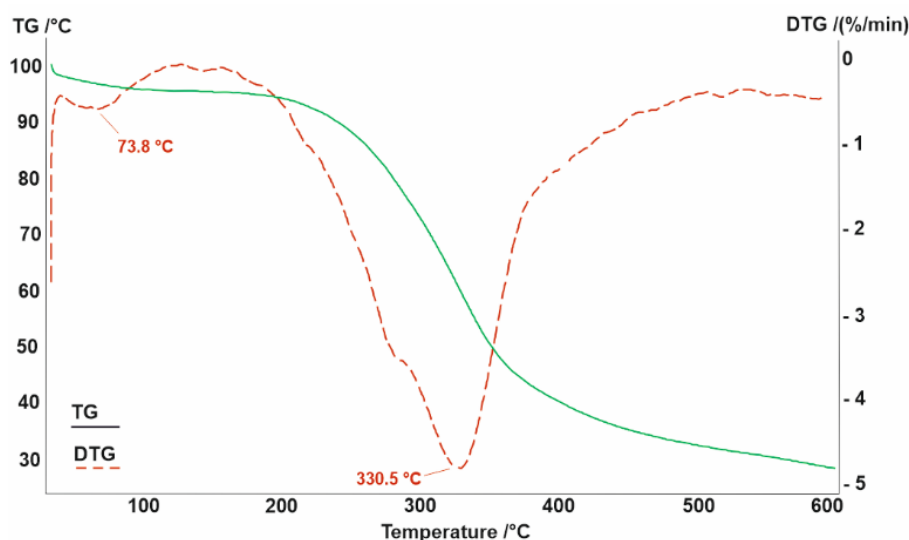


Figure 9. Thermogravimetric curve (TG) and first derivative of TG curve (DTG) of keratin extract (KE) sample.

III.1.3. Characterization of films with different percentages of added keratin extract

Pure PCL films and PCL films enriched with extracted keratin (PCL/KE) were obtained by solvent casting. Different keratin concentrations were investigated for the composite systems: 10% (PCL/KE10), 15% (PCL/KE15), 25% (PCL/KE25), and 30% (PCL/KE30). Pure PCL and PCL/KE films were characterized by simultaneous thermal analysis (TA) and scanning electron microscopy (SEM).

Table 1. Thermal behaviour data of PCL, PCL/KE10, PCL/KE15, PCL/KE25 and PCL/KE30 films obtained by DSC analyses.

Sample	T_m 1st Heating	ΔH_m 1st Heating	T_m 2nd Heating	ΔH_m 2nd Heating	χ_c
	°C	J/g	°C	J/g	%
PCL	61.5	58.93	60.7	21.53	15.8
PCL/KE10	57.7	29.73	54.8	1.80	1.5
PCL/KE15	60.4	53.01	55.5	3.30	2.9
PCL/KE25	60.1	51.96	57.8	2.17	2.1
PCL/KE30	60.6	58.66	56.3	4.29	4.5

T_m : melting temperature

ΔH_m : melting enthalpy

χ_c : Crystallinity degree

Thermogravimetric analyses were conducted in a nitrogen atmosphere, heating the samples from 40 to 600°C. The TG and DTG curves for PCL/KE films, pure PCL, and keratin are shown in Figure 10, while the main thermal parameters and degradation ranges are summarized in Table 2. Interestingly, all PCL/KE films exhibit superior thermal stability compared to both pure PCL and keratin. The integration of keratin within the PCL matrix improves the overall thermal stability of the composite systems. Among the different formulations, sample PCL/KE15 exhibits slightly higher thermal stability than the other PCL/KE films (Table 2 and Figure 10).

The thermal degradation process of films can be divided into three main stages. The first stage ($T < 200\text{ }^{\circ}\text{C}$) is mainly associated with water evaporation: in this region, PCL/KE films exhibit a weight loss of approximately 1%, while pure PCL exhibits significantly lower weight loss. This difference is attributable to the presence of water in the composite films, linked to the intrinsically hydrophilic nature of keratin. The second stage corresponds to the main thermal degradation of the films; both pure PCL and PCL/KE films show comparable final degradation temperatures, with almost complete degradation around 450°C. The third stage, characterized by a weight loss of less than 3% in the PCL/KE films, is attributed to the advanced degradation processes of the keratin component.

Table 2. Thermal behaviour of PCL, KE, PCL/KE10, PCL/KE15, PCL/KE25 and PCL/KE30 films analyzed by TG-DSC analyses.

Sample	< 200°C			200 - 450°C			450 - 600 ° C			T.W.L.	R.M.
	ΔW (%)	DTG (°C)	DSC (°C)	ΔW (%)	DTG (°C)	DSC (°C)	ΔW (%)	DTG (°C)	DSC (°C)		
PCL	0.2	-	63.8 ^a	98.4	338.3	343.4 ^a	0.3	-	-	98.9	1.1
KE	4.2	68.5-145.5	65.3 ^a	58.9	281.5-330.5	223.9 ^b - 339.6 ^a	6.8	517.0	519.4 ^a	69.9	30.1
PCL/KE10	1.1	-	59.1 ^a	96.7	340.6-378.9	342.6 ^a - 352.1 ^a - 378.9 ^a	1.7	-	492.1 ^a	99.4	0.6
PCL/KE15	0.8	-	63.3 ^a	90.1	350.3-391.8	354.7 ^a - 394.3 ^a	1.8	-	520.2 ^a	92.7	7.3
PCL/KE25	1.4	-	59.3 ^a	93.7	345.5-386.2	345.0 ^a - 362.0 ^a - 387.5 ^a	2.6	-	498.6 ^a	97.7	2.4
PCL/KE30	1.1	-	59.1 ^a	91.0	346.2-388.4	355.2 ^a - 391.7 ^a	2.1	-	491.3 ^a	94.1	5.9

ΔW = weight loss (by TG); ^a = endothermic reaction; ^b = exothermic reaction; T.W.L. = Total Weight Loss; R.M. = residual mass

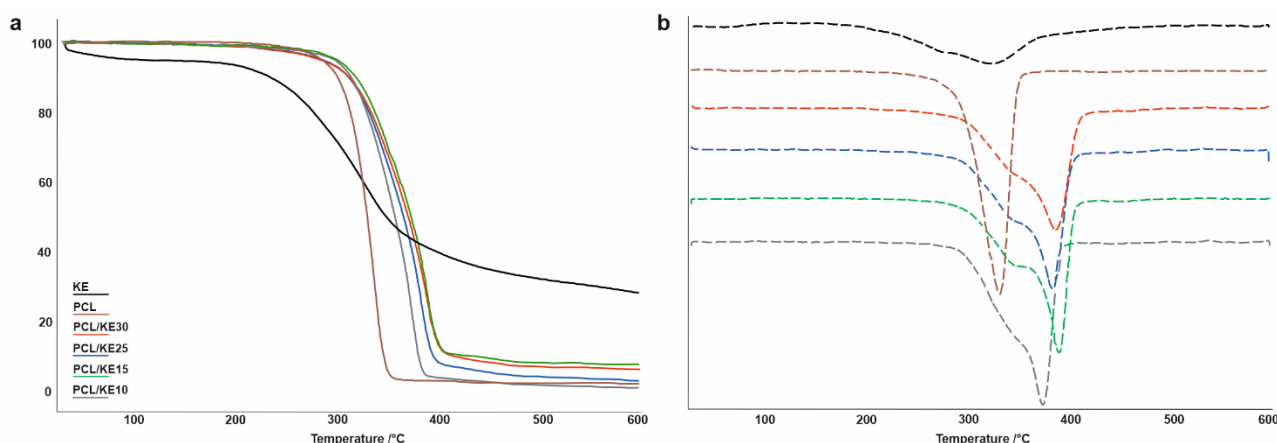


Figure 10. Thermogravimetric curve (TG) **(a)** and first derivative of TG curve (DTG) curves **(b)** of pure PCL, keratin extract (KE), and PCL/KE films.

Regarding melting behaviour, DSC analyses showed that the melting temperature, recorded during the second heating scan, is higher for the pure PCL film (60.7°C) than that of the PCL/KE films, which varies between 54.8 and 57.8°C (Figure 11; Table 1). Furthermore, the enthalpy of fusion (ΔH_m) of the composite films is lower than that of pure PCL; A similar trend was also observed for the degree of crystallinity (Table 1). In this context, the PCL/KE15 sample exhibits a slightly higher ΔH_m value than the other composite films, suggesting a peculiar behaviour that deserves further analysis.¹⁰⁴

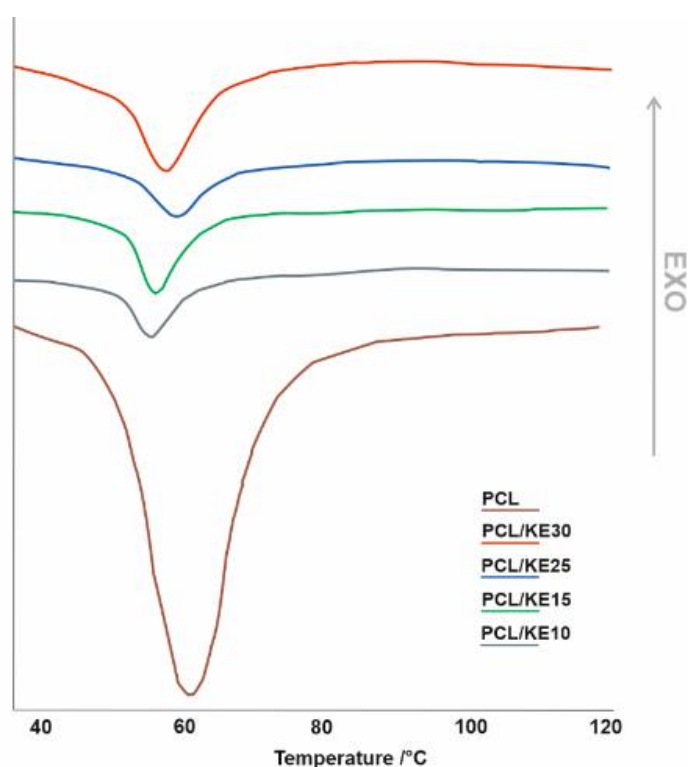


Figure 11. Differential scanning calorimetry (DSC) curves of PCL and PCL/KE films.

Finally, the PCL/KE films, along with the keratin and pure PCL, were analyzed by SEM (Figure 12). The keratin powder appears to consist of fibers of heterogeneous sizes (Figure 12a). The pure PCL film obtained by solvent casting, on the other hand, presents a smooth surface, characterized by the presence of microcracks, likely due to the solvent evaporation process during film preparation (Figure 12b).

SEM images revealed significant differences in keratin distribution within the composite films. In the PCL/KE10 sample, keratin was only partially organized and distributed unevenly across the film surface (Figure 12c). In contrast, the PCL/KE15 film exhibited more homogeneous keratin incorporation, with uniform distribution within the polymer matrix (Figure 12d). In the samples with higher keratin content, PCL/KE25 and PCL/KE30, the dispersion again appeared inhomogeneous (Figures 12e,f), with evidence of unincorporated keratin depositing on the surface of the polymer film.

SEM analysis of the cross-sectional surfaces provided further relevant information on the internal morphology of the materials (Figure 13). The pure PCL film exhibited a compact and smooth structure (Figure 13a), while the PCL/KE15 sample exhibited an irregular and jagged cut surface (Figure 13b). This morphological characteristic could be advantageous in the biomedical field, for example, for the use of films as medical patches in the wound healing process. The microstructural architecture of films plays a key role in cell-material interactions, as cells are able to sense and respond to the local geometric characteristics of the substrate.¹⁰⁵

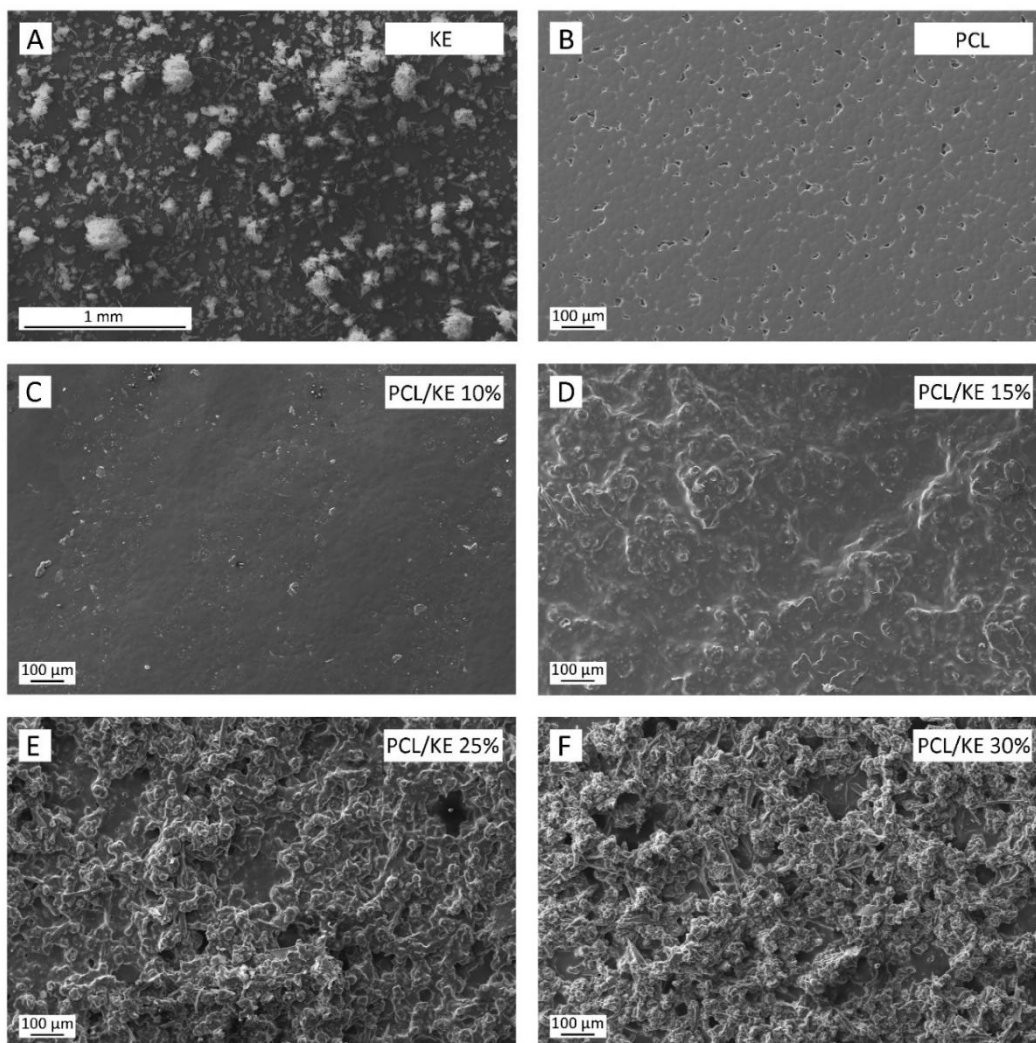


Figure 12. Scanning electron microscopy images of: **A)** KE; **B)** PCL; **C)** PCL/KE10; **D)** PCL/KE15; **E)** PCL/KE25; **F)** PCL/KE30.

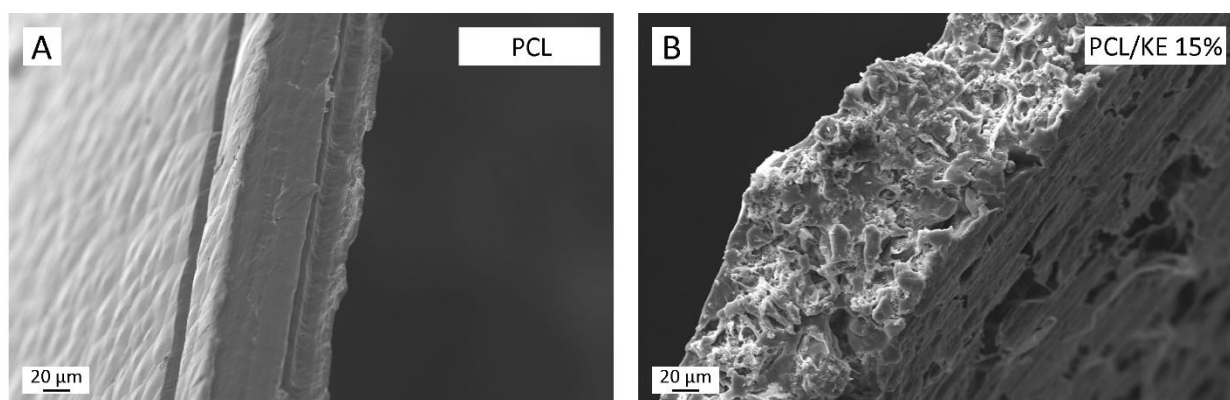


Figure 13. Scanning electron microscopy images of: **A)** cross section of pure PCL film; **B)** cross section of PCL/KE15 film.

III.1.4. HaCaTs Viability and Proliferation Study

In vitro cell viability and proliferation tests were performed using HaCaT human epidermal keratinocytes. Cell viability and proliferation were assessed using the MTT assay, a colorimetric test commonly used to estimate cellular metabolic activity as an indirect measure of cytotoxicity. Specifically, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is internalized by viable cells and reduced by mitochondrial enzymes to an insoluble blue formazan,¹⁰⁶ a process that occurs exclusively in metabolically active cells.¹⁰⁷

As shown in Figure 14, HaCaT cells grown on both pure PCL films and those enriched with different weight percentages of keratin (10%, 15%, 25%, and 30%) were viable throughout the entire 5-day culture period. Specifically, HaCaT cells grown on pure PCL films showed no significant differences compared to the control (cells grown without the film) on day 1, while statistically significant differences in proliferation rates emerged on days 3 and 5.¹⁰⁸ In contrast, cells grown on PCL/KE films, regardless of keratin content, showed significant differences compared to the control as early as day 1 and maintained these differences on days 3 and 5. Among the different formulations, the PCL/KE15 sample promoted the highest rate of cell proliferation from the early stages of culture. However, the increase in cell growth observed between days 3 and 5 was lower than that recorded between days 1 and 3, likely due to the progressive limitation of available space and nutrients, factors that can negatively affect cell proliferation in the later stages of culture.¹⁰⁸

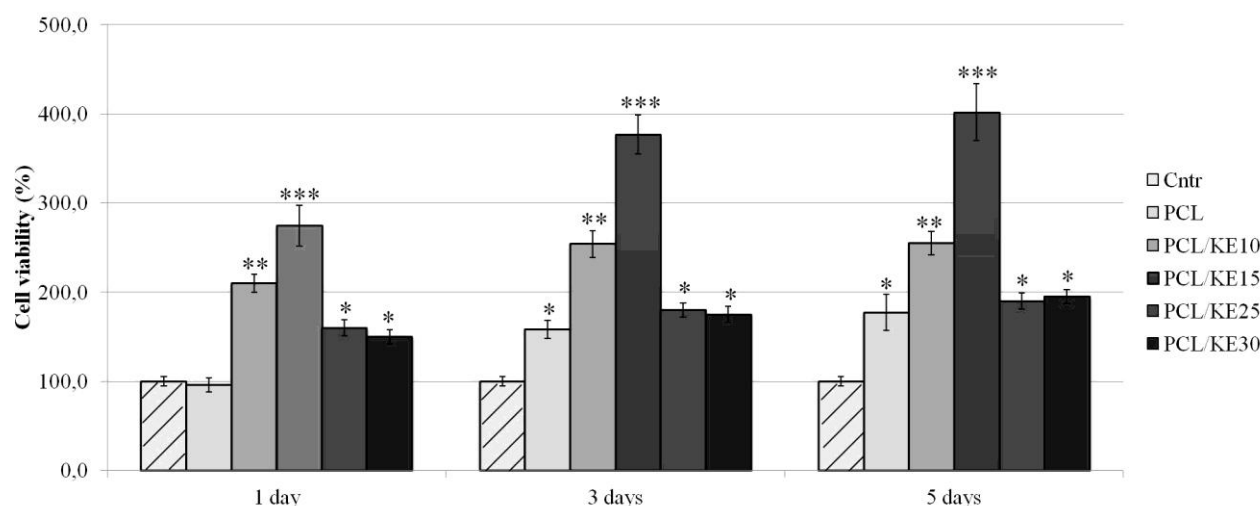


Figure 14. Viability and proliferation of HaCaTs grown on PCL films and PCL/KE films (10%, 15%, 25%, and 30%) measured by MTT assay after 1, 3 and 5 days. Data represent means $\pm$ SD. Significant differences from control were defined as * $p < 0.01$, ** $p < 0.001$ and *** $p < 0.0001$.

The results obtained are consistent with those reported in the literature, which describe the non-cytotoxicity of PCL and PCL/keratin films and scaffolds, as well as their potential to promote cell

growth and proliferation.^{89,108} However, it is interesting to note that, in the present study, PCL/KE films showed a positive effect on cell growth and proliferation starting from the first day of incubation, while previous studies reported a significant increase in cell viability and proliferation in PCL/KE systems only starting from the third day of culture. Furthermore, in previously published studies, pure polyester films and those enriched with keratin have often shown overlapping biological behaviours, suggesting a limited contribution of keratin to cell proliferation.^{89,108} The greater effect observed in the present study may be attributed to the type of keratin used. In particular, the films studied here were prepared using keratin derived from chicken feathers, while in previous works, keratin was mainly obtained from wool or human hair.^{89,108}

It is known that keratins can be classified into α - and β -keratins,¹⁰⁹ which exhibit different structural organizations at the nanoscopic level: β -keratin filaments have a diameter of approximately 3–4 nm,¹¹⁰ while α -keratin filaments have larger diameters, around 7 nm.¹⁰⁹ In this context, wool and human hair are predominantly composed of α -keratin, while feathers contain β -keratin. Based on these structural differences, it can be hypothesized that the β -keratin architecture favors cell adhesion and proliferation more, justifying the early initiation of HaCaT proliferation on PCL/KE films as early as day 1.

In particular, the β -sheet structure of keratin offers a more favorable substrate and greater stability for cell adhesion than the α -conformation.¹¹¹ Furthermore, the three-dimensional organization of β -keratin can facilitate cell migration within the matrix, allowing for more effective colonization of the material and contributing to the formation of new tissue.¹⁰² These aspects are crucial in tissue regeneration processes, in which cells must be able to move, interact, and organize to restore the functionality of damaged tissue. Finally, the higher cell growth rates observed for PCL/KE15 films can be attributed to the presence of a sufficiently high concentration of keratin, which is simultaneously homogeneously incorporated within the polymer matrix. In contrast, although PCL/KE25 and PCL/KE30 films have a higher keratin content by weight, SEM analyses revealed a non-uniform dispersion of the keratin phase, which limits the material's ability to provide effective support for cell growth and proliferation.

III.2 Porous PCL/Keratin Composite Scaffolds: Structural Features, Degradation Behaviour, and Osteogenic Response

This section extends the investigation from two-dimensional keratin/PCL films to three-dimensional porous composite scaffolds developed for bone tissue engineering applications. The PCL used to fabricate the scaffolds was synthesized following the same procedure described previously in the section (III.1.1), ensuring consistent polymer characteristics across the different material systems studied. The discussion addresses the structural characteristics of keratin/PCL scaffolds (PCL/FK), including porosity, pore interconnectivity, and spatial distribution of keratin within the scaffold architecture, along with the influence of keratin incorporation on scaffold degradation behaviour. The biological performance of the three-dimensional constructs is evaluated using MC3T3-E1 pre-osteoblasts as a model cell line, selected to reflect the intended application of these materials as internal scaffolds for bone tissue regeneration. Unlike the two-dimensional keratin/PCL films investigated in the previous section, which were designed for surface-contact applications and therefore assessed using epidermal keratinocytes (HaCaTs), the three-dimensional porous architectures developed here are conceived to operate within a volumetric and mechanically demanding environment. In this context, pre-osteoblasts represent an appropriate cellular model to investigate cell adhesion, cytoskeletal organization, and osteogenic response within an interconnected porous structure. Overall, the results presented in this section elucidate how scaffold architecture and keratin incorporation jointly modulate structure–degradation–bioactivity relationships in keratin/PCL systems, supporting their suitability for applications in bone tissue regeneration.

III.2.1 Keratin extract from chicken feathers by denaturing-reducing method with urea and L-cysteine solution

Keratin extraction was conducted following a protocol based on a reducing agent consisting of 8 M urea and 0.165 M L-cysteine, previously used for wool.¹¹² This combination was chosen for its proven effectiveness in breaking the disulfide bonds and non-covalent interactions responsible for the stability of the fibrous structure of keratin, thus overcoming the chain breaking as in the case of alkaline hydrolysis. Specifically, urea acts as a chaotropic agent, capable of disrupting hydrophobic interactions and intra- and intermolecular hydrogen bonds, while L-cysteine acts as a milder and more selective reducing agent compared to traditionally used reagents, such as β -mercaptoethanol or sodium sulfide.¹¹² Overall, this strategy allows for a controlled disintegration of the keratin matrix, yielding structurally intact protein fractions and limiting amino acid alteration. At the end of the extraction phase, the sample was dialyzed for three days against distilled water, using membranes

with a molecular cutoff of 8 kDa. This step proved essential for eliminating residual urea, L-cysteine, and other low-molecular-weight species, helping to improve the purity and stability of the protein extract.

The extraction yield obtained, 45%, is significantly higher than that previously reported for methods based on alkaline hydrolysis.⁸⁶ SDS-PAGE analysis (Figure 15) highlights the presence of a predominant band around 20 kDa, consistent with the values reported in the literature for keratin subunits present in chicken feathers.¹¹³ The appearance of a single, well-defined band suggests a high degree of molecular homogeneity of the extract and indicates the preservation of the main protein chains, without obvious signs of fragmentation.

Overall, the urea and L-cysteine-based method allows to obtain a protein extract with high yield and purity, free of low molecular weight by-products and characterized by a stable electrophoretic profile, confirming the effectiveness of this approach in the controlled disintegration of the keratin matrix of chicken feathers.

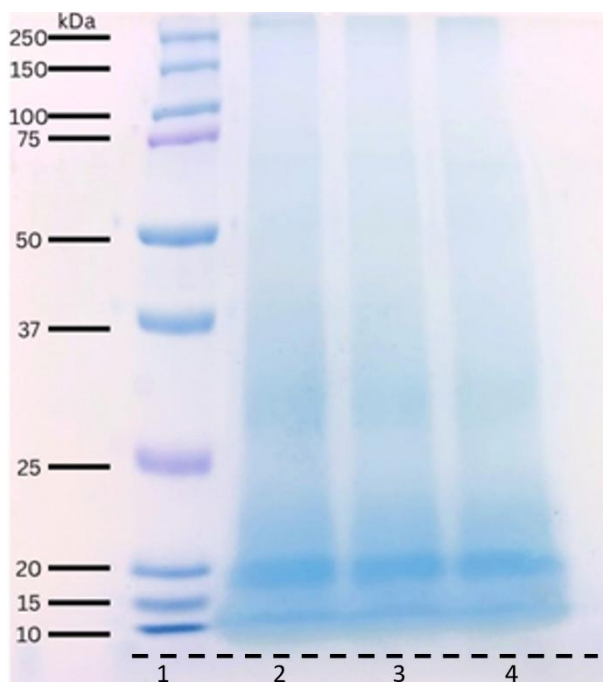


Figure 15. SDS-PAGE profile of keratin extracted from chicken feathers using urea (8 M) and L-cysteine (0.165 M), followed by dialysis (MWCO 8 kDa). Lane 1: molecular weight marker; Lane 2-4: keratin extract.

III.2.2 Preparation of porous PCL and PCL/FK scaffolds for morphological, biological and degradation evaluation studies

Porous PCL and PCL/FK scaffolds were fabricated using a solvent-based particle leaching approach to obtain three-dimensional constructs with interconnected porosity, suitable for cell colonization and tissue ingrowth. The resulting scaffolds were designed to combine a well-defined macroporous architecture with compositional differences resulting from the incorporation of keratin. After processing, the scaffolds were systematically characterized for morphological characteristics and subsequently evaluated for their biological performance and degradation behaviour using relevant *in vitro* assays, as discussed in the following sections.

III.2.3 Scanning electron microscopy (SEM) analysis of porous scaffolds

SEM analysis of the cryogenically fractured surfaces of the PCL and PCL/FK scaffolds highlighted the porous architecture achieved through the salt leaching process (Figure 16). SEM micrograph processing using Fiji/ImageJ software confirmed the presence of large, interconnected macropores with diameters ranging from 250 to 350 μm , consistent with the size of the sieved NaCl granules used as the porous agent.¹¹⁴ These pore sizes fall within the range considered optimal for bone tissue engineering applications, as pores larger than 200 μm favor cell infiltration and vascularization.¹¹⁵

Although both systems exhibit comparable macropores, significant differences in the microstructure of the porous walls were found. Pure PCL scaffolds exhibit smooth, compact, and homogeneous pore surfaces, typical of semicrystalline polyesters obtained by solvent casting and particulate leaching.¹¹⁶ In contrast, PCL/FK scaffolds are characterized by irregular and heterogeneous pore walls, with the evident presence of micropores and discontinuous surface domains. The appearance of this secondary microporosity suggests that the introduction of keratin modifies polymer-solvent interactions and evaporation kinetics during the manufacturing process, a behaviour already observed in other composite systems containing natural polymers, in which hydrophilic domains can induce phase microseparation phenomena and greater pore diversity.^{117,118} The intrinsic biochemical heterogeneity and hydrophilic nature of keratin can indeed favor local variations in evaporation mechanisms and polymer chain packing, contributing to the formation of finer pores.¹¹⁹ Overall, SEM observations indicate that PCL/FK scaffolds exhibit hierarchical porosity, resulting from the combination of macropores generated by the salt porogen and micropores induced by the presence of keratin. It is known that hierarchical porous architectures can improve mass transport, increase the available surface area, and favorably modulate subsequent cell-material interactions.¹²⁰

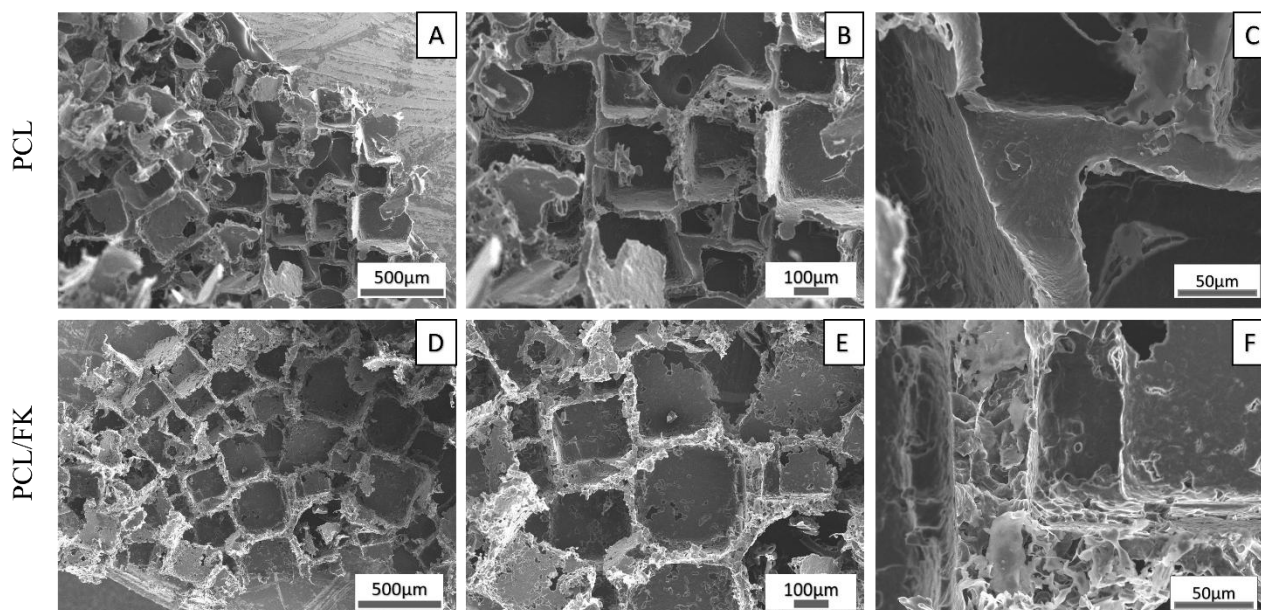


Figure 16 SEM images of cryo-fractured scaffolds prepared by salt leaching. (A-C) PCL scaffolds (D-F) PCL/FK scaffolds. From left to right, images were acquired at magnifications of 50 $\times$, 100 $\times$, and 500 $\times$,

III.2.4 Cell viability and proliferation on MC3T3-E1 cells

LDH release assay was used to assess the cytotoxic response of MC3T3-E1 cells cultured on PCL and PCL/FK scaffolds. Both materials showed no significant cytotoxicity, with cell viability maintained throughout all culture periods up to 14 days (Figure 17).

Cell viability tests confirmed the cytocompatibility of both PCL and PCL/FK scaffold types; however, viability values were consistently higher for PCL/FK scaffolds as early as day 3 of culture, suggesting improved early cell-material interactions. This result is consistent with previous studies demonstrating that integrating keratin into synthetic polymer matrices can increase surface hydrophilicity, likely through the exposure of polar amino acid residues, as well as providing biomimetic peptide sequences of the ECM that promote integrin-mediated adhesion and cell survival.^{86,121} A further increase in viability was observed on day 7, particularly on PCL/FK scaffolds, likely related to keratin's greater capacity to adsorb proteins, an aspect that may facilitate cytoskeletal organization and the formation of focal adhesions, processes essential for cell proliferation.¹²² On day 14, cell viability was further increased on both systems, albeit at a less marked rate. This trend is consistent with a condition of almost complete colonization of the scaffold surface and with the known transition of MC3T3-E1 cells from a predominantly proliferative phase to a phase of osteogenic differentiation.¹²³

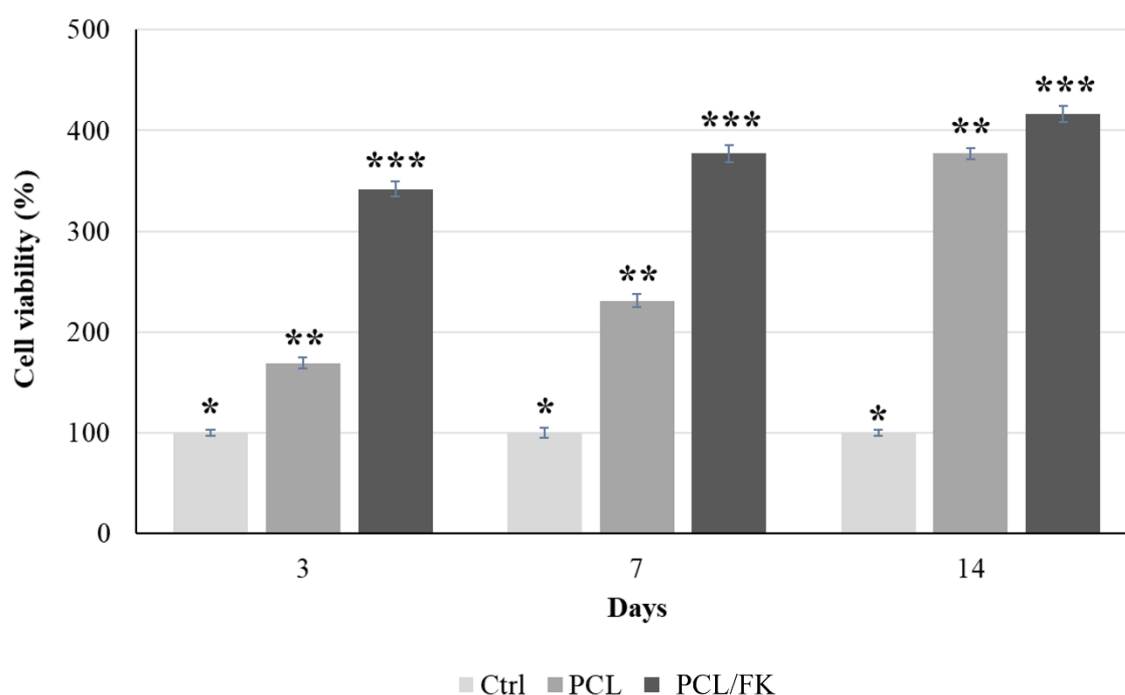


Figure 17. Viability and proliferation of MC3T3-E1 cells grown on PCL and PCL/FK scaffolds measured by LDH assay after 3, 7, and 14 days. Data represent means $\pm$ SD. Significant differences from the control were defined as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

III.2.5 Cell morphology and osteogenic differentiation

Fluorescence images obtained by cell fixation and subsequent staining with DAPI-phalloidin, combined with incubation with an anti-osteocalcin (OCN) antibody, revealed significant differences in cell morphology, cytoskeletal organization, and the degree of osteogenic maturation of MC3T3-E1 cells cultured on scaffolds (Figure 18). DAPI-phalloidin staining allowed visualization of nuclei and F-actin filaments, providing reliable information on the quality of cell adhesion and substrate recognition, considering the central role of actin networks in mechanotransduction processes and the dynamics of focal adhesions.¹²⁴ On both types of scaffolds, cells exhibited an elongated morphology, the presence of well-organized stress fibers, and protrusive structures such as lamellipodia and filopodia, indicative of effective anchoring to the substrate and adequate cytoskeletal tension. This behaviour is consistent with previous studies demonstrating that polymeric scaffolds can support cytoskeletal organization through stable mechanical signals and appropriate surface microarchitecture,¹²¹ and is in line with observations on other porous aliphatic polyesters, in which scaffold rigidity and microstructure favour cell extension, actin organization, and the formation of well-developed stress fibers, indicative of robust cell-substrate adhesion.¹¹⁴ A higher cell density was

observed on PCL/FK scaffolds, consistent with the proliferation results reported in Figure 18 and with previous studies showing that keratin-containing systems can enhance cell adhesion, metabolic activity, and surface colonization.^{82,92} OCN has been used as a late osteogenic marker, since its intracellular expression and subsequent extracellular deposition are indicative of an advanced stage of osteoblastic maturation and the ability to mineralizing.^{123,125} As expected for MC3T3-E1 cells, only weak OCN expression was detected on pure PCL surfaces in the early stages, with an appreciable increase at day 14, consistent with the canonical timing of osteogenic differentiation.¹²⁶

In contrast, cells cultured on PCL/FK scaffolds showed higher levels of OCN as early as day 7, suggesting an accelerated onset of the late differentiation phase.¹²⁶ This early OCN expression may be attributable to different microenvironmental signals, as keratin-derived biochemical motifs promote integrin-mediated adhesion and modulate intracellular signaling pathways key to osteogenic commitment, including FAK, RhoA/ROCK, and transcriptional regulators such as Runx2.¹²⁷ Furthermore, the biochemical functionality of keratin and the increased hydrophilicity of the material may promote ECM-mimetic interactions, known to accelerate osteogenic differentiation. Activation of osteogenic pathways.¹²⁸ At day 14, the PCL/FK scaffolds showed a significantly more intense OCN signal, while cells cultured on pure PCL still showed heterogeneous levels of expression, with the presence of cells in pre-maturation stages. The images in Figure 18 (F,H) also highlight small, round cells in the mitotic phase, observed more frequently on PCL/FK scaffolds at day 7 and on PCL only at day 14. The concurrence of proliferating cells and increasing OCN expression suggests that osteogenic differentiation can proceed without complete suppression of proliferation, a phenomenon already described in bioactive or ECM-mimetic contexts, in which osteogenic progenitors can proliferate and simultaneously activate differentiation programs.¹²⁹ Overall, imaging analyses indicate that PCL/FK scaffolds promote earlier and more pronounced osteogenic maturation compared to pure PCL, likely due to the combination of greater porosity, increased hydrophilicity, and biochemical signals provided by keratin, which together create a microenvironment more favourable for osteoblastic differentiation and extracellular matrix production.

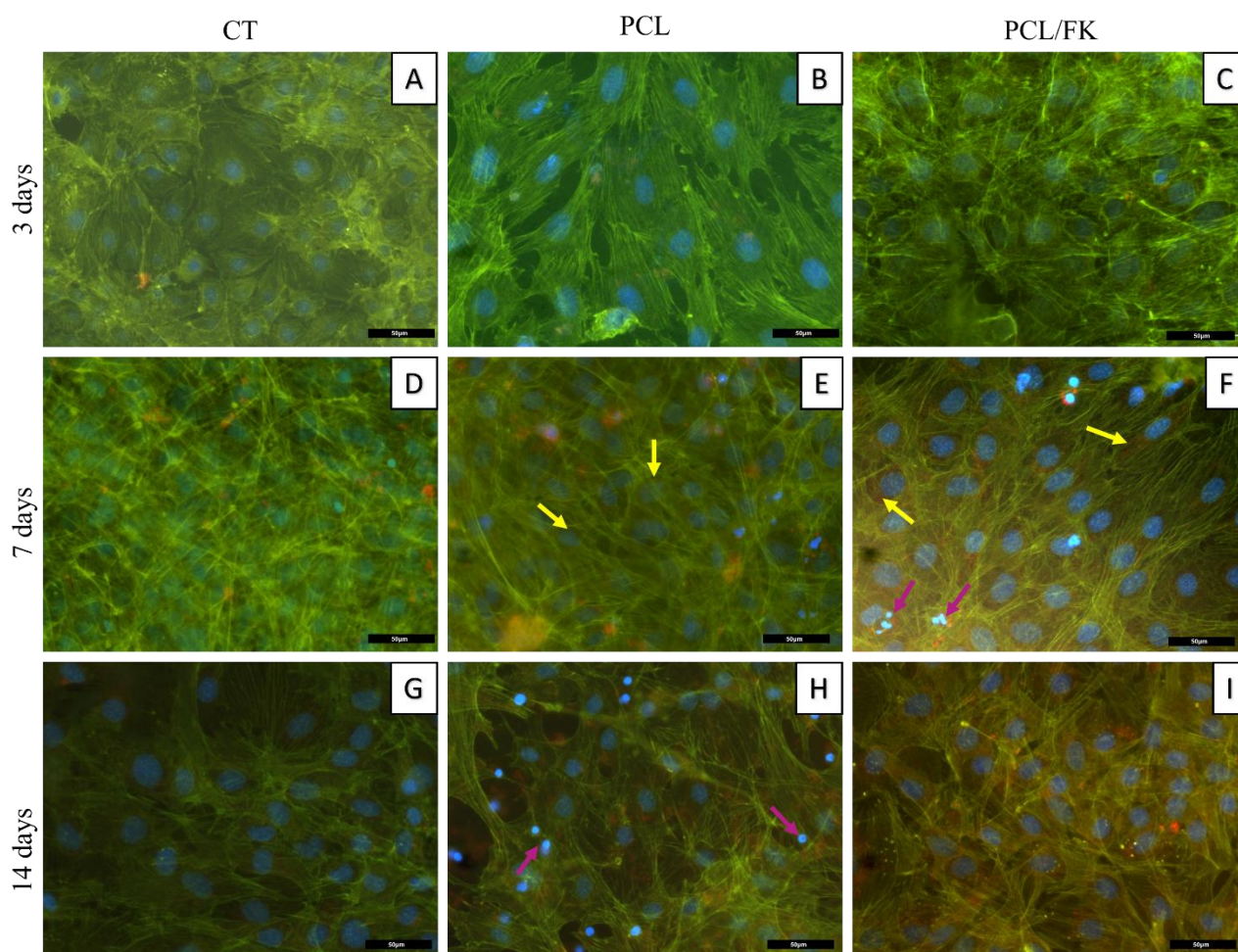


Figure 18. Fluorescence images of MC3T3-E1 pre-osteoblasts cultured on PCL and PCL/FK scaffolds for 3 (A-C), 7 (D-F), and 14 (G-I) days. Actin filaments (green), nuclei (blue), and osteocalcin (OCN, red) are shown to illustrate cytoskeletal organization, cellular distribution, and osteogenic maturation, respectively. Yellow arrows indicate regions where OCN is observable intracellularly, while purple arrows indicate small rounded mitotic cells. Scale bar: 50 μm.

III.2.6 Degradation Behaviour of PCL scaffolds

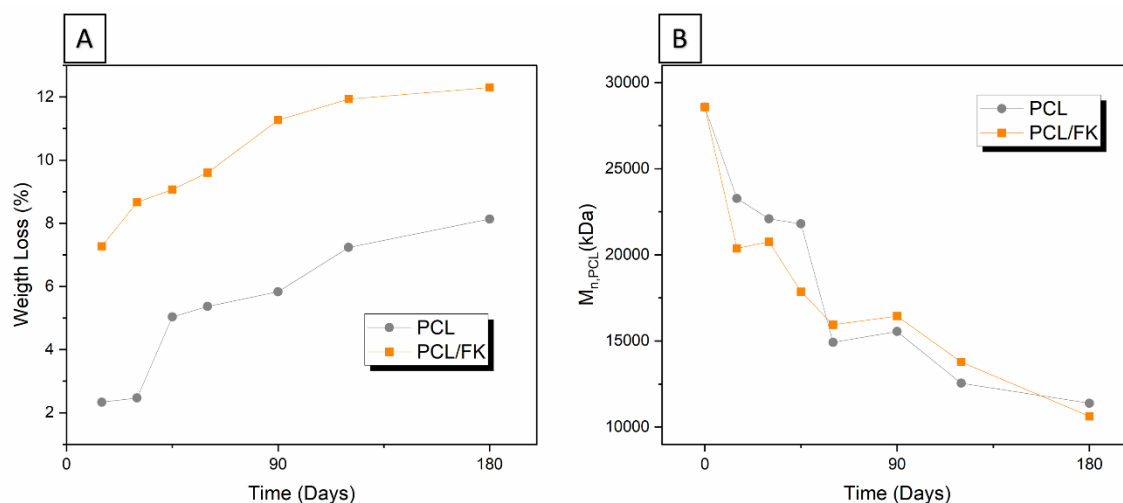


Figure 19. *In vitro* degradation profile of PCL and PCL/FK scaffolds in DPBS buffer (pH 7.4, 37 °C). (A) Percentage weight loss of PCL and PCL/FK scaffolds over time. Data represent mean $\pm$ SD ($n = 3$). (B) Evolution of the number-average molecular weight (M_n) versus time, as determined by GPC on dried samples collected at each time point.

The mass loss profiles of PCL and PCL/FK scaffolds during incubation in DPBS (pH 7.4, 37°C) are shown in Figure 19A. Weight loss was determined by recovering the samples at each time point, rinsing them thoroughly to remove residual PBS, drying them under vacuum to constant weight, and calculating the change in mass from the initial dry weight. At each incubation time, the samples were analyzed by GPC, and the evolution of the number-average molecular weight (M_n) during the degradation process is shown in Figure 19B.

Table 3. Number-average molecular weight (M_n) of PCL and PCL/FK scaffolds at different degradation times after incubation in DPBS (pH 7.4) at 37 °C.

Sample	Degradation Time (days)	M_n (kDa)	$\bar{D}$
PCL	0	28.6	1.9
	15	23.3	1.8
	30	22.1	1.7
	45	21.8	1.6
	60	14.9	2.3
	90	15.5	2.0
	120	12.5	2.4
	180	11.4	2.1
PCL/FK	0	28.6	1.9
	15	20.4	2.1
	30	20.8	1.9
	45	17.9	2.1
	60	15.9	2.4
	90	16.4	2.1
	120	13.8	2.1
	180	10.6	2.3

Both materials exhibit progressive mass loss over time, consistent with a mass hydrolysis mechanism typical of aliphatic polyesters such as PCL.¹³⁰ In the initial phases (up to 60 days), weight loss is limited, a phenomenon attributable to the limited penetration of water into the semicrystalline matrix and the slow hydrolysis of ester bonds.¹³¹ Starting at approximately 90 days, the degradation rate increases more markedly, reaching an overall weight loss of approximately 11–12% after 180 days of incubation. This increase in the later phases is consistent with the progressive accumulation of acidic degradation products, capable of locally accelerating the hydrolysis of esters in PCL-based materials.¹³² Over the entire study period, PCL/FK scaffolds showed higher degradation rates than pure PCL. This behaviour can be attributed to the increased hydrophilicity and water absorption capacity induced by the presence of keratin, which favour the diffusion of water within the material and promote the hydrolytic cleavage of the polymer chains.^{133,134} Furthermore, the composite morphology of PCL/FK scaffolds, characterized by higher overall porosity and more irregular pore walls, leads to an increase in the surface area exposed to the aqueous environment, as also highlighted by SEM analyses. It is reasonable to assume that this increased exposure does not alter the intrinsic

degradation mechanism of PCL, which proceeds predominantly through a terminal "unzipping" process rather than random chain cleavage, but rather allows a larger fraction of polymer chains to participate in the hydrolysis events over time. This results in a more rapid loss of structural integrity, while maintaining an overall gradual degradation profile compatible with tissue engineering applications. The results obtained by GPC (Figure 19B) support this interpretation, showing a continuous reduction in the number-average molecular weight for both materials over the 180-day incubation period, confirming the molecular degradation of PCL. Specifically, the M_n decreases from an initial value of approximately 28×10^3 g/mol to values below 10×10^3 g/mol after 180 days, indicating chain scission along the polymer backbone. The polydispersity index, reported in Table 3, varies in the range between 1.6 and 2.4. The molecular weight reduction kinetics are similar for both systems, suggesting that the addition of keratin does not significantly affect the intrinsic hydrolysis mechanism of PCL, but primarily affects the macroscopic degradation rate through increased hydrophilicity and porosity. Overall, these results are consistent with previous long-term degradation studies on PCL and its composites in aqueous environments, which describe pseudo-first-order hydrolytic kinetics and a transition from an induction phase to an accelerated erosion phase following oligomer accumulation.^{135,136} The behaviour observed for PCL/FK scaffolds confirms that keratin integration allows for effective modulation of water interactions and degradation kinetics, offering a favourable balance between structural stability and biodegradability, a key requirement for scaffolds intended for bone tissue engineering.

III.3 *In situ* polymerization of ϵ -CL in the presence of keratin: synthesis, characterization, and reservoir performance with thyme essential oil

This section describes experimental attempts to promote PCL growth in the presence of keratin during ϵ -CL ROP, aiming to achieve tighter association between the protein and the polyester phase compared to physically mixed systems. Unlike the materials discussed in the previous chapter, which were obtained by post-polymerization mixing of PCL and keratin, the systems studied here were designed to explore the feasibility of directly integrating keratin during the polymerization process.

Initial polymerization trials were conducted using keratin extracted according to the same protocol adopted in the previous sections, without any additional denaturation step, and using $\text{Sn}(\text{Oct})_2$ as a catalyst under conventional ϵ -CL ROP conditions. These attempts proved unsuccessful, resulting in no polymer formation or poorly reproducible results, suggesting that the presence of undenatured keratin interferes with the catalytic activity and/or polymerization mechanism. These preliminary results highlighted the critical role played by both the physicochemical state of keratin and the choice of catalyst in determining the feasibility of *in situ* polymerization.

Based on these observations, a modified strategy was pursued. Keratin was subjected to a denaturation step by treatment with a 0.1 M L-cysteine solution, with the aim of altering its supramolecular organization, thus improving its compatibility with the polymerization environment.

At the same time, the catalytic system was replaced from tin-based catalysts with aluminum-based one. A well-defined aluminum-based catalyst, $[\text{AlL}_2\text{Me}]$, containing two phenoxyimine ligands, previously reported as a highly active and robust initiator for the controlled ROP of cyclic esters.^{137,138}

This choice was motivated not only by their well-documented ability to effectively promote the synthesis of high molecular weight PCL chains, but also by broader sustainability considerations.^{138–}

¹⁴¹ In the context of green polymer chemistry, increasing attention is being paid to catalysts based on Earth-abundant and less toxic elements, and aluminum represents an interesting alternative to traditional systems.¹⁴²

The combination of keratin denaturation and the use of aluminum-based catalysts enabled the effective polymerization of ϵ -CL in the presence of keratin. The results presented in this section therefore document a systematic experimental process that led to the identification of the conditions under which ϵ -CL polymerization can proceed in the presence of keratin. The structural, thermal, and morphological characterizations discussed below are used to evaluate the effects of this *in situ* approach on material properties, providing the basis for evaluating the potential of keratin-assisted polymerization strategies.

The discussion primarily focuses on the fully characterization of the materials and the impact of keratin incorporation on the chemical structure, thermal behaviour, and morphology of the resulting PCL/Denatured Keratin (PCL/DK) materials. Spectroscopic, thermal, and morphological analyses are employed to assess the effects of keratin presence during polymerization and to distinguish materials obtained by *in situ* polymerization from their physically blended counterparts. Particular attention is given to the changes in molecular architecture and phase organization induced by the presence of keratin during polymerization, as these characteristics are expected to influence both material stability and functional performance.

For structural, thermal, and functional evaluation, the synthesized PCL/DK materials were processed into thin films by tape casting. This technique was selected to obtain flat, homogeneous films with controlled thickness, suitable for subsequent characterization and functionalization. Polymer solutions were distributed onto a flat substrate using a calibrated casting bar with a nominal gap of 200 μm , which ensures uniform spreading of the solution across the surface. Following solvent evaporation, this procedure yields self-supporting dry films with reproducible thickness and morphology, minimizing processing-induced heterogeneities and enabling meaningful comparison among different formulations.

In addition to structural and physicochemical characterization, the functionalization of PCL/DK materials with TEO is briefly explored as a proof-of-concept application. This aspect was introduced to evaluate whether the presence of keratin within the polymer matrix, resulting from polymerization carried out in its presence, could impart a reservoir-like behaviour toward low-molecular-weight compounds. To this end, thyme essential oil was selected as a model molecule due to its high volatility combined with marked antioxidant and functional potential. The objective of this investigation is therefore to evaluate whether PCL/DK systems are capable of retaining and stabilizing an intrinsically volatile compound within the polymer matrix, allowing for greater permanence and potentially exploiting its functional properties over time. This evaluation is conceived as an applied extension of the structural characterization.

III.3.1 Keratin Denaturation as a Structural Pre-treatment

Keratin denaturation was introduced as a critical pre-treatment step, aimed at modifying the protein's supramolecular organization to improve its compatibility with the PCL matrix during polymer synthesis and film processing.¹⁴³ Native keratin extracted from poultry feathers is characterized by a highly ordered hierarchical structure, stabilized by extensive intermolecular and intramolecular disulfide bonds, which confer rigidity and promote the formation of compact aggregates.¹⁴⁴ Although

these characteristics are essential for the biological function of keratin, they severely limit its dispersion and processability in polymeric systems. Denaturation with L-cysteine allows for a mild reduction, selectively cleaving the disulfide bonds ($-S-S-$) within the keratin structure. The elevated temperature accelerates the reduction process and facilitates partial unfolding of the polypeptide chains, leading to a partial loss of the protein's native secondary and tertiary organization.¹⁴⁵

Figure 20 shows the SEM images of the morphology of native keratin (NK) and denatured keratin (DK). In particular, DK displays a highly heterogeneous structure, characterized by the coexistence of differently organized domains. At higher magnifications, elongated and cylindrical structures are clearly observed alongside less ordered fragments, highlighting the intrinsic hierarchical organization of keratin and its tendency to form protein aggregates even after partial unfolding.

In contrast, the morphology of NK displays a markedly different structural organization. At low magnifications, native keratin already appears as large, compact lamellar domains, forming continuous, highly organized, plate-like structures. These lamellae dominate along the observed length scales, preventing the resolution of individual fibrillar or cylindrical substructures even at higher magnifications. The absence of discernible discrete elements suggests a strong conservation of the native hierarchical assembly, stabilized by extensive intermolecular interactions and disulfide cross-linking.

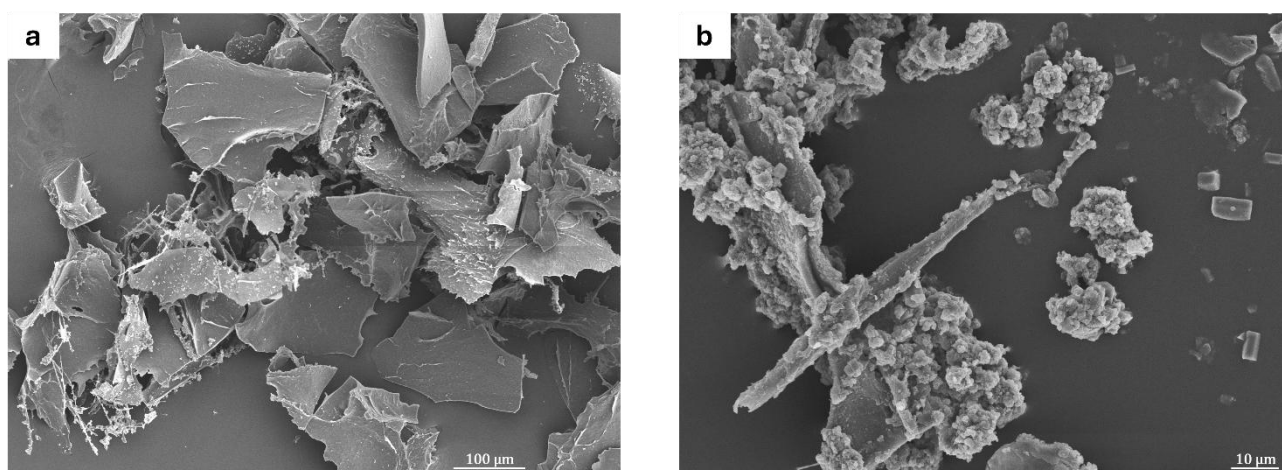


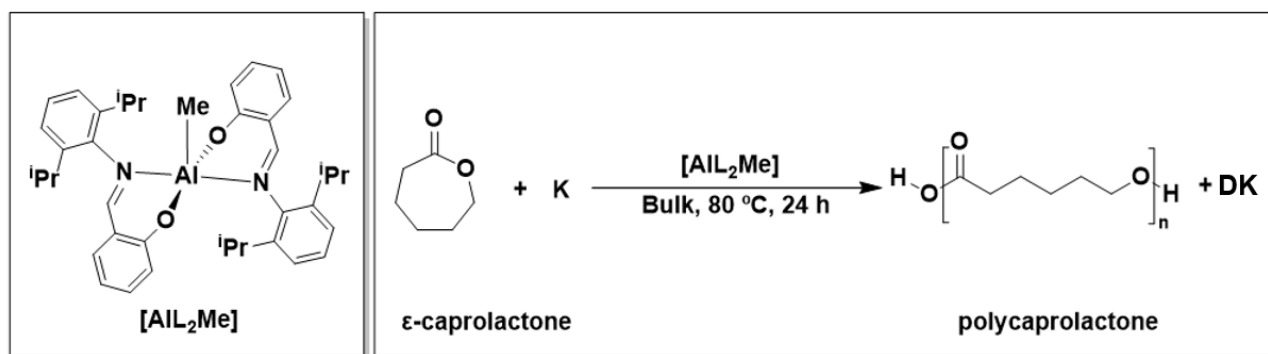
Figure 20. Scanning electron microscopy (SEM) images of native keratin (NK) (a) and denatured keratin (DK) obtained after treatment with L-cysteine (b).

A direct comparison between native and denatured keratin thus reveals a substantial reduction in structural coherence following denaturation treatment. While native keratin maintains a multilayered organization characterized by large-scale lamellar aggregations, denatured keratin exhibits a

fragmented and more open morphology, in which smaller structural units become accessible at higher magnifications, where individual cylindrical fragments and less ordered domains can be resolved.

From a materials design perspective, these structural modifications may play a crucial role in enabling the effective incorporation of keratin into the PCL matrix. The denaturation treatment may reduce the ability of keratin to retain and reorganize water into dense hydrated networks. As a result, the keratin phase may contain a lower amount of bound water, which facilitates drying and handling and, more importantly, limits catalyst deactivation during polymerization. This reduced water content minimizes catalyst poisoning effects and enables more efficient ϵ -CL polymerization, while also promoting a more uniform dispersion of keratin within the growing polymer matrix. This allows the protein phase to effectively contribute to the structural and functional properties of the material without altering the fundamental chemical structure of either component.

III.3.2 Synthesis and structural characterization of PCL/DK polymers



Scheme 2. Synthesis of PCL in presence of denatured keratin (DK) using AlL_2Me as catalyst.

The reactions were conducted using a well-defined aluminum-based catalyst, $[AlL_2Me]$, containing two phenoxyimine ligands (Scheme 2). This class of aluminum complexes is characterized by a pentacoordinated metal center with a flexible but sterically hindered coordination environment, which promotes efficient coordination of cyclic esters and subsequent monomer insertion while limiting unwanted side reactions. This aluminum-phenoxyimine catalyst has been reported to exhibit high activity in the ring-opening polymerization of cyclic esters, achieving rapid monomer conversion and a high turnover frequency (TOF) under relatively mild conditions, as exemplified by the polymerization of *rac*-lactide, which achieved quantitative conversion within hours.¹³⁷

The growing interest in aluminum-based catalytic systems reflects a broader trend in green polymer chemistry toward the use of catalysts derived from abundant metals without compromising catalytic efficiency.^{140,142}

Under these conditions, detailed in Table 4, polymerizations were conducted using 100 equivalents of ϵ -CL at 80°C for 24 hours, in the absence of solvent and without the use of a co-initiator (Scheme 2). At moderate keratin contents (up to 30 wt%), nearly complete monomer conversion was achieved, indicating that the aluminum catalyst maintains high activity even in the presence of the denatured keratin matrix. This behaviour highlights the robustness of the catalytic system and its suitability for *in situ* polymerization approaches involving bioderived components.

However, a decrease in conversion was observed when increasing the keratin content to 50 wt%. This inhibitory effect is likely attributable to mass transfer limitations resulting from the heightened solid content within the reaction medium, as previously reported for heterogeneous polymerization systems.¹⁴⁶ Specifically, the agglomeration of peptide chains creates geometric and dynamic restrictions that impede monomer diffusion towards the catalytic active centers.¹⁴⁷ This phenomenon is analogous to observations reported by previous studies, where an increase in cellulose content by 20% hindered ROP efficiency when using $\text{Sn}(\text{Oct})_2$ as a catalyst¹⁴⁸

The results of GPC demonstrate that the presence of keratin does not impede the synthesis of high molecular weight PCL. However, the keratin content influenced the chain growth, and a decrease in M_w as the amount of keratin increases was observed, while maintaining comparable monomer conversion values, as shown in Figure 21.

The dispersity (D) remained in the range between 2.6 and 3.6 across all samples, indicating a limited control over molecular weight distribution observed during ϵ -CL polymerization in the presence of keratin.

This trend could be attributable to the abundance of functional groups within the keratin structure, which may act as active sites for the initiation of new PCL chains¹⁴⁹ or to the increasing amount of water that could be present in the keratin due to the presence of hydrophilic groups, a behaviour analogous to the effects of moisture entrapped within the fibrillar networks of other biopolymers, such as chitosan.¹⁵⁰

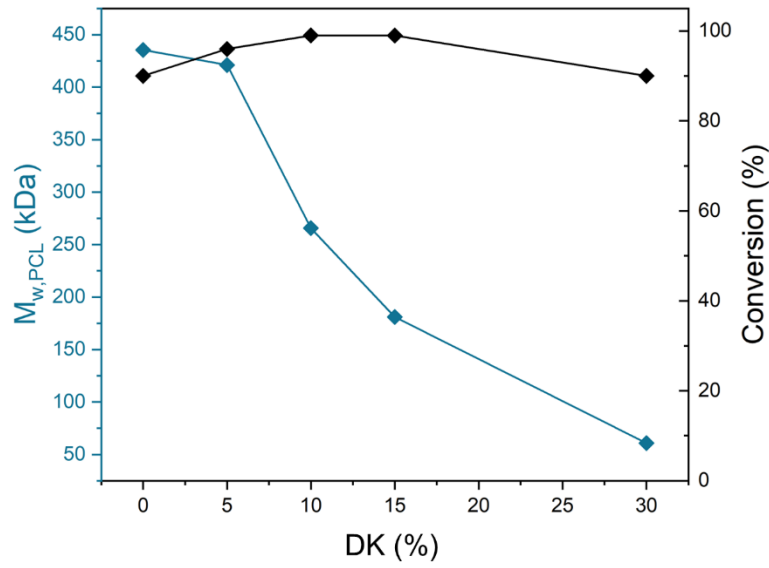


Figure 21. Weight-average molecular weight (M_w) and polymerization conversion trends as a function of keratin content (wt%)

On the other hand, the incorporation of keratin into the final materials was quantified by a mass balance approach combining gravimetric measurements with monomer conversion values determined by ^1H NMR spectroscopy. Monomer conversion (c_{NMR}) was calculated by comparing the integrals of the characteristic signals of ϵ -CL monomer with those of the polymer backbone in the ^1H NMR spectra. The actual keratin content in the dried materials (%Ker) was then calculated according to the following equation: (1)

$$(\%)Ker = \left(\frac{m_{\text{tot}} - (m_{\text{mon}} * c_{\text{NMR}})}{m_{\text{tot}}} \right) * 100$$

where m_{tot} is the final dry mass of the recovered sample, m_{mon} is the initial mass of ϵ -CL used in the synthesis, and C_{NMR} is the polymer conversion determined by ^1H NMR (expressed as a value between 0 and 1). This calculation allows estimation of the actual keratin fraction retained in the final material after polymerization and purification.¹⁵¹

As shown in Table 4, a proportional loss of keratin was recorded as the initial amount increased, likely due to reduced mixing homogeneity at high solid loadings, as the high density of the solid phase compromises initial mixing within the reaction volume. Nevertheless, the keratin content remained close to the initial values in all cases, even after the standard post-treatment required in polymerization processes; specifically, no significant losses were observed after purification with organic solvents.

Table 4. Samples composition, conversion factor, molecular weight and polydispersity index.

Sample	^a DK _i (%)	^b C _(NMR)	^c DK _f (%)	M _n (kDa)	M _w (kDa)	<i>Đ</i>
PCL	0	0.90	0	132	435.4	3.3
PCL/DK ₅	5	0.96	5	156.2	421.2	2.7
PCL/DK ₁₀	10	0.99	8.75	76.5	265.7	3.4
PCL/DK ₁₅	15	0.99	12.39	50.3	181.1	3.6
PCL/DK ₃₀	30	0.90	21.39	23.6	60.8	2.6

Reaction conditions: 24 h, 80°C, [AL2Me]:[ε-CL] 1:100, without solvent. ^aAmount of initial DK. ^bConversion factor of ε-CL obtained through ¹H NMR analysis. ^cAmount of final DK (%) Ker.

With the aim of understanding the role of keratin in ROP, various structural characterization analyses were performed. ¹H NMR spectroscopy confirmed the formation of PCL. As illustrated in Figure 22, the PCL/DK₁₀ aliquot spectrum shows the characteristic polymer signals, which exhibit no significant shifts in the presence of keratin. No keratin-related signals were detected, consistent with its inherent insolubility in the solvent used. Given the high molecular weight of the resulting chains, the signal intensity of the polymer end-groups was not detectable in the spectrum, limiting the information obtainable from solution-state NMR.

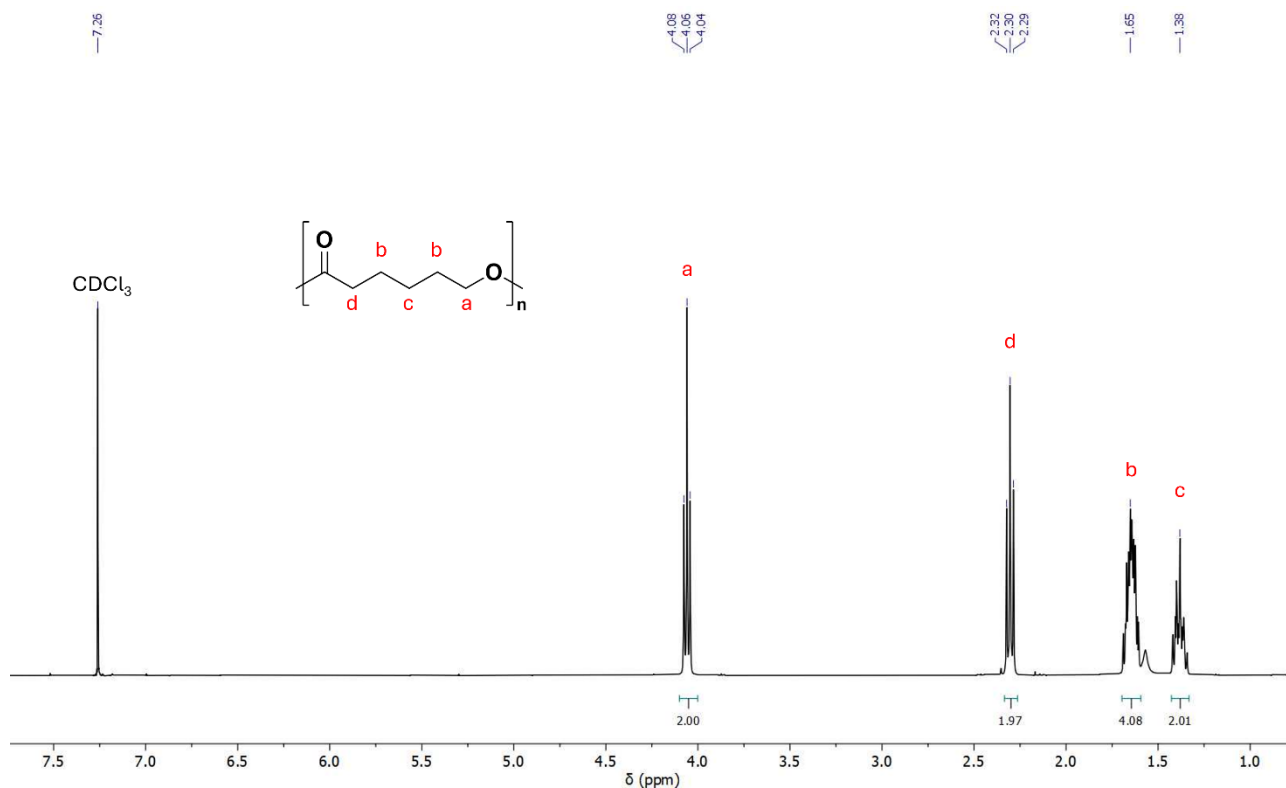


Figure 22. Solution-state ¹H NMR spectrum of the PCL/DK₁₀ sample recorded in CDCl₃.

To overcome these limitations, FTIR spectroscopy was employed to evaluate the absorbance of neat PCL, neat DK, and the synthesized PCL/DK samples, shown in Figure 23a. Initial identification of the spectra revealed the characteristic ester carbonyl stretching of PCL at 1722 cm^{-1} ¹⁵² and the vibrational modes of the protein, namely the Amide I band (C=O stretching) at 1630 cm^{-1} and the Amide II band (C–N stretching and N–H in-plane bending) at 1518 cm^{-1} .¹⁵³ According to previous studies, the position of the Amide I band in neat keratin is indicative of a predominantly β -sheet conformation.¹⁵⁴ All formulations clearly exhibit the characteristic resonance corresponding to the PCL carbonyl group, and a closer inspection of the amide region reveals progressive and composition-dependent modifications. In particular, the intensity of the Amide II signal increases as a function of keratin loading, confirming the effective incorporation of the biopolymer into the polymer matrix.

Given the overlap between the bands of the polymer and those of the protein, spectral subtraction of the pure PCL spectrum was performed to highlight spectral differences attributable to protein, the resulting spectra are shown in Figure 23b. This procedure is used in FTIR as an interpretative tool for the analysis of minority components in complex systems, with a predominantly qualitative value.¹⁵⁵ This approach enabled a clearer evaluation of the amide region, revealing significant shifts in both Amide I and Amide II bands. The Amide II band shifted from 1518 cm^{-1} in neat keratin to 1580 cm^{-1} in the PCL/DK samples, indicating a restriction of N–H vibrational modes. This shift suggests a modification of the local chemical environment of the polypeptide backbone, likely associated with strong interfacial interactions between keratin and the surrounding PCL matrix. Similarly, the Amide I band shifted from 1630 cm^{-1} to 1647 cm^{-1} , suggesting a transition from a β -sheet-dominated structure towards a more α -helical or disordered conformation.¹⁵⁶ This shift reflects alterations in the hydrogen-bonding network of the polypeptide backbone, likely induced by interactions with the surrounding PCL matrix during polymerization.^{157,158}

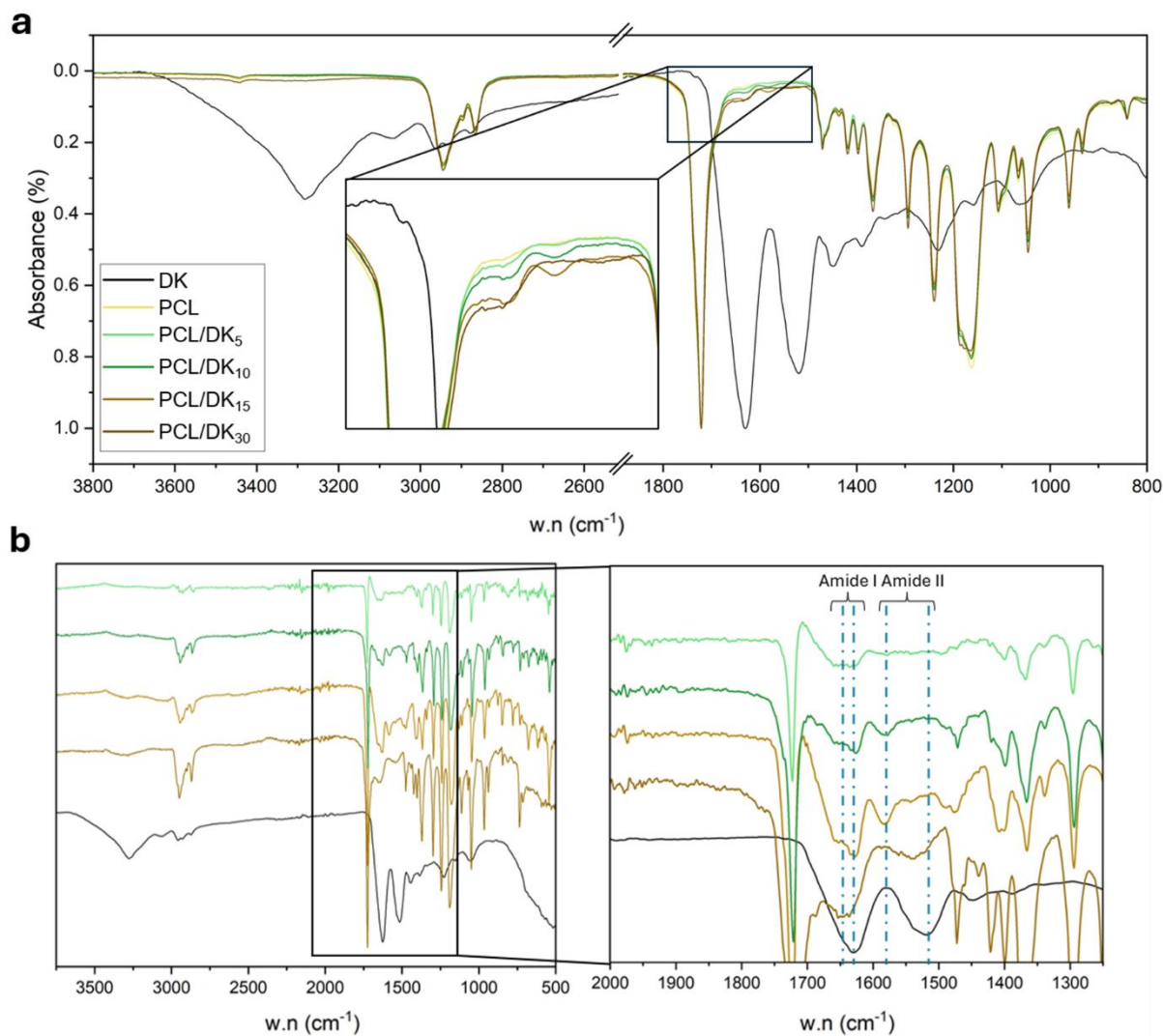


Figure 23. (a) FTIR spectra of denatured keratin (DK), neat PCL and PCL/DK samples with increasing keratin content; (b) FTIR difference spectra obtained after subtraction of the neat PCL spectrum from PCL/DK samples.

Figure 24 shows the further structural characterization completed by MALDI-TOF mass spectrometry, performed on the PCL/DK₁₀ sample. Four main series were identified (Figure 24a), two of them attributable to free PCL chains, one corresponding to the unsaturated-terminated PCL species with the general formula $[(C_6H_{10}O_2)_n] + Na^+$ ($m/z = 114.07n + 23$), and a second series attributed to hydroxyl-terminated PCL chains with the formula $[(H) + (C_6H_{10}O_2)_n + (OH)] + Na^+$ (Figure 24b).

The remaining two identified series correspond to chains initiated by keratin amino acid residues in the PCL/DK₁₀ spectrum (Figure 24c). The new main series, with formula $[(H) + (C_6H_{10}O_2)_n + (R_Arg)] + Na^+$ ($m/z = 1 + 114.07n + 156.2 + 23$), corresponds to PCL chains grown from arginine residues. The identification of these residues is consistent with the presence of type I and type II

microfibrillar protein homologs in keratin, rich in arginine-derived guanidinyll functionalities.¹⁵⁶ A smaller series has been attributed to glycine-initiated PCL chains, with the formula $[(H) + (C_6H_{10}O_2)_n + (R_Gly)] + H^+$ ($m/z = 1 + 114.07n + 75 + 1$). These results provide direct evidence, obtained by mass spectrometry, that keratin could act as a macroinitiator in the ROP of ϵ -CL, supporting the spectroscopic observations.

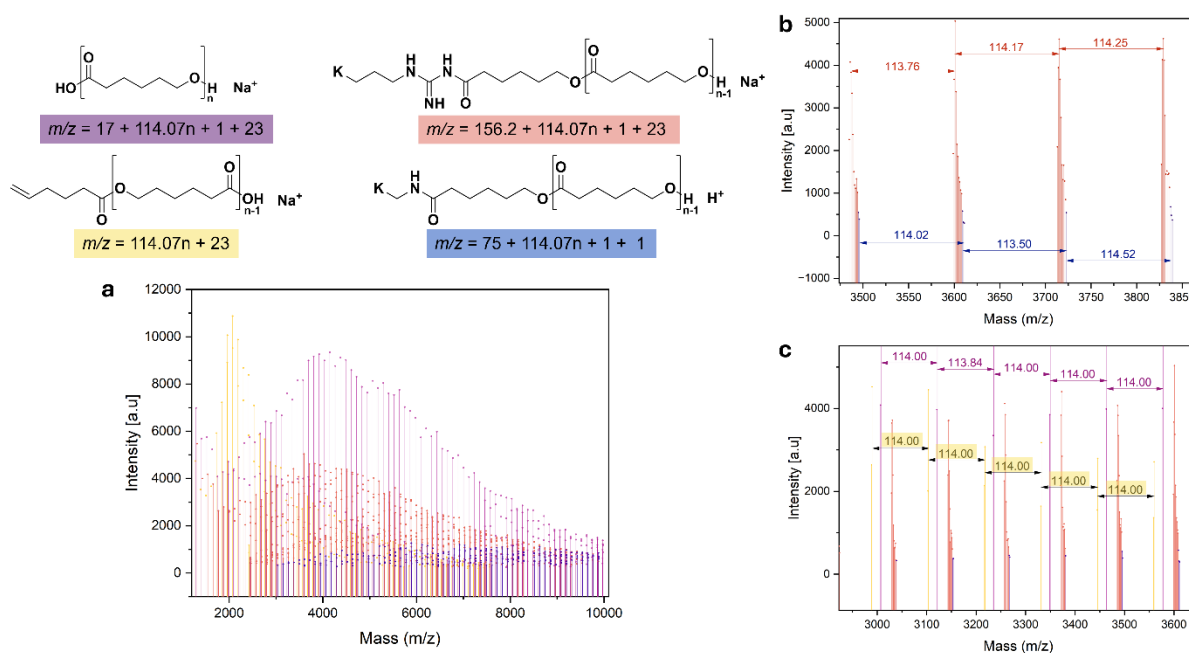


Figure 24. MALDI-TOF mass spectra of PCL/DK₁₀.

As shown in Figure 25, the solid-state ^{13}C CP/MAS NMR spectrum of the PCL/DK₁₀ sample shows characteristic resonances of PCL,¹⁵⁹ including the carbonyl carbon at 173 ppm, the methylene carbon adjacent to the oxygen at 65 ppm, and aliphatic carbons in the 25–35 ppm region. Additionally, weak signals were detected at around 135 ppm, corresponding to aromatic amino acid residues of keratin.¹⁶⁰ Since the spectrum of pure keratin showed a substantial overlap between the carbonyl resonances of keratin and PCL, spectral deconvolution was performed. The carbonyl region was successfully fitted using a Lorentzian component (75%), attributed to the crystalline domains of PCL, and a Gaussian component (25%), associated with the amorphous conformations of keratin.¹⁶¹

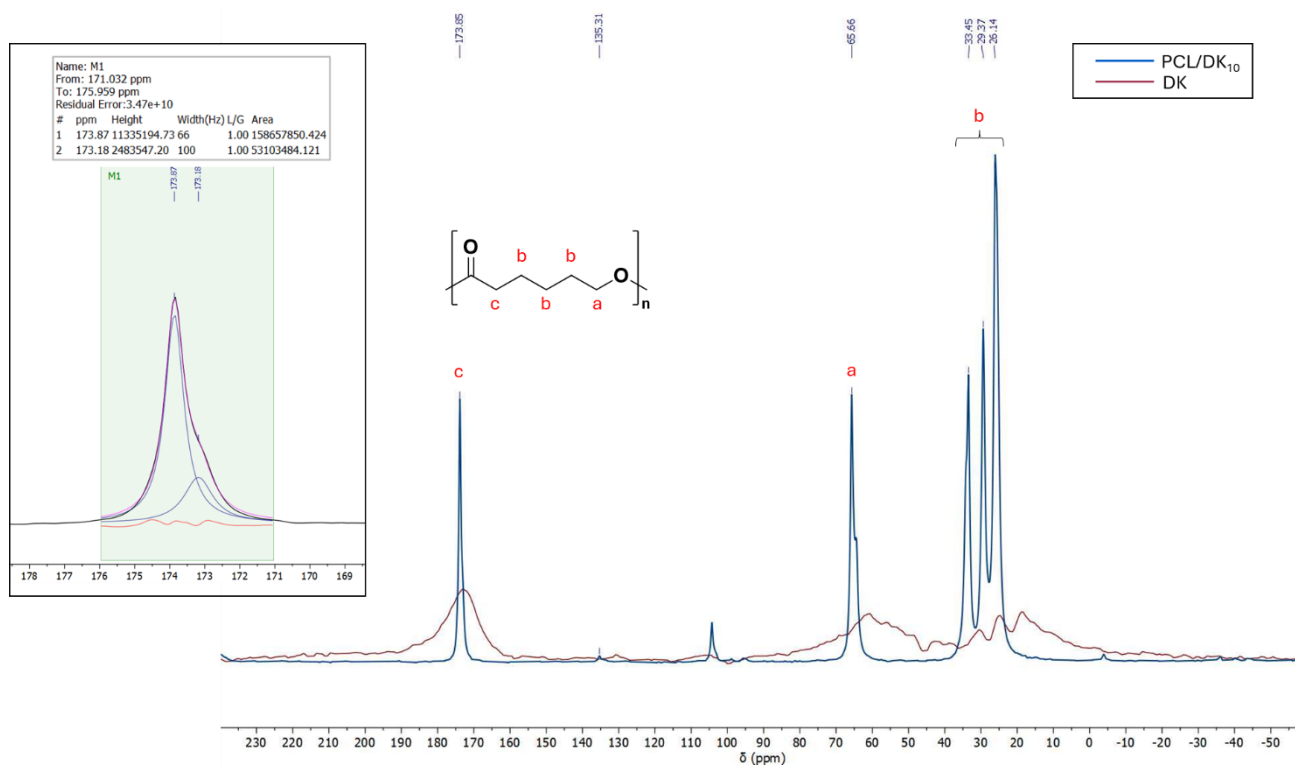


Figure 25. Solid-state ^{13}C CP/MAS NMR spectrum of the DK and PCL/DK₁₀ sample.

In particular, the full width at half maximum (FWHM) of the protein-associated disordered component was significantly broader (≈ 100 Hz) than that of the ordered PCL phase, providing physical evidence of increased conformational heterogeneity and increased molecular mobility.¹⁶² This broadening suggests that keratin incorporation perturbs the local chemical environment of the polymer matrix, a behaviour analogous to that reported for grafting protocols involving natural fibers such as cellulose.¹⁶³

III.3.3 Thermal characterization of PCL/DK polymers

The thermal stability of pure PCL and PCL/DK films was studied by thermogravimetric analysis (TGA) (Figure 26a) in an inert atmosphere, and the corresponding degradation temperatures were extracted from both the weight-loss curves and their first derivatives (DTG) (Figure 26b). The key thermal parameters, including the temperature at 5% weight loss ($T_{5\%}$), the maximum degradation temperature of the PCL phase ($T_{max, \text{PCL}}$), and the degradation temperatures associated with the keratin component ($T_{max, \text{DK}}$), are summarized in Table 5. Pure PCL exhibits a single, well-defined thermal degradation step, with a $T_{5\%}$ of 273°C and a maximum degradation rate centered at approximately 327°C.¹⁶⁴ This behaviour is consistent with the thermal decomposition of the polyester backbone and confirms the high purity and homogeneous nature of the synthesized PCL matrix. . The presence of a single degradation event also indicates the absence of low-molecular-weight impurities or residual monomers. After incorporation of DK, a marked change in the thermal degradation profile is observed. Even at low keratin contents (5 wt%), the $T_{max, \text{PCL}}$ shifts significantly toward higher temperatures, reaching values close to 350-356 °C. This improvement in thermal stability persists for formulations containing up to 30 wt% keratin, with variations within a relatively narrow temperature range (± 6 °C). This increase in degradation temperature suggests a stabilizing effect of the protein phase on the polyester matrix.

Table 5. Samples composition, conversion factor, molecular weight and polydispersity

Sample	T_g (°C)	T_m (°C)	ΔH_m (J g ⁻¹)	χ_c (%)	$T_{5\%}$ (°C)	$T_{max, \text{PCL}}$ (°C)	$T_{max, \text{K}}$ (°C)
PCL	-61.7	55.7	52.7	38.7	273	327	
PCL/DK ₅	-62.4	55.6	49.8	36.5	294	356	305
PCL/DK ₁₀	-62.1	55.8	49.4	36.2	281	353	230
PCL/DK ₁₅	-62.1	56.0	49.3	36.2	250	344	246 - 331
PCL/DK ₃₀	-61.5	50.6	43.7	32.0	303	354	244- 307 - 340

The DTG curves of the PCL/DK samples reveal the presence of multiple degradation events. In samples containing 5–30 wt% keratin, two main degradation peaks can be distinguished: one associated with the PCL-rich phase and a second attributed to the keratin component, which typically occurs at lower temperatures (≈ 230 – 250 °C). In some formulations, additional shoulders or minor peaks are observed, reflecting the complex thermal behaviour of DK and its heterogeneous structure. The effect of keratin on the thermal degradation behaviour of PCL appears to follow a two-stage

trend. At low to moderate keratin contents (up to 15 wt%), the degradation temperature of the PCL phase increases compared to neat PCL, suggesting a stabilizing effect associated with reduced chain mobility and the formation of a more thermally resistant interfacial region between the polymer and the protein phase.¹⁶⁵ In contrast, at higher keratin loadings (30 wt%), this stabilizing effect is no longer observed, and the degradation temperature of the PCL fraction does not further increase, indicating that excessive protein content may disrupt the polymer matrix continuity and limit the effectiveness of interfacial stabilization. Similar stabilization effects have been reported in PCL-based composites containing bio-based fillers, where physical confinement, interfacial interactions, or partial shielding of the polymer chains delay the onset of thermal decomposition.^{166,167} In the current system, DK can act as a thermally stable dispersed phase that limits heat and mass transfer during degradation, thus increasing the apparent thermal resistance of the PCL matrix.

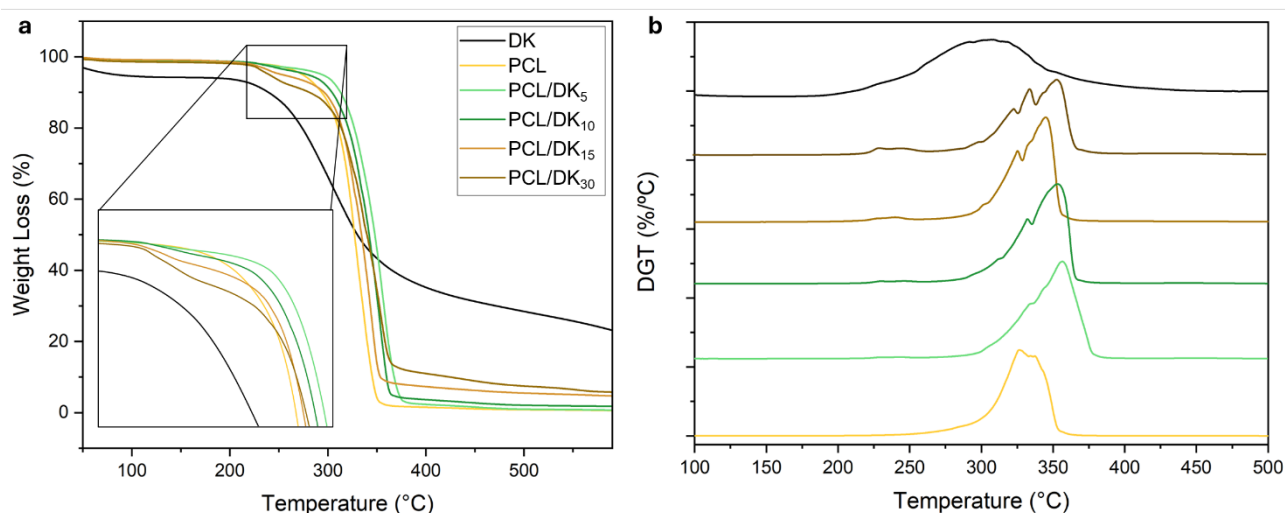


Figure 26. (a) Thermogravimetric (TG) and (b) derivative thermogravimetric (DTG) curves of neat PCL and PCL/DK samples with increasing keratin content.

Overall, the TGA results indicate that the incorporation of DK leads to an improvement in the thermal stability of the PCL phase at low to moderate keratin contents (up to 15 wt%), while no further enhancement is observed at higher loadings (30 wt%), highlighting a concentration-dependent effect. These results highlight the existence of an optimal compositional window in which the protein phase effectively interacts with the polymer matrix, helping to improve thermal resistance without compromising the integrity of the polyester network. From an application perspective, this behaviour is particularly relevant for processing and end-use conditions, where increased thermal stability at

moderate filler loadings can provide an additional advantage for the development of functional PCL-based biocomposites.^{168,169}

The thermal transitions and crystalline behaviour of pure PCL and PCL/DK films were studied by DSC, and the main thermal parameters derived from the second heating scan are reported in Table 4.

Pure PCL with a number-average molecular weight (M_n) of approximately 132 kDa exhibits a glass transition temperature (T_g) of about $-62\text{ }^{\circ}\text{C}$ and a melting endotherm centered at $55.7\text{ }^{\circ}\text{C}$, with a melting enthalpy of 52.7 J g^{-1} corresponding to a degree of crystallinity (χ_c) of 38.7%. These values are fully consistent with literature reports describing the influence of molecular weight on the crystallization behaviour and thermal transitions of PCL^{170,171} After DK incorporation, the glass transition temperature of the materials remains essentially unchanged for all formulations up to 30 wt% protein content. The T_g values are confined within a narrow range (-62.4 to -61.5°C), indicating that the segmental mobility of the amorphous PCL phase is not significantly affected by the presence of keratin. These results indicate that the protein phase does not exert a plasticizing effect on the polyester matrix, nor does it induce significant molecular confinement phenomena in the amorphous regions. This conclusion is supported by the absence of typical behaviours associated with the presence of plasticizers, widely reported in the literature, such as a reduction in the glass transition temperature and an increase in segmental mobility.¹⁷² Similarly, the melting temperature of PCL is only marginally affected by the addition of keratin. For formulations containing up to 30 wt% DK, the T_m values remain close to those of pure PCL ($\approx 56^{\circ}\text{C}$), and no additional melting peaks or polymorphic transitions are detected. This behaviour indicates that the crystalline structure of PCL is preserved and that keratin does not promote the formation of alternative crystalline phases or significantly alter the lamellar organization of the polymer.

Regarding the degree of crystallinity, a careful analysis reveals that χ_c does not undergo significant changes for formulations containing up to 15 wt% keratin, remaining within the experimental uncertainty of the value for pure PCL. Even at 30 wt% DK, the observed reduction in crystallinity is relatively limited and does not reflect a substantial alteration in the semicrystalline character of the polymer matrix. These results indicate that, within this composition range, keratin incorporation does not significantly affect the ability of PCL chains to organize into crystalline domains.

Overall, as show in Figure 27 the DSC results demonstrate that, up to 30 wt% keratin content, protein incorporation does not induce significant changes in the fundamental thermal transitions or crystalline organization of PCL. This result indicates that the presence of keratin, despite being chemically and structurally distinct from polyester, does not alter the intrinsic structural characteristics of PCL chains. In this compositional window, the PCL segments involved in the crystalline domains retain their

ability to fold and pack in a manner comparable to pure polymer, suggesting that the keratin phase primarily influences the material at the mesoscopic or interfacial level rather than at the chain microstructure level. This behaviour indicates that the introduction of the protein phase does not compromise the structural identity of the polyester backbone. Conversely, the preservation of PCL crystallinity up to moderate keratin contents supports the idea that the polymer chains remain structurally comparable to pure PCL, while the protein phase contributes to changes in morphology and functionality without fundamentally altering the polymer crystal architecture.

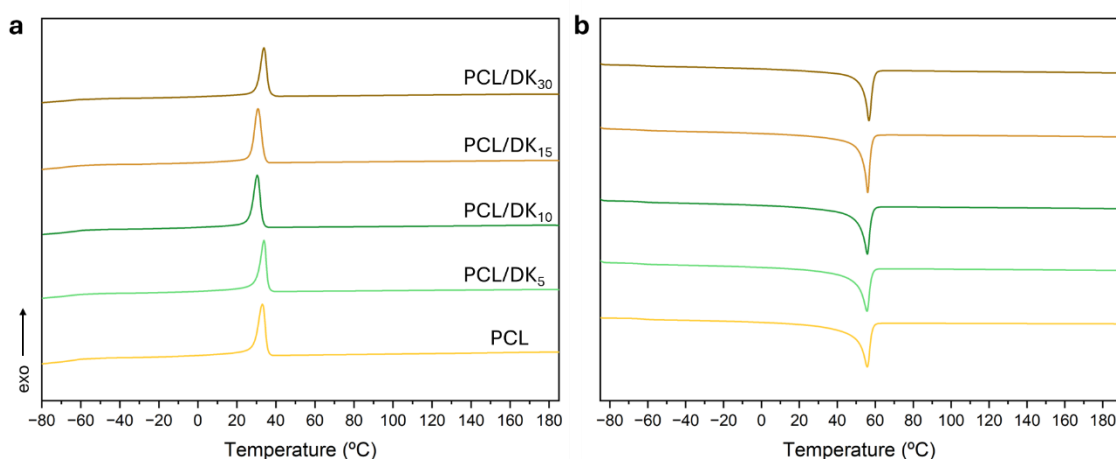


Figure 27. DSC thermograms (a) second heating scan and (b) cooling of neat PCL and PCL/DK films

III.3.4 Morphological characterization of PCL/DK polymers by SEM

The morphology of pure PCL and PCL/DK films was studied by scanning electron microscopy (SEM) on cryo-fractured surfaces to assess the dispersion of the protein phase and the quality of the polymer-filler interface. Representative micrographs are shown in Figure 28.

The cryo-fractured surface of pure PCL appears homogeneous, compact, and relatively smooth, which is consistent with the semicrystalline nature of the polymer and a uniform stress distribution during fracture. No signs of phase separation or structural heterogeneity are observed, confirming the morphological uniformity of the pure matrix. In PCL/DK films, fracture morphology strongly depends on keratin content.

For moderate keratin contents (PCL/DK₅, PCL/DK₁₀, and PCL/DK₁₅), the protein phase appears finely dispersed within the PCL matrix, and keratin fragments are embedded in the elongated PCL domains. In these samples, the polymer matrix appears to deform around the protein inclusions during fracture, suggesting a strong interfacial adhesion between the two phases. Similar to what reported in the literature, a fracture that predominantly involves the matrix rather than the interface is indicative

of an effective interface and adequate load transfer.^{173,174} This morphology suggests effective stress transfer between the polymer matrix and the dispersed protein phase, indicating the development of interfacial interactions during polymer formation rather than post-synthesis mixing, and supports the effectiveness of the *in situ* polymerization approach in promoting homogeneous dispersion.

As discussed in the previous section (III.1.3) of this thesis, physically blended PCL/keratin systems containing 15 wt% keratin exhibit heterogeneous cryofracture surfaces characterized by phase separation.

At higher keratin contents (PCL/DK₃₀), the fracture surface becomes progressively more heterogeneous. Although the protein phase remains well distributed, larger and more irregular domains are observed, indicating the onset of partial aggregation. Nonetheless, the polymer matrix still appears continuous, and no clear evidence of interfacial detachment or void formation is detected. This observation suggests that, despite the increased heterogeneity, the structural integrity of the composite is preserved up to 30 wt% keratin.

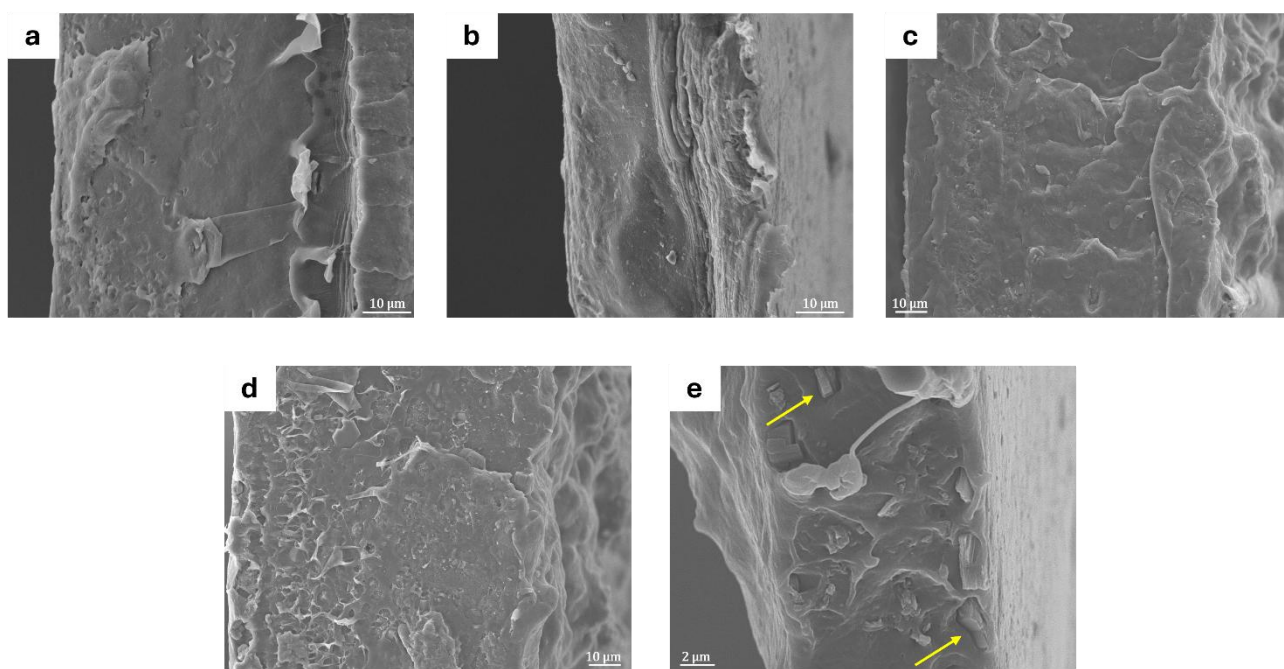


Figure 28. SEM images of cryo-fractured surfaces of (a) neat PCL and (b–e) PCL/DK films containing increasing amounts of denatured keratin ((b) 5%; (c) 10%; (d) 15% and (e) 30 wt%).

Overall, SEM analysis demonstrates that the incorporation of DK significantly alters the material's morphology, introducing a dispersed protein phase and promoting the development of an extended polymer-protein interface. Importantly, up to 15 wt% keratin, the composites exhibit a relatively homogeneous morphology with good interfacial adhesion, supporting the conclusion that the protein

phase alters the material primarily at the mesoscopic level. This morphological evolution is consistent with TGA data, which show increased thermal stability at moderate keratin contents. Together, these results confirm that the *in situ* synthesis strategy allows for effective morphological control without compromising the intrinsic structural organization of the PCL chains.

III.3.5 Preparation of TEO-loaded PCL and PCL/DK thin films by TAPE-casting

PCL and PCL/DK thin films and TEO-enriched films (PCL/TEO and PCL/DK/TEO) were prepared using a solvent-based tape-casting approach. Briefly, ROP-derived polymers, consisting of pure PCL and PCL/DK systems containing different keratin contents (5–30 wt%), were dissolved in chloroform under magnetic stirring at room temperature until complete dissolution. For TEO-enriched samples, thyme essential oil was added to the polymer solution at a concentration of 10% by weight of the total polymer mass. The mixtures were then stirred for an extended period of 3 hours to promote homogeneous dispersion of the essential oil within the polymer solution. Both TEO-free and TEO-containing formulations were processed under the same conditions.

The resulting solutions were cast onto flat glass substrates and spread using a calibrated 200 μm tape-casting bar to control film thickness. Solvent evaporation was allowed to proceed slowly at room temperature under a fume hood for 24 hours, followed by further vacuum drying overnight to remove residual solvent. After drying, the films were carefully peeled from the substrate and stored in a desiccator at room temperature prior to characterization. These thin films were subsequently used to evaluate antioxidant activity by DPPH and TBARS assays, as reported in the next section.

III.3.6 Time-dependent antioxidant behaviour of PCL and PCL/DK films assessed by DPPH and TBARS assays

The antioxidant activity of PCL-based films was initially assessed using the DPPH radical scavenging assay to study the release behaviour of TEO from polymer matrices, which was used as a signal molecule for antioxidant activity.^{175–177} Films without TEO showed negligible DPPH inhibition at all time points studied, regardless of keratin content, confirming that neither PCL nor keratin exhibit intrinsic radical scavenging activity.

In contrast, TEO-containing films showed a clear time-dependent increase in DPPH inhibition (Figure 29a). At day 0, all formulations showed very low scavenging activity, with only slight differences between samples. A statistically significant difference (** $p < 0.01$) was observed between the PCL/TEO control and the PCL/DK₅/TEO formulation, suggesting a slightly higher initial availability

of antioxidant species in the presence of low keratin content. However, no significant differences were detected between the keratin-containing formulations at this early stage, indicating comparable initial release behaviour.

After 1 day of incubation, DPPH inhibition increased for all TEO-containing films. The PCL/DK₁₀/TEO and PCL/DK₁₅/TEO formulations showed significantly greater scavenging activity than the PCL/TEO control (***, $p < 0.001$).

As observed in other systems where keratin is used as a functional reservoir, the effects are strictly time-dependent.^{178–180} Therefore, with longer incubation times (3 and 6 days), the influence of keratin content becomes more pronounced. On day 3, PCL/DK₁₀/TEO and PCL/DK₁₅/TEO films showed significantly greater DPPH inhibition than both the PCL/TEO control and the PCL/DK₅/TEO formulation (***, $p < 0.001$). This time point corresponds to the maximum radical scavenging activity observed for all samples. On day 6, a decrease in overall scavenging activity was observed, consistent with the progressive depletion and subsequent oxidation of active compounds such as TEO; however, the keratin-containing films, particularly those with 10 and 15% keratin by weight, maintained significantly greater antioxidant activity than the keratin-free control (***, $p < 0.001$).

These results indicate that keratin does not act as an antioxidant *per se*, but plays a key role in modulating the release kinetics of TEO. The progressive appearance of statistically significant differences at medium and long incubation times supports a controlled release mechanism, in which keratin delays the diffusion and volatilization of the active compounds, thus prolonging their availability.

The antioxidant efficacy of the films was further evaluated under real-world food storage conditions using fresh chicken meat using the TBARS assay, which quantifies lipid peroxidation during refrigerated storage. As expected, the effects observed in the TBARS assay, expressed in malonic dialdehyde (MDA) (nmol), occurred with a time delay compared to the results of the DPPH assay. While the DPPH assay provides an instantaneous measure of radical scavenging activity, reflecting the immediate availability of antioxidant species released from the films, the TBARS assay captures the cumulative action of these compounds over time in a real lipid matrix.

Fresh chicken meat samples wrapped with TEO-free films showed TBARS values comparable to the keratin-free control at all time points, confirming the limited protective effect of the polymer matrix alone. In contrast, all TEO-containing films significantly reduced lipid oxidation, in agreement with previous studies reporting the effectiveness of thyme essential oil in inhibiting lipid peroxidation in meat-based systems, as assessed by TBARS analysis.¹⁸¹ This protective effect is mainly attributed to the presence of phenolic compounds in TEO, such as thymol, carvacrol, and *p*-cymene, which are

known to play a key role in retarding lipid oxidation through radical scavenging and chain-breaking mechanisms.¹⁸²

Figure 29b shows that at day 0, no statistically significant differences were observed between the TEO-containing samples (ns), indicating similar immediate antioxidant protection regardless of keratin content. After 1 day of storage, samples wrapped with PCL/DK₁₀/TEO and PCL/DK₁₅/TEO films showed lower TBARS values than the PCL/TEO control.

At 3 and 6 days of storage, the effect of keratin became highly significant. All keratin-containing films showed significantly lower TBARS values than the PCL/TEO control (*, **, ***, $p < 0.05$ – 0.01 – 0.001). Notably, at these later time points, the PCL/DK₁₅/TEO formulation showed the best profile in terms of reducing lipid peroxidation, indicating an improvement in oxidative inhibition dependent on keratin content.

The TBARS results strongly corroborate the trends observed in the DPPH assay, as the combined use of free radical scavenging and lipid oxidation inhibition measurements allows for a more comprehensive assessment of antioxidant behaviour in polymer films and food matrices.¹⁸³ While DPPH measurements reflect the immediate release and availability of antioxidant species in solution, TBARS directly demonstrates their effectiveness in suppressing lipid oxidation within a real food matrix over time. The persistence of significantly lower TBARS values over extended storage times indicates that keratin enhances the long-term protective action of thyme essential oil, potentially by regulating its retention and release from the polymer matrix.

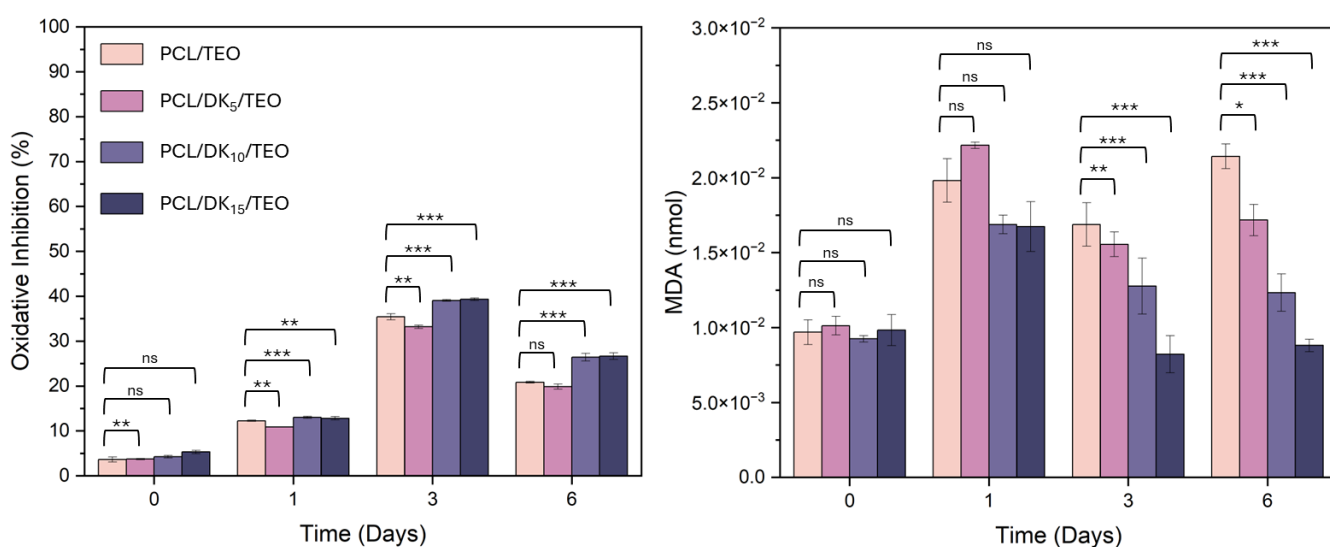


Figure 29. (a) DPPH radical scavenging activity and (b) TBARS values of PCL/DK films loaded with TEO as a function of incubation time. Data are reported as mean $\pm$ SD (n = 3); *p < 0.05, **p < 0.01, ***p < 0.001; ns = not significant.

Overall, keratin contents between 10 and 15 wt% effectively enhance the persistence and efficacy of TEO, supporting the hypothesis of the role of denatured keratin as a functional reserve phase capable of imparting controlled release behaviour to PCL-based biodegradable films.

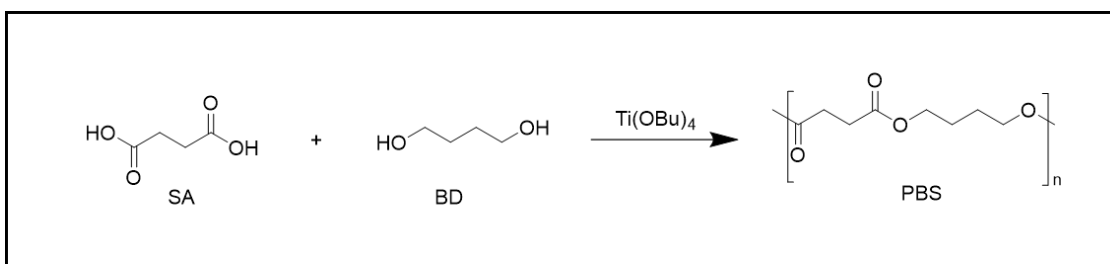
III.4 Keratin integration strategies in polycondensation reactions for poly(butylene succinate)

This section discusses the properties of PBS-based and unsaturated-PBS (u-PBS) materials synthesized in the presence of keratin via polycondensation, with the aim of evaluating how keratin incorporation during stepwise polymerization influences the behaviour of the resulting material.

The discussion focuses on the correlation of compositional and structural changes with the macroscopic properties of the material rather than its molecular architecture. Particular attention is paid to the combined effect of keratin content and the modification of the backbone structure through the incorporation of *cis*-2-butene-1,4-diol, with the aim of identifying systematic trends and property modulations resulting from these two design variables. The results presented in this section aim to assess whether the incorporation of keratin during polycondensation can effectively modulate the properties of PBS-based materials, even in the absence of direct evidence of chemical bonding, and to evaluate the feasibility of extending keratin integration strategies across different polymerization routes.

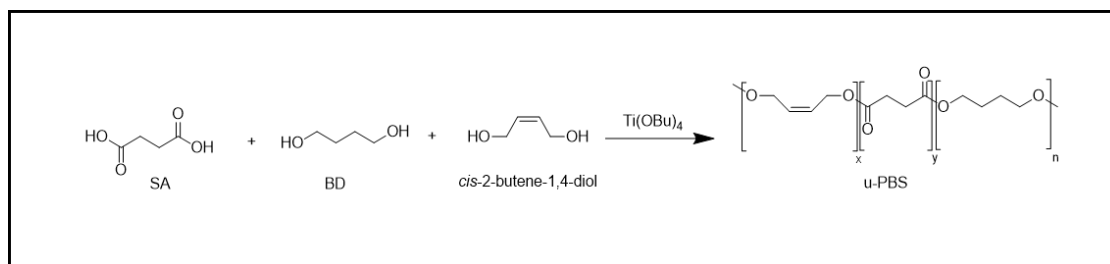
III.4.1 PBS synthesis, NMR characterization and molecular weight analysis

The synthesis of poly(butylene succinate) (PBS) was carried out via polycondensation of succinic acid (SA) and 1,4-butanediol (BD), following a procedure adapted from literature reports,^{184,185} as schematically illustrated in Scheme 3



Scheme 3. Reaction scheme for the synthesis of poly(butylenesuccinate) (PBS)

In parallel, a structural modification was made to PBS to introduce unsaturations into the polymer backbone (Scheme 4). To this end, part of the 1,4-butanediol was replaced with *cis*-2-butene-1,4-diol (10% molar ratio).



Scheme 4. Reaction scheme for the synthesis of unsaturated poly(butlenesuccinate) (u-PBS)

PBS and unsaturated PBS (u-PBS)-based materials were successfully synthesized via melt polycondensation. All reactions were catalyzed by titanium(IV) butoxide ($\text{Ti}(\text{OBu})_4$, 0.2 mol % relative to SA), a commonly used catalyst for aliphatic polyester polycondensation, ensuring effective esterification and polycondensation under melt conditions. Keratin, obtained using the extraction and processing protocol described in Section III.1.2, was introduced during polymerization at different weight fractions (1–2 wt %) to evaluate its influence on chain formation and final material properties. For keratin-containing systems, the maximum reaction temperature was limited to 190 °C in order to prevent degradation of feather-derived keratin, whose main thermal decomposition step occurs at approximately 220 °C.¹⁸⁶

In contrast, keratin-free PBS and u-PBS systems were processed at higher temperatures, up to 230 °C, as reported in Table 6, while all other reaction parameters were kept comparable. Under these conditions, polymer formation was achieved for all formulations, yielding PBS and u-PBS-based materials with measurable molecular weights, as reported in Table 6. Although the presence of keratin leads to a reduction in number-average molecular weight compared to the corresponding keratin-free polymers, the attainment of polymer chains with significant molecular weights confirms that $\text{Ti}(\text{OBu})_4$ remains catalytically active even in the presence of the biopolymer phase.

The chemical structure of the pure polymers and copolymers was confirmed by ^1H NMR spectroscopy (Figure 30a-b). Pure PBS (Figure 30a) displayed characteristic resonances associated with the succinate and butylene repeating units. In particular, the signal at δ 2.62 ppm (a) was assigned to the methylene protons of the succinic acid units, while the resonances at δ 4.11 ppm (b) and δ 1.70 ppm (c) correspond to the methylene groups of the 1,4-butanediol (BD) segments. In the case of unsaturated PBS (u-PBS, Figure 30b), the ^1H NMR spectrum showed additional signals at δ 5.73 ppm (e) and δ 4.70 ppm (d), which were attributed to the vinylic CH and CH_2 protons of the *cis*-2-butene-1,4-diol units, respectively. The presence of these resonances confirms the successful incorporation of unsaturated moieties into the polymer backbone. As observed for PBS, the signals at δ 2.62 ppm, δ 4.11 ppm, and δ 1.70 ppm were also present in the u-PBS spectrum and were assigned to the succinic

acid and BD repeating units, indicating preservation of the main polyester backbone structure upon copolymerization.

The solution-state ^1H NMR spectra of keratin-containing samples were qualitatively similar to those of the corresponding keratin-free polymers, indicating that the presence of keratin during polycondensation does not prevent the formation of polyester chains. No keratin-related signals were detected in the ^1H NMR spectra of the composite systems. This behaviour is attributed to the insolubility of keratin in the organic solvents used for NMR sample preparation, which selectively dissolve the polyester phase. Consequently, solution-state NMR reflects exclusively the structure of the soluble polymer component, while the presence of keratin cannot be directly observed using this technique.

To further investigate the solid materials, solid-state NMR analysis was performed. Figure 30c reports the solid-state NMR spectrum of the neat polymeric systems, while Figure 30d shows the spectra of keratin-containing materials together with that of neat keratin. The solid-state NMR spectra of the polymeric samples exhibit signals consistent with the expected polyester backbone, confirming that the polymer chains retain a coherent and well-defined structure in the solid state. Importantly, no additional distinct resonances attributable to keratin were detected in the spectra of the composite materials. This result is consistent with the low keratin content introduced during polymerization and with the dominance of the polyester phase in the overall spectral response. Even under solid-state conditions, where insolubility effects are overcome, the contribution of keratin remains below the detection threshold or spectrally overlapped with the polymer matrix.

Overall, the combined solution-state and solid-state NMR analyses confirm that polyester synthesis proceeds efficiently in the presence of keratin and that the resulting materials exhibit polymer chain structures comparable to those of the neat systems. While NMR does not provide direct evidence of specific molecular-level interactions between keratin and the polyester chains in these systems, it robustly demonstrates that keratin incorporation does not disrupt chain formation or the structural integrity of the polymer backbone.

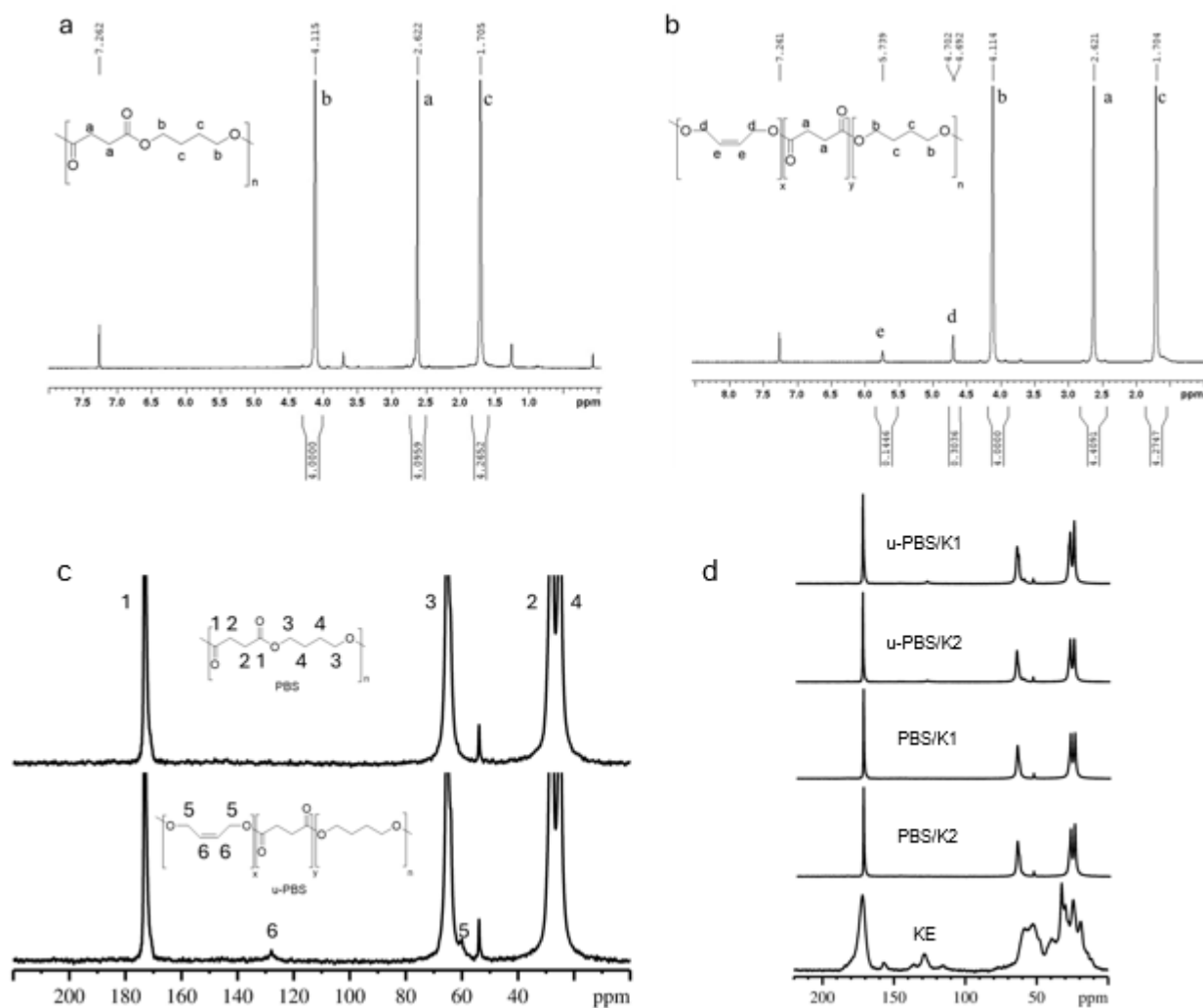


Figure 30. ^1H NMR spectra of (a) PBS and (b) u-PBS recorded in CDCl_3 , solid-state NMR of (c) PBS and u-PBS and (d) their keratin-containing formulations and pure keratin spectra.

The molecular weights of the synthesized materials were determined by size-exclusion chromatography (SEC) after dissolving the samples in chloroform. As shown in Table 6, neat PBS exhibited number-average molecular weights in the range of approximately 37 kDa, while u-PBS displayed slightly lower values. The incorporation of keratin led to a reduction in molecular weight for both PBS and u-PBS-based systems; however, polymer chains with measurable molecular weights were obtained in all cases, indicating that keratin does not inhibit the polycondensation process.

In PBS-based systems, the presence of keratin (PBS/K) resulted in M_n values typically in the range of 8–10 kDa, accompanied by broader molecular weight distributions. In contrast, keratin-containing u-PBS materials maintained relatively higher molecular weights, reaching up to approximately 16 kDa at moderate keratin contents. This behaviour suggests that backbone modification through *cis*-2-butene-1,4-diol incorporation may partially mitigate the molecular weight reduction induced by

keratin, potentially by affecting melt viscosity, chain mobility, or reaction kinetics during polycondensation.

In addition to molecular weight, the polydispersity index ($\bar{D}$) provides insight into the breadth of the molecular weight distributions. As reported in Table 6, neat PBS and u-PBS samples exhibited relatively narrow distributions, with $\bar{D}$ values around 1.8, typical of melt polycondensation processes under controlled conditions. In contrast, keratin-containing systems showed higher $\bar{D}$ values, ranging from approximately 2.0 to 2.4, indicating increased heterogeneity in chain length distribution. This broadening can be attributed to the increased complexity of the reaction environment in the presence of keratin, which may introduce local variations in reactivity and chain growth without preventing overall polymer formation.¹⁸⁷

In addition, the synthesis yields reported in Table 6 indicate that the polycondensation process proceeds efficiently both in the absence and in the presence of keratin. Neat PBS and u-PBS samples exhibited high yields, typically above 95%, confirming the robustness of the polymerization protocol. Although a slight decrease in yield was observed for keratin-containing systems, particularly at higher protein loadings, the values remained consistently above 90% in all cases. This modest reduction can be reasonably attributed to processing-related losses and to the increased complexity of the reaction mixture, rather than to inhibition of polymer formation.¹⁸⁷ Overall, the yield data further support the conclusion that keratin incorporation during polycondensation does not prevent effective polymer synthesis.

Table 6 Composition, reaction conditions, and molecular weight data of PBS and u-PBS-based polymers.

Run/ Samples name	Keratin (%) wt/wt	BD/ <i>cis</i> -2- butene-1,4-diol (molar ratio) in product	T(°C)	M _n (kDa)	<i>D</i>	Yield (%)
1. PBS	-/-	-/-	170-230	37, 1	1.8	96%
2. u-PBS	-/-	93/7	170-230	29.3	1.8	95%
3. PBS/K1	1%	-/-	170-190	10,7	2.4	91%
4. u-PBS/K1	1%	94/6	170-190	10,5	2.0	91%
5. PBS/K2	2%	-/-	170-190	8,4	2.3	93%
8. u-PBS/K2	2%	92/8	170-190	16,1	1.5	90%

Overall, the combined ¹H NMR and GPC results indicate that keratin can be incorporated during PBS polycondensation without suppressing polymer chain formation.

III.4.2 FTIR analysis of PBS and u-PBS-based systems with keratin

Fourier transform infrared (FTIR) spectroscopy was employed to investigate the chemical structure of PBS and u-PBS-based materials. Figure 31 reports the FTIR spectra of the investigated systems, organized to facilitate a clear comparison between polymer families and compositions: panel (a) shows neat PBS and PBS-based materials containing 1 and 2 wt% keratin together with the FTIR spectrum of feather-derived keratin (KE) used for polymer synthesis; panel (b) reports neat u-PBS and the corresponding keratin-containing formulations, again including the spectrum of KE as a reference; while panel (c) compares the spectra of neat PBS and u-PBS.

In all samples, the spectra are dominated by absorption bands characteristic of aliphatic polyesters, indicating preservation of the PBS backbone regardless of unsaturation or keratin incorporation. In particular, the strong absorption band centred at approximately 1711–1713 cm⁻¹, attributed to the stretching vibration of the ester carbonyl groups, is observed with comparable position and shape in both PBS and u-PBS-based materials (Figure 31a–c).¹⁸⁸

This confirms that ester bond formation proceeds efficiently during polycondensation and that no chemical degradation or significant alteration of the polyester backbone occurs in the presence of keratin.

Comparison of pure PBS and pure u-PBS (Figure 31a) reveals a faint but detectable shoulder at approximately 1680 cm^{-1} in the u-PBS spectra, which can reasonably be attributed to the presence of C=C functionalities introduced by the unsaturated diol, as already established by ^1H NMR spectroscopy.

The effect of keratin incorporation is best assessed by comparing PBS-based materials within the same polymer family (Figure 31b) and, separately, u-PBS-based materials (Figure 31c). In both cases, the FTIR spectrum of pure keratin (KE) provides a direct reference for identifying protein-related contributions. In PBS-based samples, the addition of keratin leads to modest changes in the regions associated with protein functional groups, particularly within the Amide I ($1650\text{--}1700\text{ cm}^{-1}$) and Amide II ($1600\text{--}1450\text{ cm}^{-1}$) regions. In the Amide I region, particularly at higher keratin contents, such as 2 wt%, a slight increase in absorbance is observed following keratin incorporation, consistent with the position of the Amide I band in the spectrum of pure keratin.¹⁵³ However, due to the strong overlap with the intense ester carbonyl band of PBS, a quantitative assessment or clear separation of the protein contribution is not possible.

However, no significant changes are observed in the C=C-related feature at approximately 1680 cm^{-1} (Figure 31b), indicating that no interaction at the molecular level occurred between the unsaturated u-PBS materials and keratin. Importantly, the lack of systematic peak shifts in this region suggests that keratin-polyester interactions remain predominantly physical and do not involve the formation of new covalent bonds detectable by FTIR spectroscopy.

In the fingerprint region ($1300\text{--}1000\text{ cm}^{-1}$), which includes the C–O–C stretching vibrations of the ester groups, all samples show nearly identical spectral features (Figure 31a,b). Similarly, the C–H stretching region ($3100\text{--}2800\text{ cm}^{-1}$) shows comparable profiles across all compositions, with only slight variations in relative intensity attributable to differences in polymer composition and keratin content. These observations further confirm that the fundamental chemical structure of the polyester backbone is preserved following keratin incorporation and/or unsaturation introduced by *cis*-2-butene-1,4-diol.

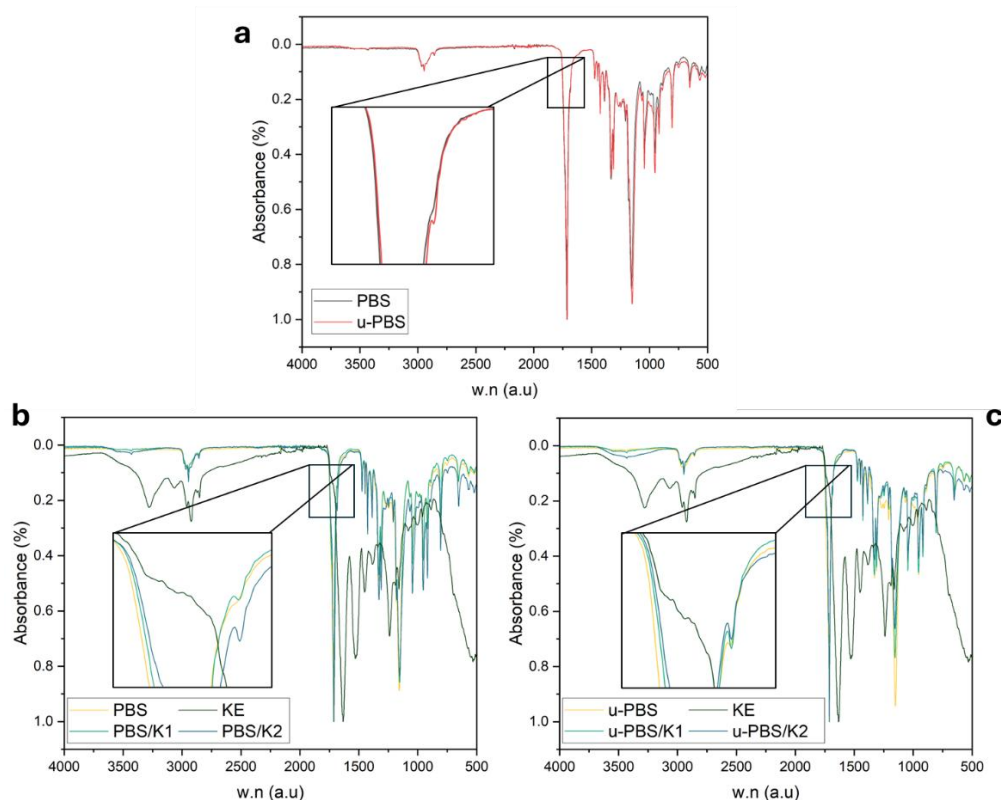


Figure 31. FTIR spectra of PBS and u-PBS-based materials and KE. (a) Comparison between the FTIR spectra of neat PBS and neat u-PBS. (b) FTIR spectra of neat PBS and PBS-based materials containing 1 and 2 wt% KE, reported together with the spectrum of KE as a reference (c). FTIR spectra of neat PBS and u-PBS-based materials containing 1 and 2 wt% KE, reported together with the spectrum of KE.

Overall, FTIR analysis indicates that unsaturation of the PBS backbone and keratin incorporation primarily affect the materials at a structural and organizational level rather than through the formation of new chemical bonds. These results are fully consistent with NMR, SEC, and morphological analyses and support the role of keratin as a structural modifier influencing chain organization and material properties without altering the fundamental chemical structure of PBS-based systems.

III.4.3 Rheological analysis assessment

Rheological analysis in the oscillatory regime, conducted using frequency sweeps (Figure 32), allows us to evaluate the combined effect of the chemical structure of the polymer backbone and the presence of keratin on the viscoelastic behaviour of PBS and u-PBS-based systems. Complex viscosity (η^*) is a parameter sensitive to entanglement density, chain mobility, and the possible presence of structural heterogeneities introduced during polycondensation.

Pure PBS exhibits a nearly constant complex viscosity across the entire frequency range investigated, demonstrating quasi-Newtonian behaviour. This behaviour is typical of semicrystalline aliphatic polyesters with relatively high molecular weights and well-developed entanglement distributions, as widely reported in the literature for PBS obtained by bulk polycondensation.^{189,190} This response suggests a relatively stable melt structure, in which the chain dynamics is dominated by viscous contributions and does not exhibit marked shear thinning phenomena in the frequency range considered.¹⁹¹ In contrast, u-PBS exhibits a clear dependence of complex viscosity on frequency, with a progressive decrease in η^* as ω increases. This shear-thinning behaviour can be attributed to the introduction of structural irregularities along the polymer chain due to the presence of unsaturated units derived from *cis*-2-butene-1,4-diol, as already observed in other unsaturated polyester resin systems in the literature.¹⁹² The literature reports that the introduction of unsaturations or structurally asymmetric comonomers in aliphatic polyesters reduces chain packing efficiency and entanglement density, favouring greater segmental mobility under dynamic stress.¹⁹³ Consequently, u-PBS exhibits a more frequency-sensitive rheological response than linear PBS.

The introduction of keratin during polycondensation could further modify the rheological behaviour of the systems. Overall, all samples containing keratin show a reduction in complex viscosity compared to pure polymers, indicating that the presence of the biopolymer interferes with the continuity of the polymer matrix and the formation of an efficient entanglement network. This effect is particularly evident in u-PBS/K systems, which exhibit the lowest η^* values across the entire frequency range analyzed. The combination of a chemically irregular backbone with unsaturations and the protein phase therefore appears to amplify the reduction in melt viscosity, suggesting a synergistic effect on chain mobility.

In PBS/K systems, however, a dependence of complex viscosity on keratin content is observed. In particular, PBS/K2, containing a greater amount of keratin than PBS/K1, shows higher viscosity values, especially at low frequencies. This behaviour can be interpreted as the effect of a higher concentration of protein phase, which introduces additional physical constraints and contributes to an increase in flow resistance. In the literature, similar behaviours have been observed in polymer systems loaded with biopolymers or natural fillers, where low concentrations favour a plasticizing or network-thinning effect, while higher concentrations can lead to the formation of a more interconnected structure or aggregation phenomena that increase melt viscosity.¹⁹⁴

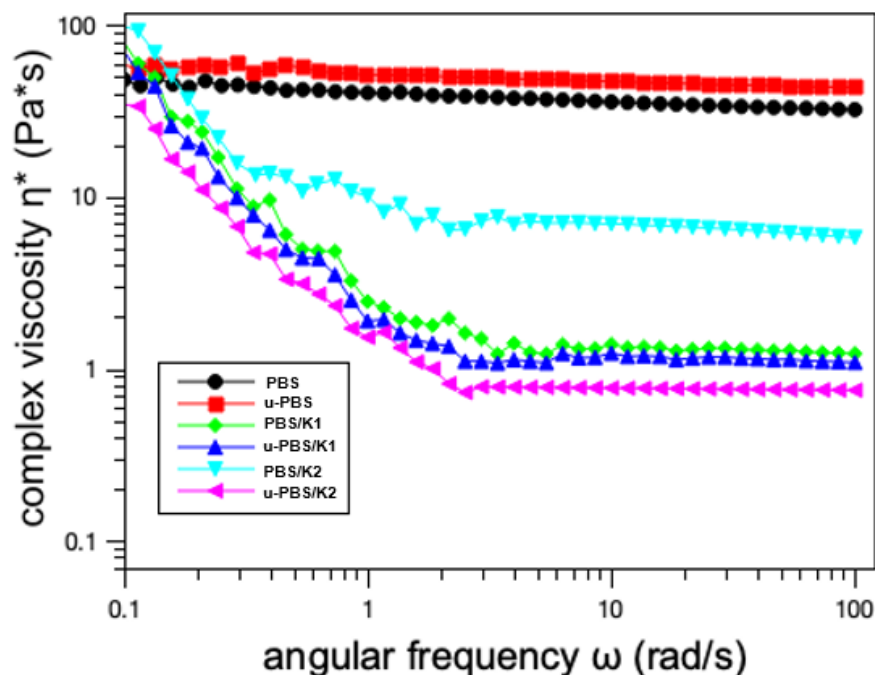


Figure 32. Frequency sweep results showing the complex viscosity (η^*) as a function of angular frequency (ω) for PBS, u-PBS, and keratin-containing systems, measured at 140 °C under oscillatory shear.

Overall, the rheological results indicate that keratin acts as a structural modifier rather than a rigid reinforcement, influencing the dynamics of the polymer chains as a function of both its concentration and the chemical structure of the polyester backbone. The combined effect of chain and keratin unsaturations leads to systems characterized by increased mobility and a more pronounced viscoelastic response, characteristics that are consistent with the molecular weight data.

III.4.4 Surface morphology and wettability of PBS and u-PBS-based films

Films of all the samples were prepared by solvent casting to obtain flat, homogeneous surfaces suitable for both scanning electron microscopy (SEM) observation and contact angle measurements. Samples containing PBS, u-PBS, and keratin were dissolved in chloroform, and the resulting solutions were cast onto flat substrates. Solvent evaporation was allowed to proceed under controlled conditions at room temperature, resulting in the formation of continuous films with uniform thickness.

Contact angle measurements were performed on the as-cast film surfaces, allowing for a direct assessment of surface wettability. Conversely, for SEM analysis, solvent-cast films were subjected to cryogenic fracture in liquid nitrogen to expose the internal morphology and cross-sectional characteristics of the materials while minimizing plastic deformation. This approach allows for

reliable observation of phase organization and fracture behaviour without introducing artifacts associated with mechanical shear.

The solvent casting approach was chosen to minimize processing-induced surface roughness and ensure comparable surface characteristics across the different formulations. Overall, this preparation strategy allows surface morphology and wettability to primarily reflect the material's intrinsic composition and phase organization, rather than processing history effects. The resulting films were therefore considered suitable for evaluating both morphological characteristics by SEM and surface wettability by static water contact angle measurements, as discussed in this section.

The images reported in Figure 32 show representative static water contact angle measurements for PBS, u-PBS, and the corresponding keratin-containing formulations (PBS/K and u-PBS/K systems), providing insight into the surface wettability and hydrophobic character of the materials. Contact angle analysis is widely used to probe surface composition and molecular organization in polymeric systems, as it is highly sensitive to the chemical nature and orientation of functional groups present in the outermost surface layers.^{195,196}

Figure 33 shows neat PBS exhibits a contact angle of approximately 53°, indicative of a moderately hydrophobic surface, in good agreement with values reported in the literature for aliphatic polyesters such as PBS and PBSA, where ester functionalities and methylene-rich backbones lead to intermediate wettability.¹⁹⁷ The u-PBS sample shows a slightly higher contact angle (≈59°), suggesting a modest increase in surface hydrophobicity upon introduction of unsaturated diol units. Similar effects have been reported for chemically modified PBS systems, in which backbone irregularities and reduced crystallinity promote surface enrichment of hydrophobic segments.¹⁹⁸

Upon incorporation of keratin, a further but limited increase in contact angle is observed across all formulations. Keratin-containing samples display contact angle values ranging from approximately 68° to 73°, regardless of whether the polymer backbone is saturated (PBS) or unsaturated (u-PBS). This moderate increase indicates a partial reduction in surface wettability, while preserving an overall intermediate hydrophobic character.⁹¹ Comparable trends have been observed in polyester-based systems containing proteins or other natural polymers, where the addition of biopolymeric phases induces only subtle changes in surface energy due to incomplete surface coverage and competing polar–nonpolar interactions.¹⁹⁹

The relatively small magnitude of the contact angle increase suggests that keratin does not dominate the surface chemistry of the films. Instead, keratin is likely embedded within the polymer matrix, with only partial exposure of hydrophobic amino acid residues at the surface. At the same time, the presence of polar peptide groups and residual functional moieties may counterbalance hydrophobic contributions, preventing a transition toward highly hydrophobic behaviour. Similar surface responses have been reported for keratin-containing polymer blends and composites, where protein incorporation modifies wettability without inducing strong surface segregation.^{200,201}

Moreover, the absence of pronounced differences among keratin-containing samples indicates that neither keratin content in the investigated range nor backbone unsaturation leads to significant phase separation or preferential migration of keratin to the surface. This behaviour is consistent with reports on PBS-based composites, where surface properties reflect a balance between polymer matrix chemistry and filler distribution rather than sharp compositional gradients at the interface.^{202,203}

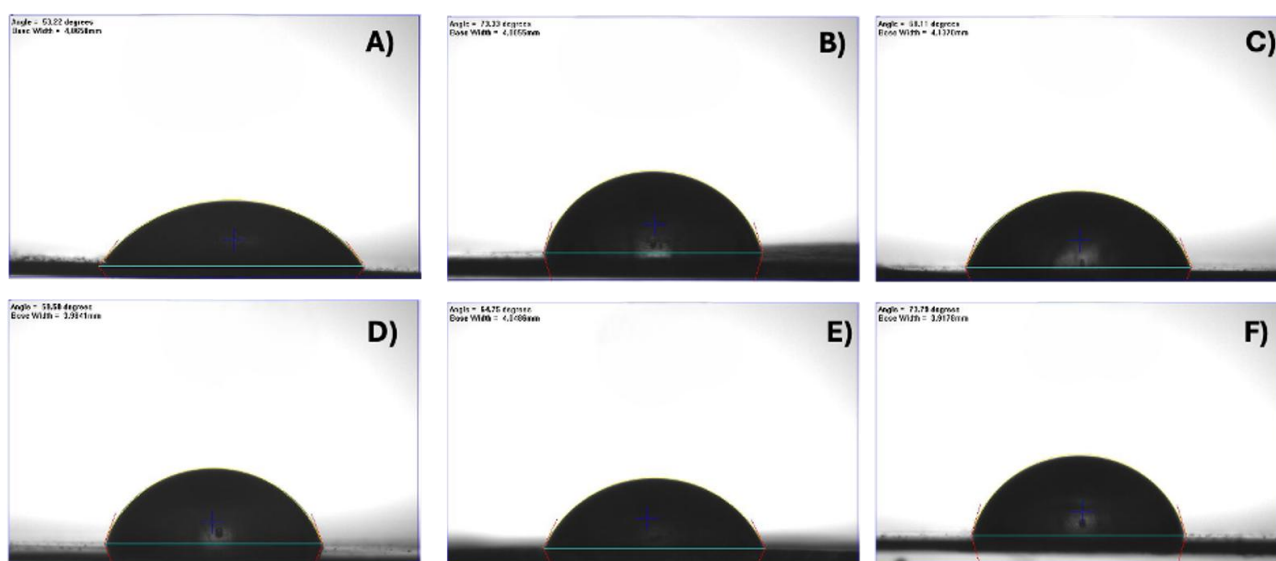


Figure 33. Static water contact angle images of (A) PBS, (B) PBS/K1, (C) PBS/K2, (D) u-PBS, (E) u-PBS/K1, and (F) u-PBS/K2 films.

Overall, contact angle measurements demonstrate that both keratin incorporation and unsaturated diol modification slightly modulate the surface wettability of PBS-based materials without inducing drastic changes in hydrophobicity, an effect that is reasonable and expected given the low concentrations of keratin added. This moderate tuning of surface properties is consistent with the bulk structural, thermal, and rheological observations discussed in previous sections and may be advantageous for applications requiring controlled interfacial interactions rather than extreme wetting behaviour.²⁰⁴

Figure 34 shows SEM micrographs of cryogenically fractured surfaces of PBS, u-PBS, and the corresponding keratin-containing films, allowing us to evaluate the effect of keratin incorporation and backbone modification on the internal morphology of the solvent-cast materials. Cryofracturing was used to expose the bulk morphology while minimizing plastic deformation, thus providing insights into phase organization and fracture behaviour.

Pure PBS exhibits a relatively compact and homogeneous fracture surface, characterized by a continuous morphology with limited evidence of heterogeneity. The fracture profile appears smooth and dense, consistent with the semicrystalline nature of PBS and the relatively high viscosity and crystallinity observed for this system.²⁰⁵ This morphology is indicative of a compact polymer matrix with limited internal discontinuities. After keratin incorporation, significant changes in fracture morphology are observed. PBS/K1 exhibits a more structured and rougher surface, with the appearance of lamellar features and localized deformation patterns. These features suggest that the presence of keratin interferes with the uniformity of the PBS matrix, introducing heterogeneities that alter crack propagation during fracture. At higher keratin content (PBS/K2), this effect becomes more pronounced, with an increasingly irregular morphology and a greater density of surface features. This evolution is consistent with the rheological results, which indicate composition-dependent changes in melt structure and chain mobility.²⁰⁶

The u-PBS sample already exhibits a more heterogeneous fracture surface than pure PBS, reflecting the reduced crystallinity and increased chain irregularity induced by main chain unsaturation. The fracture morphology of u-PBS appears less compact, with more pronounced deformation characteristics, consistent with its lower viscosity and greater shear thinning behaviour observed in rheological measurements. After keratin incorporation, the effect of backbone unsaturation and the presence of proteins becomes strongly composition dependent. In u-PBS/K1, the fracture surface exhibits a higher density of cracks and sharp discontinuities, with evident fracture planes and localized brittleness characteristics. This morphology suggests a reduced stress-dissipating capacity of the matrix, likely associated with a lower molecular weight and reduced chain entanglement, as also indicated by the SEC and rheological results. In contrast, u-PBS/K2 exhibits a relatively more homogeneous and compact fracture surface, with fewer sharp cracks and a smoother fracture profile. Despite the increased keratin content, the morphology appears less fragmented than that observed for u-PBS/K1, likely related to the higher molecular weight recorded for u-PBS/K2, suggesting a partial redistribution of stress during fracture. This behaviour may be attributed to a more uniform dispersion

of the keratin phase or the formation of a more interconnected microstructure at higher keratin loading, which limits crack propagation.²⁰⁷

It is important to note that no large-scale phase separations or macroscopic keratin aggregates were observed in any of the samples, indicating that keratin is reasonably well distributed within the polymer matrix at the concentrations studied. Instead, the morphological changes appear to result from microstructural heterogeneity rather than complete phase segregation. Similar fracture morphologies have been reported in PBS-based and polyester systems containing natural polymers or proteins, where the biopolymer phase acts as a structural modifier that alters fracture propagation and internal organization without forming distinct, continuous domains.²⁰⁸

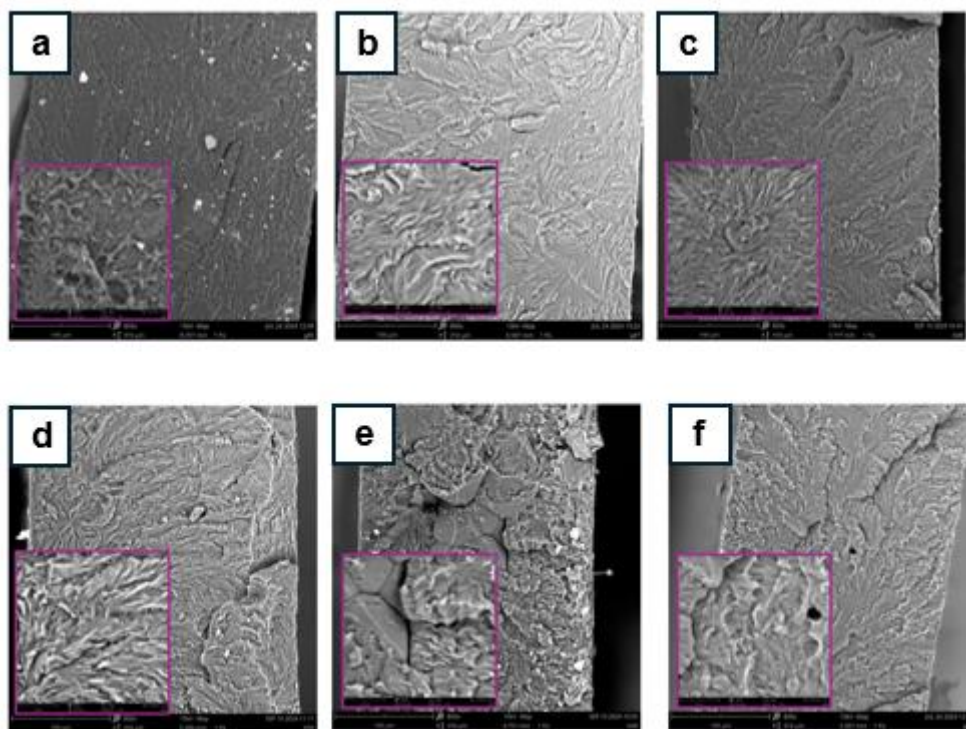


Figure 34 SEM micrographs of cryogenically fractured surfaces of solvent-cast films: (a) PBS, (b) PBS/K1, (c) PBS/K2, (d) u-PBS, (e) u-PBS/K1, and (f) u-PBS/K2.

Overall, SEM analysis confirms that both keratin incorporation and backbone unsaturation significantly influence the internal morphology of PBS-based films. The progressive increase in surface roughness and structural heterogeneity observed in keratin-containing samples provides a morphological basis for the changes in rheological behaviour discussed in the previous sections, supporting the interpretation of keratin as a modifier of matrix organization rather than a discrete reinforcing phase.

Chapter IV

EXPERIMENTAL SECTION

IV.1.1 Synthesis of PCL

The PCL was synthesized by ROP of ϵ -CL in the presence of $\text{Sn}(\text{Oct})_2$ as catalyst under nitrogen atmosphere. In detail, 1 mL of ϵ -CL (9.0 mmol) were introduced in a three neck flask and then 2.2 mL of a 9 mM solution of $\text{Sn}(\text{Oct})_2$ in toluene was added. The resulting mixture was placed in an oil bath at 130 °C and stirred for 24 h. After that time, the mixture was cooled to room temperature, dissolved in dichloromethane, and the resulting mixture was poured in hexane. The precipitated polymer was recovered by filtration and dried *in vacuo*. Yield: 98%.

^1H NMR (400 MHz, CDCl_3): δ = 4.04 (t, 2H, $-(\text{CH}_2)_4\text{CH}_2\text{O}-$), 2.29 (t, 2H, $-\text{CH}_2-\text{C}(\text{O})\text{O}-$), 1.65–1.61 (m, 4H, $-\text{CH}_2-$), 1.39–1.33 (m, 2H, $-\text{CH}_2-$).

^{13}C NMR (100 MHz, CDCl_3): δ 24.76, 25.72, 28.54, 34.31 ($-\text{OCO}(\text{CH}_2)_4-$), 64.33 ($-(\text{CH}_2)_4\text{CH}_2\text{O}-$), 173.73 ($-\text{COO}-$).

IV.1.2 Synthesis of Keratin/PCL biocomposites (PCL/DK)

Keratin/poly(ϵ -CL) (PCL/DK) materials were synthesized by ROP of ϵ -CL carried out in the presence of denatured keratin (DK). The polymerizations were conducted under solvent-free conditions. In a typical reaction, ϵ -CL (100 equivalents relative to the catalyst) and a predetermined amount of DK (5-30%) were introduced into the reaction vessel, followed by the addition of the aluminum-based catalyst [AlI_2Me]. The reactions were conducted at 80 °C for 24 hours in an inert atmosphere without the use of an external co-initiator. Finally, the reaction mixtures were cooled to room temperature and purified by dissolution-precipitation using cold hexane to remove residual unreacted monomer and catalyst. The purified polymers were dried under vacuum to constant weight.

^1H NMR (400 MHz, CDCl_3): δ = 4.04 (t, 2H, $-(\text{CH}_2)_4\text{CH}_2\text{O}-$), 2.29 (t, 2H, $-\text{CH}_2-\text{C}(\text{O})\text{O}-$), 1.65–1.61 (m, 4H, $-\text{CH}_2-$), 1.39–1.33 (m, 2H, $-\text{CH}_2-$).

Gel permeation chromatography (GPC) was used to determine the number-average (M_n) and weight-average (M_w) molecular weights and dispersity ($\mathcal{D}$) of pure PCL and PCL/DK samples. Measurements were performed using THF as the eluent and calibrated with polystyrene standards.

Solid-state ^{13}C CP/MAS NMR experiments were performed on selected samples using a cross-polarized magic-angle spinning setup. The spectra were recorded to identify the characteristic resonances of PCL and keratin. The carbonyl region was deconvoluted using Lorentzian and Gaussian functions to distinguish ordered from disordered components.

MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight) mass spectrometry was performed on pure PCL and PCL/DK₁₀ samples to study the end-group structures of the polymers. The spectra were acquired in positive ion mode using sodium or protonated species as cationizing agents. The resulting mass distributions were analyzed to identify linear and cyclic PCL chains and to assess features potentially associated with polymer growth in the presence of keratin.

IV.1.3 Synthesis and structural characterization of biocomposites based on PBS and u-PBS with keratin integration in the polycondensation reaction phase

Poly(butylene succinate) (PBS) was synthesized via the melt polycondensation of succinic acid (SA) and 1,4-butanediol (BD) using titanium(IV) butoxide as a catalyst. In a typical synthesis, succinic acid (0.500 g, 4.23 mmol) and 1,4-butanediol were added to a round-bottom flask at a molar ratio of 1:1.2 (BD: 0.458 g, 5.08 mmol). Titanium(IV) butoxide ($\text{Ti}(\text{OBu})_4$) was added as a 0.0300 M solution in toluene at a concentration corresponding to 0.2 mol% of the succinic acid (0.00846 mmol).

The reaction mixture was heated to 180°C under nitrogen and stirred continuously for 2 hours to promote esterification. Subsequently, the temperature was gradually increased to 230°C and the reaction was allowed to proceed for an additional 5 hours to promote polycondensation. At the end of the reaction, the system was cooled to room temperature and quenched by adding approximately 20 mL of chloroform. The resulting solution was precipitated in 100 mL of cold methanol, and the recovered polymer was dried under vacuum at room temperature for 24 hours before characterization.

Unsaturated poly(butylene succinate) (u-PBS) was synthesized following a similar polycondensation procedure, with partial replacement of the 1,4-butanediol with *cis*-2-butene-1,4-diol to introduce unsaturation along the polymer chain. Specifically, the diol component consisted of a mixture of BD and *cis*-2-butene-1,4-diol, maintaining an overall molar ratio of SA:BD:*cis*-2-butene-1,4-diol = 1:1.1:0.1.

The same catalytic system ($\text{Ti}(\text{OBu})_4$, 0.2 mol% of SA) and the same reaction protocol were used. The esterification was carried out at 180°C under nitrogen, followed by a polycondensation step at 230°C. Processing and purification were performed as described for PBS. The incorporation of unsaturated units into the polymer chain was confirmed by ^1H NMR analysis.

In parallel, formulations containing keratin were synthesized in an analogous manner, with the only difference being the temperature in the second polycondensation step, which was maintained at 190°C to prevent protein degradation. Keratin was gradually introduced into the reactor at constant temperature to ensure uniform dispersion within the growing polymer matrix. The resulting products were processed and purified similarly to keratin-free systems.

^1H NMR spectra were recorded at 298 K using Bruker AM 300 (^1H : 300 MHz), Bruker AVANCE 400 (^1H : 400 MHz), and Bruker ASCEND 600 (^1H : 600 MHz) spectrometers, depending on sample availability and resolution requirements. Polymer samples (≈ 10 mg) were dissolved in 0.5 mL of deuterated chloroform (CDCl_3). Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal reference. Residual chloroform signals ($\delta = 7.26$ ppm for ^1H) were used for spectral calibration. The reported NMR data include chemical shift, signal multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad), and integration. Spectral acquisition and processing were performed using Bruker TopSpin software (v2.1).

Molecular weight distributions were determined by SEC using an Agilent system equipped with a refractive index detector and a 5 μm Mixed-C PLgel column. Chloroform was used as the eluent at a flow rate of 1.00 mL min^{-1} . Polymer samples were dissolved in chloroform, filtered through 0.22 μm PTFE membranes, and injected with an injection volume of 20 μL . Measurements were performed at 35°C.

Molecular weights were calculated using a universal calibration based on polystyrene standards with narrow molecular weight distributions. Number-average molecular weight (M_n) and dispersity (D) values were determined accordingly.

FTIR spectra were collected to confirm the chemical structure of PBS and u-PBS and to identify characteristic ester and unsaturated functional groups and functional groups attributable to the embedded keratin. Measurements were performed in attenuated total reflectance (ATR) mode in the range of 4000–400 cm^{-1} with a resolution of 2 cm^{-1} , averaging 24 scans per spectrum.

IV.2.1 Extraction of keratin from chicken feathers by alkaline hydrolysis in 0.1M NaOH solution

Chicken waste feathers were cleaned with distilled water and degreased with a solution of SDS at 10%. Feathers were subsequently desiccated in an oven at 60°C for 48 h. Ten grams of feathers were added to 150 mL of 0.1 M NaOH and let soak overnight with stirring. Subsequently, the mixture was filtered with a cotton cloth and the liquid residue was acidified with a 10% trichloroacetic acid solution to adjust the pH at 2.4. The precipitate was gathered and cleaned three times with milli-Q water. The keratin protein sample was stocked at −20°C for 24 hr and then freeze-dried. The extraction yield of the keratin content from feathers was calculated using Equation (2):

$$Y(\%) = \left[\frac{m_{dry}}{m_0} \right] * 100$$

Where m_0 = initial weight of cleaned feathers; m_{dry} = weight of freeze-dried residue.

IV.2.2 Extraction of keratin from chicken feathers by denaturing-reducing extraction in 8M urea and 0.165M L-cysteine solution

Chicken waste feathers were cleaned with distilled water and degreased with SDS 10% solution. Feathers were subsequently desiccated in an oven at 60°C for 48h. Ten grams of feathers were added to 150mL of 8M Urea 0.165M L-cysteine and soaked for 7h at 70°C with stirring. Subsequently, the mixture was filtered with a cotton cloth and the pH was adjusted at 10.5 with 1M NaOH solution. The obtained solution was filtered and cleaned by dialysis against distilled water for 3 days at room temperature. Dialysis was performed using a Spectra/Por® dialysis membrane with a molecular weight cut-off (MWCO) of 8 kDa. The distilled water was changed three times a day. The keratin protein sample was stocked at −20°C for 24h and then freeze-dried. The extraction yield of the keratin content from feathers was calculated using the Equation (2).

IV.2.3 Keratin denaturation in 0.1M L-cysteine solution

Keratin was extracted from poultry feathers according to the procedures described previously in section IV.2.2. Native keratin (NK) was obtained after cleaning, drying, and mechanically processing the raw feathers, which were used as the extraction matrix. Denatured keratin (DK) was prepared by treating native keratin with 0.1M L-cysteine as a mild reducing agent to selectively cleave intermolecular and intramolecular disulfide bonds. The treatment was carried out at 80°C for 24 hours under controlled conditions to promote partial unfolding of the protein structure while avoiding

extensive degradation of the backbone. At the end of the reaction, the denatured keratin was thoroughly washed to remove residual reagents and dried before further use.

IV.2.4 Determination of the effective keratin content in PCL/DK biocomposites

Monomer conversion was determined by ^1H NMR spectroscopy by comparing the integrals of the characteristic signals of the ϵ -CL monomer with those of the polymer backbone. The final keratin content in the PCL/DK polymers was calculated by mass balance, combining gravimetric data with the conversion values obtained from NMR analysis, according to the following equation (1)

Where:

$$(\%)Ker = \left(\frac{m_{(tot)} - (m_{(mon)} * c_{(NMR)})}{m_{(tot)}} \right) * 100$$

- $m_{(tot)}$: final dry mass of the sample (g)
- $m_{(mon)}$: initial mass of ϵ -CL used in the synthesis (g)
- $c_{(NMR)}$: polymer conversion determined by ^1H NMR (value between 0 and 1)
- $(\%)Ker$: actual percentage by weight of keratin in the dried material

IV.2.5 Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight of the feather keratin extract was evaluated by SDS–PAGE electrophoresis using a 12% polyacrylamide gel under denaturing conditions according to the procedure of Laemmli.²⁰⁹ Sample preparation for electrophoretic analysis was performed following previously described procedures.^{210,211} Briefly, each keratin extract was dissolved in deionized water at a concentration of 35 mg/mL. An aliquot of approximately 15 μL of the protein solution was then mixed with 5 μL of loading buffer and heated to boiling temperature for 10 minutes to ensure complete protein denaturation. For electrophoretic analysis, 10 μL of molecular weight protein marker and 20 μL of the denatured keratin samples were loaded into separate wells of the gel. The electrophoresis run was initially conducted at 80 V for 30 minutes, followed by a second run at 120 V for 90 minutes to achieve adequate separation of the protein bands. After electrophoresis, the gel was washed twice with distilled water and then stained with Coomassie Brilliant Blue. The destaining step was performed overnight using a solution containing 10% glacial acetic acid and 10% methanol until well-

defined protein bands were obtained. Finally, the stained gel was acquired and documented using a ChemiDoc imaging system (Bio-Rad, Milan, Italy).

IV.3.1 Preparation of the two-dimensional polymer film

Two-dimensional polymer films were obtained using the solvent casting technique. Both pure PCL films and PCL composite films enriched with extracted keratin (PCL/KE) were prepared using circular molds with an internal diameter of 1 cm. To prepare the pure PCL films, 100 mg of the synthesized PCL was dissolved in 1.4 mL of chloroform at room temperature under continuous stirring. The resulting solution was then cast into the molds using a glass syringe and evaporated under a fume hood for 24 hours to allow complete removal of the solvent. The PCL/KE composite films were prepared with different keratin contents, equal to 10% (PCL/KE10), 15% (PCL/KE15), 25% (PCL/KE25), and 30% (PCL/KE30) by weight. In this case, 85 mg of synthesized PCL was initially dissolved in 1.4 mL of chloroform at room temperature under magnetic stirring; subsequently, the amount of keratin powder corresponding to the desired percentage was added. The resulting mixture was then cast into molds and subjected to the same solvent evaporation process used for pure PCL films.

IV.3.2 Preparation of three-dimensional porous polymer scaffold

The 3D porous scaffolds were fabricated using a salt leaching technique to achieve a highly interconnected architecture, suitable for cell infiltration and nutrient exchange. As shown in Figure 35, two different systems were prepared: one consisting of pure PCL and a PCL-based composite containing 15% by weight of keratin extract (PCL/FK). To fabricate the scaffolds, PCL or PCL/FK were dissolved in chloroform (CHCl_3) until homogeneous solutions with a polymer concentration of 12% by weight were obtained. The solutions were then mixed with sieved sodium chloride, with particle sizes ranging from 75 to 500 μm , used as a porogen in a polymer/salt ratio of 1:10. This ratio was selected to ensure an adequate compromise between mechanical integrity and pore interconnectivity. The resulting viscous mixtures were cast into cylindrical molds made of borosilicate glass tubes (Pyrex, 12 $\times$ 75 mm) and left to dry slowly at room temperature for a week in covered containers, to limit rapid evaporation of the solvent and prevent structural collapse. Once solidification was complete, the samples were removed from the molds and sectioned to the desired dimensions. NaCl was removed by immersing the scaffolds in deionized water at room temperature; the distilled wash water was replaced every 24 hours for a total period of 5 days. The salt leaching rate was monitored by measuring the electrical conductivity of the wash water. In the initial stages of

the process, the conductivity was approximately 1350 $\mu\text{S}/\text{cm}$, indicating a high concentration of dissolved salt, then progressively decreased over time until reaching values below 5 $\mu\text{S}/\text{cm}$ after 72 hours, comparable to those of deionized water. These values confirm the complete removal of the porogen. After the leaching process, the scaffolds were vacuum-dried at room temperature for 24 hours to eliminate residual moisture before the subsequent characterization and biological evaluation phases.

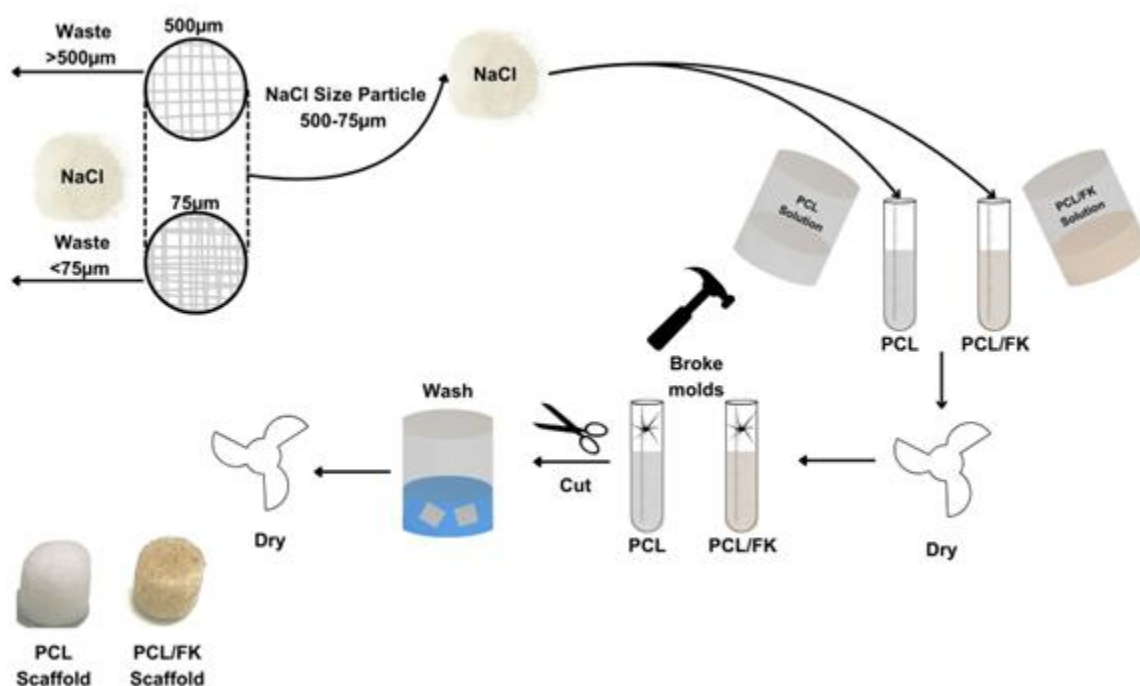


Figure 35 Graphical representation of the production process of PCL and PCL/FK scaffolds

IV.3.3 PCL/DK Film Processing by Tape Casting and thermal and morphological characterization

PCL and PCL/DK films were prepared by tape casting using a solvent-based technique. The polymers obtained by ring-opening polymerization, containing pure PCL or PCL mixed with DK at different weight percentages (5, 10, 15, 20, and 30%), were first dissolved in chloroform under magnetic stirring at room temperature until completely dissolved. In the enriched samples, TEO was added to the polymer solution at a concentration of 10% by weight of the polymer mass and homogenized for 3 hours to ensure uniform dispersion.

The resulting solutions were cast onto a flat glass substrate and leveled using a calibrated 200-micron squeegee to control the film thickness. The solvent was allowed to evaporate slowly at room temperature under a fume hood for 24 hours. The films were then further dried under vacuum

overnight to remove residual solvent. After drying, the films were carefully peeled off the substrate and stored in a desiccator at room temperature before characterization.

Thermogravimetric analysis was conducted in an inert atmosphere to evaluate the thermal stability of pure keratin, pure PCL, and PCL/DK samples. Measurements were performed from (range temperature) at a constant heating rate. The temperature at 5% weight loss ($T_{5\%}$), maximum degradation temperatures (T_{max}), and derived thermogravimetric curves (DTG) were extracted from the data.

Differential scanning calorimetry (DSC) was used to study thermal transitions and crystallinity of materials. Samples were subjected to heating-cooling-heating cycles, and thermal parameters were extracted from the second heating cycle. The glass transition temperature (T_g), melting temperature (T_m), enthalpy of fusion (ΔH_m), and degree of crystallinity (χ_c) were determined using standard procedures.

The morphology of native keratin, denatured keratin, and pure PCL and PCL/DK films were examined by scanning electron microscopy (SEM). PCL-based samples were cryofractured in liquid nitrogen to obtain representative cross-sections, while keratin powders were dispersed in an insoluble solvent, carefully dried, and applied to appropriate stubs. All samples were subsequently coated with a conductive gold layer before image acquisition. SEM micrographs were acquired at various magnifications to evaluate morphology, phase dispersion, interfacial adhesion, and fracture morphology.

IV.3.4 Rheological, morphological, and surface characterization of PBS and u-PBS-based systems with keratin

For rheological, morphological, and surface analyses, the polymer samples were processed under controlled conditions to ensure reproducibility and comparability across different formulations. Rheological measurements were performed on compression-molded samples, obtained by hot-pressing the polymers at temperatures above their melting point, followed by cooling to room temperature under pressure to minimize the effects of thermal history.

The viscoelastic properties of the materials were assessed using dynamic oscillatory melt rheometry. A DHR-2 rheometer (TA Instruments) with a 25 mm parallel-plate geometry was used for the tests at a temperature of 140°C and a plate spacing of 1.5–2 mm in a nitrogen environment. Stress and strain amplitude tests were initially performed, with an initial stress of 10 Pa and a strain of 1×10^{-5} , up to

a final strain of 2 at 0.628 rad/s. Complex viscosity (η^*) and phase angle (δ) were then measured as a function of angular frequency (ω) ranging from 0.01 to 100 rad/s.

For morphological and surface characterization, films were prepared by solvent casting. Samples containing PBS, u-PBS, and keratin were dissolved in chloroform and cast onto flat substrates, allowing the solvent to evaporate at room temperature to obtain continuous films with uniform thickness. Scanning electron microscopy (SEM) analysis was performed on cryogenically fractured films to observe their internal morphology and fracture behaviour. Samples were immersed in liquid nitrogen and fractured to expose fresh cross-sectional surfaces, which were then sputter-coated with a thin conductive layer prior to SEM observation. The surface morphology and microstructure of the materials were characterized using a FEI QUANTA 200 scanning electron microscope (SEM) (Thermo Fisher Scientific, Waltham, MA, USA).

The wettability of the sample surfaces was assessed by water contact angle (WCA) testing at room temperature using an FTA 1000 instrument (First Ten Ångströms, UK). Four μL of deionized water was poured onto the surface of each sample using an automated droplet dispensing system, with droplet images acquired over a time interval ranging from 0.033 to 300 seconds.

This combined experimental approach allows for the correlation of the viscoelastic behaviour of the melt, internal morphology, and surface properties, providing a comprehensive assessment of the effects of backbone modification and keratin incorporation on the structure-property relationships of PBS-based materials.

IV.4.1 In vitro cell culture study of HaCaTs cells on two-dimensional films

The viability and proliferation of HaCaT keratinocytes cultured on PCL and PCL/KE15 films were assessed using the MTT assay.²¹² PCL and PCL/KE15 films were placed in 48-well polystyrene culture dishes and sterilized by ultraviolet (UV) irradiation for 30 minutes. Subsequently, the samples were quickly rinsed with DPBS containing 1% penicillin/streptomycin solution.²¹³ HaCaT cells were then seeded onto the films at a density of 6.0×10^4 cells per well and cultured for periods of 1, 3, and 5 days. To assess cell viability, 40 μL of MTT solution (5 mg/mL) was added to each well. After 4 hours of incubation at 37°C in a humidified atmosphere containing 5% CO₂, the culture medium was removed and the formazan crystals formed were solubilized by adding DMSO, shaking the plates for 15 minutes in the dark. Finally, the absorbance of the solutions was measured at a wavelength of 570 nm using a spectrophotometer (Infinite F200 PRO, Tecan, Grödig, Austria). The percentage of cell viability was measured according to Equation (3)

$$\% \text{ cell viability} = \frac{Abs_{sample}}{Abs_{control}} \times 100$$

where Abs_{sample} was the absorbance of cells seeded on the films and $Abs_{control}$ was the absorbance of HaCat cells incubated in the culture medium.⁸⁹

IV.4.2 In vitro cell culture study of MC3T3-E1 cells on 3D scaffolds

Murine pre-osteoblastic cells MC3T3-E1 Subclone 4 (ATCC® CRL-2593™, Manassas, VA, USA) were used as a cellular model. Cultures were maintained in Minimum Essential Medium Alpha (α -MEM; Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Merck, Darmstadt, Germany) and 1% penicillin/streptomycin (10,000 U/mL / 10 mg/mL; Gibco). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and monitored daily by inverted phase-contrast light microscopy. Cell passage was performed twice weekly when approximately 80% confluence was reached. The viability and proliferation of MC3T3-E1 cells cultured on PCL and PCL/FK scaffolds were assessed by the lactate dehydrogenase (LDH) release assay (Roche Diagnostics, Mannheim, Germany).²¹⁴ The scaffolds, approximately 1 mm thick, were placed in 24-well polystyrene culture dishes, sterilized by ultraviolet (UV) irradiation for 30 minutes, and then rinsed in sterile DPBS containing 1% penicillin/streptomycin before cell seeding.²¹⁵ MC3T3-E1 cells were seeded at a density of 3.0×10^4 cells per well, either directly on the surface of the dishes (control condition) or on the scaffolds. Osteogenic differentiation was induced by supplementing the complete culture medium with L-ascorbic acid (50 μ g/mL), promoting extracellular matrix maturation and progression toward the osteoblastic phenotype. The culture medium was replaced every 3 days, and the cells were maintained in culture for periods of 3, 7, and 14 days. At each time point, cytotoxicity was assessed by measuring the LDH activity released into the culture medium by damaged cells. Subsequently, total cell viability and proliferation were determined by inducing complete cell lysis, releasing intracellular LDH. Under these conditions, LDH activity was proportional to the number of viable cells present on the scaffolds and was therefore used to quantify cell proliferation, comparing it to the control condition. Aliquots collected after cell lysis were analyzed for LDH activity by measuring absorbance at 492 nm using a microplate spectrophotometer (Infinite F200 PRO, Tecan, Grödig, Austria). Cell counts were calculated using an LDH calibration curve obtained according to the manufacturer's instructions and normalized to the control.

IV.5 *In vitro* degradation study of porous 3D scaffolds

The *in vitro* degradation behaviour of the scaffolds was studied under physiologically relevant conditions, using phosphate-buffered saline (PBS, pH 7.4) maintained at 37°C. For each formulation (PCL and PCL/FK), 10 mg samples were accurately weighed and incubated in triplicate in DPBS at 37°C with constant agitation in a thermoshaker to simulate the movement of physiological fluids. The degradation process was monitored at predetermined time intervals of 15, 30, 45, 60, 90, 120, and 180 days. At each time point, the samples were recovered from the solution, gently rinsed with deionized water to remove any residual salts, and then vacuum-dried at room temperature for 48 hours until a constant weight was reached. The weight loss (WL %) was determined according to Eq (4):

$$\text{Weight Loss (\%)} = \left[\frac{W_0 - W_t}{W_0} \right] * 100$$

where W_0 and W_t represent the initial and the remaining dry weight after degradation time t , respectively.

In parallel, gel permeation chromatography (GPC) was performed on the dried specimens at each time point to determine the PCL molecular weight (M_n).

IV.6.1 DPPH Radical Scavenging Assay

The antioxidant activity of the films was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, adapted to solid polymeric materials. Rectangular samples (approximately 1×1 cm) were cut from each film formulation and immersed in 10 mL of a freshly prepared DPPH solution in methanol (0.1 mM). The samples were incubated in the dark at room temperature under static conditions. At predetermined storage times (0, 1, 3, and 6 days), the standard incubation time of 30 min was considered for time 0. Aliquots of the DPPH solution were collected, and the absorbance was measured at 517 nm using a UV-Vis spectrophotometer. A DPPH solution without the film was used as a control. The percentage of DPPH radical scavenging activity was calculated according to the following equation: (5)

$$\text{DPPH Inhibition (\%)} = \frac{A_0 - A_t}{A_0} * 100$$

where A_0 is the absorbance of the control solution and A_t is the absorbance measured in the presence of the film.

All measurements were performed in triplicate, and the results were expressed as mean $\pm$ standard deviation. This test allowed us to evaluate the time-dependent release of antioxidant compounds from

the films, highlighting the role of keratin as a modulating phase rather than an intrinsic antioxidant component.

IV.6.2 Lipid Peroxidation Assay (TBARS)

The effectiveness of films in inhibiting lipid oxidation in foods was evaluated using the thiobarbituric Acid Reactive Substances (TBARS) assay, using chicken meat as a model system. Fresh chicken breast was cut into uniform portions (approximately 10 g), wrapped with different film formulations (with and without TBARS), and stored at 4°C for up to 6 days. At selected storage times (0, 1, 3, and 6 days), lipid peroxidation was quantified by measuring the TBARS, expressed as nmol of malondialdehyde (MDA) per gram of sample. Briefly, 0.3 g of meat was homogenized with a 0.2% phosphoric acid solution, followed by a reaction with thiobarbituric acid under acidic conditions and heating. After cooling, the samples were centrifuged, and the absorbance of the supernatant was measured at 532 nm using a UV-Vis spectrophotometer. A calibration curve was prepared using 1,1,3,3-tetramethoxypropane as the MDA standard. TBARS values were expressed as nmol MDA/g⁻¹ of sample. Each measurement was performed in triplicate. This test provided an *in situ* assessment of the antioxidant performance of the films, allowing a correlation between the radical scavenging activity measured by DPPH and the actual inhibition of lipid oxidation in a real food matrix.

IV.7. Statistical analysis

All quantitative data are reported as mean $\pm$ standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (one-way ANOVA) to evaluate differences among multiple groups. When statistically significant differences were detected, Tukey's honestly significant difference (HSD) post hoc test was applied for pairwise comparisons between samples.

A p-value lower than 0.05 was considered statistically significant. The levels of significance are reported as follows: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***), while non-significant differences are indicated as ns. All experiments were performed using at least three independent replicates ($n = 3$), unless otherwise stated. Statistical analyses were carried out using appropriate data analysis software.

Chapter V

Conclusions

This doctoral thesis has investigated the integration of feather-derived keratin into degradable polyester matrices, with the overarching objective of valorizing an abundant agro-industrial waste within sustainable polymeric materials. A progressive research strategy was adopted, moving from physically blended systems to polymerization-assisted approaches, in order to systematically assess the role of keratin as a functional and structural modifier across different material architectures and polymerization routes.

In the first part of the work, keratin/PCL materials obtained by physical mixing were successfully developed as two-dimensional films and three-dimensional porous scaffolds. In film-based systems, keratin incorporation was shown to significantly influence surface morphology, thermal behaviour, and surface-mediated biological response. *In vitro* studies using HaCaT keratinocytes demonstrated that keratin-containing films support cell viability and proliferation, with optimal performance observed at intermediate keratin contents. These findings highlight the effectiveness of physical blending as a simple and scalable strategy to enhance the biological performance of otherwise bioinert PCL surfaces. The extension of this approach to three-dimensional scaffolds further demonstrated that keratin can be successfully incorporated into porous architectures suitable for bone tissue engineering, supporting MC3T3-E1 pre-osteoblast adhesion and proliferation while modulating scaffold morphology and degradation behaviour.

Despite the effectiveness of physical blending, the limitations associated with phase separation and interfacial stability motivated the exploration of polymerization-assisted strategies. The introduction of keratin during the ring-opening polymerization of ϵ -CL enabled the synthesis of PCL/keratin materials obtained *in situ*, providing a tighter association between the protein and the growing polymer chains. Although the complexity of the system and the chemical nature of keratin posed challenges in achieving direct molecular-level evidence of covalent bonding, the *in situ* approach resulted in materials with distinct structural, thermal, and morphological features compared to physically blended counterparts. These results demonstrate that keratin can actively participate in polymer formation processes without inhibiting polymerization, opening new perspectives for the design of multifunctional biodegradable materials.

The functional potential of keratin-containing PCL systems was further explored by exploiting keratin as a reservoir phase for volatile bioactive compounds. The incorporation of thyme essential oil revealed that keratin does not act as an intrinsic antioxidant but plays a key role in modulating the retention and release of low-molecular-weight active molecules. The time-dependent antioxidant behaviour observed in both DPPH and TBARS assays confirms that keratin can extend the functional lifetime of volatile compounds, supporting the concept of keratin-based reservoir materials for applications such as active packaging or controlled-release systems.

Finally, the thesis explored the transferability of keratin integration strategies to polycondensation-based systems through the synthesis of PBS-based materials. The incorporation of keratin during melt polycondensation demonstrated that polymer chain formation can successfully proceed in the presence of the protein, albeit with a reduction in molecular weight. While direct molecular-level interactions between keratin and the growing polyester chains cannot be conclusively demonstrated in this system, the successful synthesis of polymeric materials with defined molecular weights enabled a systematic evaluation of their thermal, mechanical, rheological, and morphological properties. Structural, rheological, and surface analyses confirmed that keratin acts as a structural modifier within PBS and unsaturated PBS matrices, influencing chain organization, viscoelastic response, and surface wettability without altering the fundamental chemical structure of the polyester backbone.

In conclusion, this work demonstrates that feather-derived keratin can be successfully exploited as a multifunctional component in biodegradable polyester systems, acting as a bioactive surface modifier, a structural organizer, and a functional reservoir phase depending on the processing strategy and material architecture. The systematic comparison of physical blending and polymerization-assisted approaches provides valuable insights into the advantages and limitations of each strategy and establishes a foundation for future developments in keratin-based sustainable materials. Further work may focus on improving control over polymer–protein interactions, expanding functionalization strategies, and translating these materials toward specific biomedical or packaging applications.

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