Universidade do Minho
Escola de Engenharia

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The Potential of Multifunctional Proteins and Peptides for Hair Protection and Coloring

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The Potential of Multifunctional Proteins and Peptides for Hair Protection and Coloring

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Trabalho efetuado sob a orientação do
Professor Doutor Artur Manuel Cavaco Paulo
e do
Doutor Artur Jorge Araújo Magalhães Ribeiro

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STATEMENT OF INTEGRITY

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I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

O Potencial de Proteínas Multifuncionais e Péptidos para a Proteção e Coloração de Cabelos

Resumo

O cabelo é um elemento importante na aparência das pessoas, sendo uma das características físicas que podemos controlar e alterar facilmente. No entanto, os procedimentos cosméticos em conjugação com as rotinas diárias e insultos ambientais podem danificar e enfraquecer irreversivelmente as fibras. A indústria cosmética utiliza diferentes formulações contendo proteínas, hidrolisados de proteínas, péptidos ou aminoácidos, com benefícios comprovados para o cabelo. Estes produtos, apesar de serem utilizados para alterar e/ou melhorar a forma, cor, volume, brilho e tato do cabelo, são limitados a uma única função. O desenvolvimento de formulações multifuncionais é então visto como uma etapa promissora para o desenho de produtos cosméticos avançados. As novas formulações vão transmitir mais que uma propriedade ao cabelo e vão responder às necessidades do mercado e dos consumidores. O objetivo principal desta tese foi o desenvolvimento de formulações multifuncionais à base de proteínas, capazes de alterar e/ou melhorar as propriedades do cabelo enquanto eliminam ou minimizam os potenciais danos causados às fibras.

Neste trabalho, uma proteína de fusão baseada na sequência de um péptido derivado da queratina (KP) e numa proteína cromogénica ultramarina (UM), foi explorada com sucesso como agente para colorir cabelo. A proteína KP-UM mostrou-se capaz de colorir cabelo descolorado, entre rosa e roxo dependendo da formulação base, enquanto melhorou as suas propriedades mecânicas, demonstrando assim o seu potencial para ser incorporada em novas formulações cosméticas sustentáveis

O uso de proteínas para a proteção do cabelo contra procedimentos com altas temperaturas foi investigado através de proteínas baseadas na sequência da γD-cristalina (sequência original e mutante) e o péptido KP. Os cabelos tratados com as proteínas e depois sujeitos a temperaturas elevadas (200°C), apresentaram resultados melhorados relativamente à perda de água e à conversão das estruturas da queratina de α-hélice para folhas-β, confirmando o carater protetor das proteínas.

Partículas à base de proteínas foram também desenvolvidas para melhorar diferentes propriedades do cabelo. Partículas com queratina e fibroína de seda e Poloxamer 407 na sua constituição foram testadas em cabelo asiático virgem e descolorado, com um efeito positivo na proteção, recuperação de propriedades mecânicas, estabilidade térmica e na suavidade do cabelo. Partículas à base de queratina e zeína foram desenvolvidas para encapsular fragrâncias e serem utilizadas como perfume para o cabelo. As formulações libertaram a fragrância ao longo do tempo e melhoraram as propriedades mecânicas e o grau de hidratação do cabelo.

Filmes preparados com queratina extraída de cabelo humano foram desenvolvidos no decorrer desta tese e o efeito de diferentes tratamentos térmicos (convencional e ohmico) nas propriedades dos filmes de queratina foi avaliado por diferentes técnicas. Os filmes apresentam diferenças de acordo com a origem da queratina, pH da solução de queratina e tratamento térmico. A aplicação das proteínas KP-Cryst sobre os filmes de queratina demonstrou a capacidade destes para serem usados como modelo de cabelo nas avaliações de novos produtos cosméticos.

As novas formulações aqui desenvolvidas mostram um grande potencial para serem incluídas em novos produtos cosméticos para cabelo com grande impacto socioeconómico.

Palavras chave: cabelo, cor, formulações cosméticas, péptido, proteína.

The Potential of Multifunctional Proteins and Peptides for Hair Protection and Coloring

Abstract

Hair is an important element of humans' physical appearance, being one of the personal features that we can have direct control and easily change. However, cosmetic procedures, daily hair care routines and environmental insults can irreversibly damage and weaken the hair fibers. Hair cosmetic industry has developed several formulations containing proteins, proteins hydrolysates, peptides and amino acids, with proved benefits to the hair. These products despite being used to change and/or improve the hair shape, color, volume, gloss, shine and feel, are limited to one function. The development of new multifunctional formulations is thus seen as very promising for the design of advanced hair cosmetic products. The new formulations will impart more than one feature to the hair and will respond to the market and consumer's needs. The main objective of this thesis was the development of advanced multifunctional protein-based formulations capable to change and/or improve several hair's properties while avoiding or minimizing the damages potentially caused to the fiber.

In this work, a fusion protein based on the sequences of a keratin-based peptide (KP) and an ultramarine chromogenic protein (UM), was successfully explored as a hair coloring agent. The KP-UM protein was able to color overbleached hair, from pink to purple depending on the base formulation, while recovering its mechanical properties, demonstrating its potential to be incorporated in new ecofriendly cosmetic formulations.

The use of proteins for hair protection against high temperature procedures was investigated using fusion proteins based on the sequence of the human eye γD-crystallin (wild-type and mutant) and the KP peptide. Hair treated with the KP-Cryst Wt and KP-Cryst Mut proteins and then subjected to iron application (200°C), presented an improved performance regarding water loss and conversion of keratin α-helix structure into β-sheets, confirming the protective character of the tested proteins.

Protein-based particles were also developed to improve different hair properties. Particles with keratin, silk fibroin and poloxamer 407 in its constitution were tested on virgin and overbleached Asian hair, with a positive effect on the protection, recovery of hair mechanical properties, thermal stability and hair smoothness. Keratin and zein based particles were developed to encapsulate fragrances to be used as hair perfumes. These formulations released the fragrances over time and improved the hair's mechanical properties and hair's hydration degree.

Films prepared with keratin extracted from human hair were also developed during this thesis and the effect of different heating treatments (conventional and ohmic) on the keratin films' properties was evaluated by different techniques. The films presented different properties according the keratin source, keratin solution pH and heating treatment. The application of KP-Cryst proteins over the keratin films demonstrated their capacity to be used as a substitute to the hair when evaluating new cosmetic products.

The new formulations here developed show great potential to be included in new hair cosmetic products with a high socio-economic impact.

Keywords: color, cosmetic formulation, hair, peptide, protein.

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List of Symbols and Abbreviations

CD – Circular Dichroism

CH – Conventional Heating

CIE – Commission International de l'Eclairage

Cryst – Crystallin

Cys – Cysteine

DC – Detergent Compatible

DMSO – Dimethyl sulfoxide

DSC – Differential Scanning Calorimetry

DLS – Dynamic Light Scattering

FITC – Fluorescein Isothiocyanate

FTIR – Fourier-Transform Infrared Spectroscopy

GC-MS – Gas chromatography-mass spectrometry

GFP – Green Fluorescent Protein

HPH – High Pressure Homogenization

I – Improvement

K – Keratin

K/S – Kubelka-Munk relationship

Kan – Kanamycin

Ker – Keratin

KP – Keratin-based Peptide

MALDI-TOF – Matrix-Assisted Laser Desorption/Ionization – Time of Flight

MTT – 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

Mut – Mutant

Mw – Molecular Weight

NTA – Nanoparticle Tracking Analysis

OB – Overbleached

OH – Ohmic Heating

PBS – Phosphate Buffer Saline

PDI – Polydispersity Index

PMF – Potential of Mean Force

Pol_{407} /P – Poloxamer 407

R – Recovery

rpm – rotation per minute

RT – Room Temperature

SD – Standard Deviation

SDS-PAGE – Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

SEC – Size-exclusion Chromatography

SEM – Scanning Electron Microscopy

SF – Silk Fibroin

STEM – Scanning Transmission Electron Microscope

TB-AIM – Terrific Broth - Auto Induction Medium

TFA – Trifluoroacetic acid

TGA – Thermogravimetric Analysis

UM – Ultramarine

up – ultra-pure

UV – Ultra-Violet

Wt – Wild Type

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Chapter I

Motivation and Thesis Outline

Chapter I - Motivation and Thesis Outline

Abstract

In this chapter are introduced the scope and the main objectives of this doctoral thesis. The motivation of the study about the use of proteins to change and/or improve the hair properties is provided, including the identification of the main objectives of the thesis. A brief introduction to the thesis organization is also present in order to provide a clear outline of the thesis.

1.1 Scope and Objectives

Hair is a proteinaceous biomaterial with a complex structure and one of the defining characteristics of humans. Some of the hair functions include body protection against environmental factors such as cold or heat and the ultraviolet light, playing a role in the body thermoregulation and in human social and sexual interaction. Hair alterations such as color, length or shape are selected according to society stimulus, cultural influence, fashion and personal taste. However, most of the cosmetic procedures used to change hair physical properties can irreversibly damage and weaken the hair fibers. Hair cosmetic industry as developed several products for hair care containing proteins, protein hydrolysates, peptides and amino acids. Yet, the great potential of having more than one hair feature changed in just one product, together with a growing environmental consciousness, makes the development of green multifunctional cosmetic formulations very appealing for hair cosmetic industry.

The main objective of this doctoral thesis was the development of new cosmetic formulations capable to change and/or improve hair properties using proteins as the main active agents, while avoiding or minimizing the damages caused to the fiber. Specifically, the main goals were:

Bioproduction, purification and characterization of a chromogenic fusion protein and the study of its capacity to simultaneously color and strength the hair fiber;

Bioproduction, purification and characterization of fusion proteins composed by a keratin-based peptide and the human eye γD-crystallin, and the study of their protective role against high temperature procedures, when applied in the hair;

Production and characterization of protein-based particles and their effect on hair properties like strength, softness, humidity, and smell;

Development of a hair model using keratin films and the effect of heating treatments on the final properties of the films.

1.2 Thesis Outline

In agreement with the aims of the present doctoral thesis, this document is divided into eight chapters, five of which describe experimental research published or in the process of submission in international peer reviewed journals. The content of each chapter is summarized below.

Chapter I describes the motivation and the main objectives of this doctoral thesis and presents an introduction to the thesis format.

Chapter II is based on a review paper and present an overview of the existing hair cosmetic formulations and their effect on hair properties. Initially, the chapter is focused on a brief introduction of the hair as the main subject of the thesis. The types of hair and properties according their ethnic origin; the hair chemical composition and the hair structure are revised. A special focus is dedicated to the use of proteins, peptides, proteins hydrolysates and amino acids as active ingredients in hair cosmetic formulations to change hair properties like color, shape, volume, strength and conditioning.

Chapter III describes the use of a chromogenic protein fused with a keratin-based peptide to color overbleached Asian hair. The chromogenic protein selected for this work was the blue ultramarine and the keratin-based peptide was added to the protein sequence to increase its specificity and binding to the hair fibers. Different formulations and different protein concentrations were tested to color overbleached Asian hair. The effect of the fusion protein on hair fibers properties was evaluated using different techniques.

In **Chapter IV** is reported the effect of two proteins based of the human eye γD-crystallin on virgin Asian and Caucasian hair. The two proteins, wild-type and mutant, were conjugated with a keratin-based peptide to increase protein binding to the hair. In this study, it was demonstrated the positive effect of the protein pretreatment on hair fibers before and after hair exposure to thermal damage, demonstrating the protective capacity of these proteins against high temperature procedures.

The **Chapter V** demonstrates the use of keratin-based particles for the protection and restoration of virgin and overbleached Asian hair mechanical and thermal properties. This chapter also describes the effect of different concentrations of silk fibroin and Poloxamer 407 on particles' physical characteristics and stability, and particles' capacity to bind to the hair. Cytotoxicity characterization tests were performed on human keratinocytes with no signs of cell metabolic toxicity for any tested concentration and time point.

In **Chapter VI** is described the development of particles composed by different ratios of keratin and zein, for the controlled release of fragrances in hair. It was evaluated the effect of pH on particles' physical properties and stability over time, with a surfactant behavior being observed for the keratin. The release profile of the two tested fragrances (menthol and linalool) from the Keratin:Zein particles was also assessed by GC-MS, for two temperatures (25 and 37°C). The particles had the ability to protect the fragrances during storage and a controlled released dependent on the temperature was observed.

In **Chapter VII** is discussed the effect of heating on the properties of keratin films prepared with keratin extracted from virgin or overbleached human hair. Two different heating methods, conventional and ohmic, were tested in keratin solutions with a pH range between 7 and 10. The protocol for films formation and insolubilization was also optimized. Besides the effect on films' properties, the capacity of these films to be used as a hair model was also assessed by the application of proteins over the keratin-films.

In the final **Chapter VIII**, is presented a general conclusion of the work and the main contributions of the thesis to the state of art of hair cosmetic science. Future perspectives regarding the use of fusion proteins, keratin-based particles and keratin films in cosmetic science and cosmetic industry are also presented.

Chapter II

The Role of Proteins and Peptides in Hair Cosmetic Formulations: an Overview

Chapter II – The Role of Proteins and Peptides in Hair Cosmetic Formulations: an Overview

Abstract

Physical appearance is nowadays related with a deep notion of beautifulness with people constantly seeking for a younger look. The number of products developed to manipulate and improve a person's appearance are of great deal in today's society. According to society stimulus, cultural influence, fashion and personal taste, we can change the hair properties relatively to its length, shape, color, volume, shine and softness. However, daily hair care routines, environmental insults and, particularly, cosmetic procedures and products can induce irreversible changes in hair, damaging and weakening the hair fiber. The development of new sustainable eco-friendly hair cosmetic products, which do not compromise the properties of the hair fibers, is thus seen of great interest for product formulators, cosmetic industry partners and end users. This review focuses, initially, on the hair structure, hair composition and hair properties according to different ethnicities. An extensive revision on protein-based cosmetic formulations (proteins, proteins hydrolysates and amino acids) and their effect on hair's properties is also addressed.

This chapter is based on the following scientific publication:

Ana Tinoco, Madalena Martins, Artur Cavaco-Paulo and Artur Ribeiro, The Role of Proteins and Peptides in Hair Cosmetic Formulations: an Overview, To be submitted.

2.1 Human Hair

Human hair is an element that plays an important role in our appearance and ultimately reflects our personality, thoughts and beliefs. The hair, as a biological feature, is responsible for the protection of the body against environmental factors like cold, heat, sunlight and wetness; being also involved in the body thermoregulation [1,2]. Although hair has no vital function, it plays a very important role in the self-image of both men and women of all ages, with an impact in individual, social and sexual interactions [2,3].

Human hair has the same chemical nature as all epidermal appendages specific to mammals, such as coats or furs, wool, feathers, nails, horns and hoofs [4]. Hair is mainly composed by proteins (65-95% of the hair total weight), and by water, lipids, pigments and trace elements [4–6]. Keratins represent the great majority of the proteins that constitute the hair, and is characterized by its high content of cysteine residues. This unique sulfur-containing amino acid is able to establish cross-linking between protein chains via disulfide linkages, which makes the hair fiber highly resistant to mechanical shocks and chemical attacks [4,7].

2.1.1 Hair types according to ethnic origin

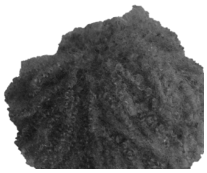
A great diversity of hair types can be observed around the world, with differences regarding shape, color, ellipticity, thickness and length. Natural hair color can vary from black to light blond and the hair shape can be classified as straight, wavy, curly or frizzy [8,9]. Considering the combination of different hair fiber features (color, shape and cross sectional parameters), hair can be categorized into three major groups: African, Asian or Caucasian **(Table II.1)** [10].

Comparing the diameter of the three ethnicities, the Asian hair is the thickest (hair fiber diameter around 100 μm), followed by the African hair (hair fiber diameter around 80 μm), while the Caucasian hair is the thinnest (hair fiber diameter around 50 μm) [11,12]. Although the hair diameter of the Asian and Caucasian ethnicities are uniform along the hair fiber, the African hair present a high degree of irregularity [12]. Regarding the hair shaft cross section geometry, it varies between a circular and an oval-flat shape for the Asian and the African hairs, respectively. The hair shaft cross section of Caucasian hair has an intermediate structure between the Asian and the African ethnicities, presenting a round or an oval form [8,12,13].

Hair shape vary not only between the different hair ethnicities, but it can also differ between different hair shafts of the same individual. Moreover, the fusion of different ethnicities has contributed to the appearance of a mixed racial background where the same person can present several hair characteristics

[14]. Although in the last years it was thought that the hair shape was controlled by the hair cross section shape, new *in vitro* growth studies and computer-aided 3D reconstruction suggested that hair follicle shape is the responsible for the hair shape [11]. Generally, Asian hair presents a straight tertiary structure, African hair tends to be frizzy while Caucasian hair can vary from straight to wavy and curly [8]. The highly curly behavior observed for the African hair make it more brittle and more sensitive to excessive handling, when compared to the other types of hair [8]. This sensibility is also justified by the mechanical properties of African hair cortex that is the less resistant to mechanical stress, followed by the Caucasian and the Asian hair [12]. Also, the African hair has a lower moisture content when compared to the other hair types [11]. Regardless of its ethnicity, all types of hairs present the same biochemical composition, morphology and molecular structure [8,10,11].

Table II.1 - Human hair morphological characteristics according to hair's ethnic origin.

Type of Hair	Thickness	Shape	Ellipticity	Example
African	≈ 80 μm	Curly/frizzy	Flat-oval	
Asian	≈ 100 μm	Straight	Round	
Caucasian	≈ 50 μm	Straight/wavy/curly	Oval	

2.1.2 Chemical Composition of Hair

The human hair is a very complex fiber composed by different morphological components and chemical species – **Table II.2** [1,6]. Basically, hair is composed by proteins and lipids, which correspond to 65-95% and 1-9% of hair dry weight, respectively [15,16]. Other constituents include water, pigments and trace elements [6].

Table II.2 - Chemical composition of human hair.

Components	Percentage	References
Protein	65-95%	[7,15,16]
Lipids	1-9%	[16–18]
Water	12-13%	[6,17]
Pigments	0.1-5%	[19,20]
Trace elements	<1%	[15,17,18]

2.1.2.1 Proteins

Keratin is a fibrous structural protein rich in cysteine, able to form helicoidal complexes, and is the main constituent of hair [7,17]. Due to its high resistance to degradation the keratin complexes present in the hair are usually classified as hard keratins [7]. This protein is composed of about 18 types of amino acids with a high content of cysteine residues. The hair cysteine content vary between gender and ethnicities, according to the diet and throughout the hair fiber [17]. This high cysteine content allows the formation of many disulfide bonds, creating a fiber with a strong and durable structure, and excellent mechanical, chemical and thermal properties [15,18]. Human hair keratins are mainly organized in a α-helix conformation and can be divided in two major families according to its charge [1, 6]. Type I keratins present smaller molecular weights, normally between 44 to 46 kDa, and due to its acidic isoelectric point, between 4.5-5.5, are also known as acidic keratins. Type II keratins have an isoelectric point between 6.5-7.5 and are formed by larger proteins, with molecular weights ranging from 50 to 60 kD [15]. Due to the higher proportion of type I keratins, hair is negatively charged under most of the pH conditions and treatments [21].

Together with keratins, the keratin-associated proteins (KAP) constitute the major structural components of the hair fiber [22]. KAPs are relatively small proteins with hydrophobic characteristics and, considering their chemical properties, the KAPs can be divided into three groups: high sulfur proteins (<30 mol% cysteine content), the ultrahigh sulfur proteins (>30 mol% cysteine content), and the high glycine/tyrosine proteins [23,24]. The KAP are located in the matrix surrounding the keratin-associated filaments, and may be responsible for the rigidity of the hair shaft due to extensive disulfide bond cross-linking with the keratin-associated filaments [22].

The highly organized structure of keratin provides hair a substantial resistance against environmental offenses and daily cosmetic procedures, such as friction, tension, perming, combing, chemical assault and UV radiation [15,18].

2.1.2.2 Water

Hair physicochemical properties are affected by the water content present in the hair fiber. The ability of the hair to be stretched, combed and styled are also influenced by hair internal and external water [6,25]. The hair fibers are hygroscopic elements capable of absorbing large amounts of water [15]. For example, the time required for the hair to absorb 75% of the maximum possible amount of water is around four minutes [6]. The keratin shows a high water absorption capacity, with the water interacting with the amino and guanidine groups of keratin [6], peptide bonds and acidic/basic side chains [15]. In normal conditions, 25°C and 65% of relative humidity, the hair water content varies between 12-13% [17]. The more notorious structural difference on hair after water absorption is the increase in fiber diameter in more than 15% when completely saturated [15]. During fiber swelling, the water adsorbs to the hydrophilic matrix of the cortical cells, deforming the microfibrils structure, culminating in an increase in the fiber diameter [15,18]. Hairs' water absorption capacity is also dependent on the quantity of lipids in hair and the pH of the surrounding milieu [18].

2.1.2.3 Lipids

The lipids present in the hair can be classified as external lipids when they have origin in the sebaceous glands during the contact of hair with the scalp; or as internal lipids when they occur internally in the hair, as remains of the cell membranes [17,18]. The internal lipids, located in the intercellular spaces, represent about 1-3% of the hair mass and consist, fundamentally, of fatty acids and polar lipids [18]. Almost no differences were determined between the internal and external lipids composition, with the free fatty acids being the main component. Neutral lipids including wax, glyceride, cholesterol and squalene were also reported as hair lipid components [17]. The main difference between the internal and the external lipids is the presence of the 18-methyleicosanoic acid, covalently attached via thioester linkages to the cuticle's surface of the hair fiber. This lipid acts as a hair lubricant, decreasing the friction between the hair fibers, and its presence affects some hair properties like shine, feel, dryness and combing [15].

2.1.2.4 Pigments

The color of human hair can vary from black to brown, red or white, with different tones in each color, being the final shade determined by the quantity and quality of the melanin pigments present in hair [19]. In human hair, there are two chemically distinct forms of melanin pigments, eumelanin and pheomelanin **(Figure II.1),** that differentiate between them regarding the color, solubility properties and sulfur content

[20]. Eumelanin is a black to brown pigment, insoluble in acid and alkali solutions, while pheomelanin is a yellow to reddish-brown pigment soluble in alkali solutions [20]. Regarding the pigment composition, eumelanin contains 6.9% of nitrogen and almost no sulfur, while pheomelanin has 8-11% and 9-12% of nitrogen and sulfur, respectively [19,20]. Despite the differences between the pigments, both are synthesized from the same precursor, dopaquinone. When dopaquinone experiences a series of redox reactions, it origins the eumelanin molecule. However, if dopaquinone contacts with sulfhydryl compounds, it can act as a precursor for pheomelanin [19].

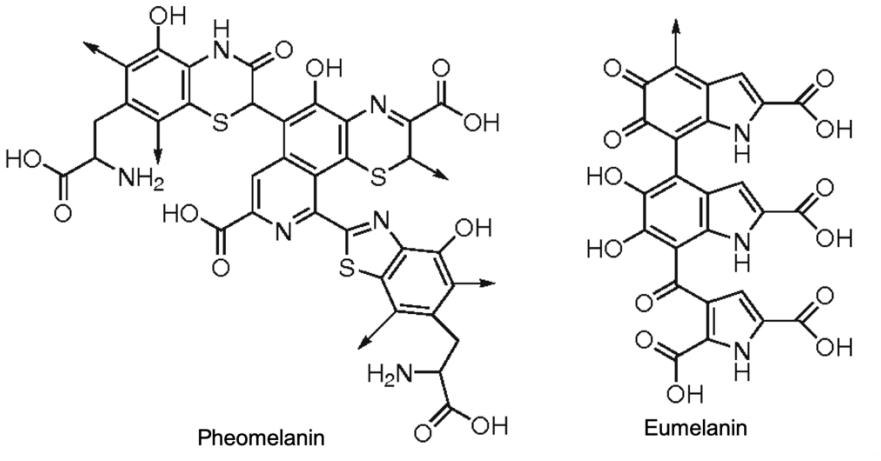


Figure II.1 - Chemical structure of pheomelanin and eumelanin. Adapted from Wakamatsu, K. and Ito, S., 2002 [26].

2.1.2.5 Trace Elements

Besides the proteins, lipids and water, hair also possess in its constitution variable amounts of different inorganic elements, normally lower than 1% of the hair dry weight [15]. The more common metals found in hair include alkaline (Na and K), alkaline earth (Ca, Mg and Sr) and other different metals like (Ca, Pb, Fe, Hg, Cd, Se, Zn, Mn and As) along with metalloids (Si and P) [15,17,18]. The trace elements can be classified as endogenous or exogenous depending on its origin. The endogenous elements are originated from the human body and are incorporated in the hair structure during its synthesis within the hair follicle [27]. Exogenous elements are usually derived from atmospheric dust and contaminants, water and hair cosmetic products [18,27].

2.1.3 Hair Structure

The hair is formed by two distinct structures, the follicle and the shaft, corresponding respectively to the living and nonliving part of the hair. In addition, the hair shaft is composed by three different main regions **(Figure II.2):** the cuticle, the cortex and the medulla [1,2,4].

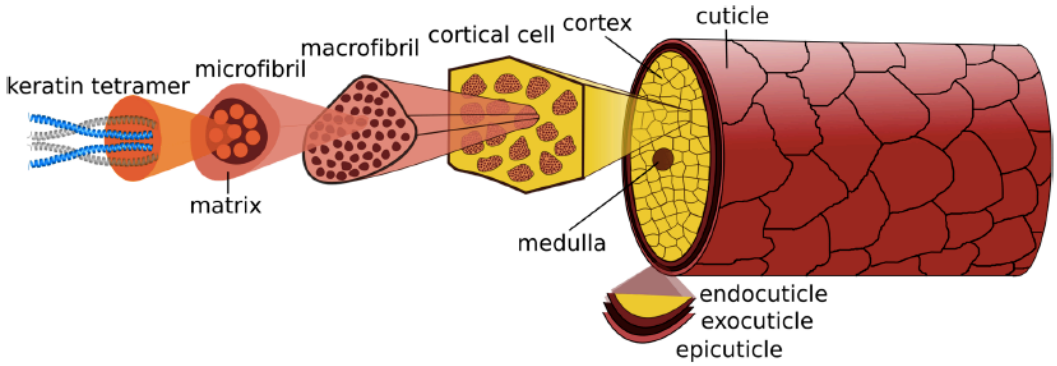


Figure II.2 - Schematic cross section of a hair fiber. Diagram adapted from Cruz *et al.* 2016 (Cruz *et al.*, 2016).

2.1.3.1 Cuticle

The cuticle covers the entire hair from the root to the tip and act as a chemical and physical barrier protecting the inner cortex from external chemical and environmental damage [1,15]. The cuticle layer contains 6-10 layers of overlapping keratinized scales [3,15], each with a thickness of 0.5-1.0 μm and a length of approximately 45 μm [17]. Cuticular cells overlap in a scale-like structure, with the cuticle's proximal end linked to the cortex while the cuticle's free edges are directed upward to the hair tip [3,7]. The shape and orientation of the keratinized scales are responsible not only for limiting the friction between the hair shafts [15] but also for the hair luster and texture [3]. However, the cuticle scales are prone to damage promoted by frictional forces like combing, brushing, shampooing, blow-drying or by alkaline compounds used for cuticular disruption in several cosmetic treatments [3].

The cuticle cell can be divided into four sublamellar layers: epicuticle, A-layer, exocuticle and endocuticle [2]. The epicuticle is a lipid layer that contributes to the hair lubricity and act as a barrier against environmental factors and the penetration of compounds into the hair [5,15]. Being the outer layer of the cuticle, the epicuticle represents the hydrophobic layer responsible for the hair surface properties [28]. Below the epicuticle is the A-layer that comprises almost 30% of hair cystine content and also plays an important role in hair protection against chemical, physical and environmental insults [7,15]. The A-layer is highly cross-linked being responsible for the cuticle structural strength and rigidity [15]. Despite the

exocuticle presents a lower cysteine content, the proteins in this layer are mainly cross-linked by disulfide bonds [10,17]. The endocuticle possesses the proteins with lower cystine content and is composed mainly by remaining cell organelles [4,12]. The cellular membrane complex is responsible to maintain the structure of the cuticle, gluing the cuticle keratinized cells together. The proteins which constitute the cellular membrane complex present a cystine content around 2% [15].

2.1.3.2 Cortex

The cortex is the major component of the human hair fiber whose axis is parallel to the hair fiber growth direction, representing 90% of the hair mass [5,6]. Its structure and structural organization are responsible for the mechanical strength of the hair fibers [6,7]. It possesses long cylindrical assembled cortical cells surrounded by the cellular membrane complex [1]. In addition, the cortical cells are composed by macrofibrils surrounded by the intermacrofibrillar matrix and melanossomes [4,6]. Each macrofibril is composed by several microfibrils, and these are composed by keratin intermediate filaments arranged in a spiral form inserted in a sulfur-rich matrix containing KAPs [2,7,15]. The KAPs play an important role in the formation of a strong hair shaft through the disulphide bond crosslinking with the keratin intermediate filaments [24,29].

2.1.3.3 Medulla

The medulla is located in the center zone of the hair fiber and can be continuous, occur intermittently along the hair shaft or can even be absent [5,6]. These structures constitute a small percentage of the hair mass and are only present in the thickest hair fibers [2], consisting of specialized cells containing amorphous material and air spaces [1]. The medulla is formed from trichohyalin protein and contains glycogen and melanosomes [7]. Its function remains undetermined however it is known that medulla provides an insulating capacity to the hair [28,30] and has also a role in the light scattering, improving hair brightness [31].

2.2 Hair Cosmetic Products

Cosmetic products are used every day by thousands of people all over the world, and the application of these products is becoming an important daily routine. Humans use cosmetic products to improve self-esteem, to clean different parts of the body, to slow down the aging process, to enhance beauty and to protect the skin and the hair from the damage caused by chemicals compounds, ultraviolet light, pollution and other environmental factors [17,18].

Cosmetics products can be classified according to their use and area of application, such as decorative cosmetics, skin care, oral care, sun care, and perfumes. Regarding hair cosmetics products, they include shampoos, conditioners, colorants and hair styling preparations for temporary or permanent waving and straightening [17].

Despite the increasing consciousness regarding the safety and environmental risks of hair cosmetic products, there is still a great number of formulations which contain toxic and hazardous chemicals for humans and environment. Some cosmetic ingredients used in hair dying and hair modeling (straightening and curling) can be classified as poisonous or deleterious and may cause, after continuous exposure, allergic reactions, sensitization, skin irritation and hair loss [32]. Formaldehyde or formaldehyde-related compounds, are highly used in straightening formulations, and have been associated with adverse reactions such as eye and respiratory irritation, headaches and dizziness (nervous system problems), nausea, chest pain, vomiting, and rash [33]. Lead acetate, a recognized color additive for hair coloration, is a well-known neurotoxin related with several disorders like mental retardation, behavioral problems, nerve damage, Alzheimer's disease, Parkinson's disease and possibly schizophrenia [34]. The linkage between hair dying and hair straightening ingredients with the increasing risk to develop cancer has also been reported by many authors. Gago-Dominguez *et al.*, 2001, demonstrated the increasing risk in the development of bladder cancer for women that uses permanent dye [34], while Eberle *et al.*, 2019, reported a higher breast cancer risk associated with the use of cosmetic compositions for hair straightening or permanent dying [35]. In addition, cosmetic compositions can have a negative impact in the environment. The effect of hair formulations in the environmental pollution are mainly due to the presence of harmful chemicals like ammonia, p-phenylenediamine, lauramide DEA, benzene, toluene and colored compounds capable to contaminate the water systems by exposure to waste or waste by-products, affecting both local and distant ecosystems.

The society increasing concern about the environment and the desire to have a more natural lifestyle stimulated the research on more natural compounds for hair cosmetics. These new compounds are aimed to substitute the synthetic organic compounds currently used in several hair cosmetic products. The design and production of new eco-friendly hair formulations are thus seen has an excellent solution to mitigate or even avoid the negative impact of some cosmetic compounds.

Lately, proteins, proteins hydrolysates, peptides or even fusion proteins have been explored as a new class of eco-friendly products.

2.2.1 Hair cosmetic products with proteins and peptides

The term 'protein' includes a variety of polymeric compounds with a wide range of molecular weights, amino acidic sequence, secondary structures, and roles in biological processes [36]. In cosmetic industry, most of the proteins and protein derivatives are obtained from animals or plants, and, in a lesser extent, from algae or fungi [36]. The principal criteria for the selection of protein source is the protein availability and the production costs. However, the increasing aversion for animal-derived compounds and the ecological interests have now an important role in this decision [36,37]. The beneficial effects obtained from the protein or protein-rich substances are related with the proteins' capacity to bind water molecules, creating a suitable environment for healthy hair. In the case of protein hydrolysates, the beneficial effects are also dependent on their amphoteric and buffering properties [37].

The development of protein-based hair cosmetic formulations takes advantage of proteins properties and functions, which can be translated into the hair, to improve, recover and change some of the hair properties. Many authors have been recognizing the beneficial effects of using protein-rich substances in hair cosmetic formulations (**Table II.3**), and cosmetic products with proteins, protein hydrolysates, peptides and amino acids in their composition are currently used for: (i) hair strength and protection, (ii) hair shape and volume, (iii) hair coloring, (iv) hair smoothness, (v) hair gloss, and (vi) hair moisture.

2.2.1.1 Proteins and peptides to improve hair strength and protection

Human hair is a highly structured fiber divided in three main regions (cuticle, cortex and medulla), being mainly composed by keratin, a tough fibrous protein with a high sulfur content [38]. The cortex is responsible for hair properties like strength and elasticity, while the cuticle is the outmost layer of hair and is responsible for the protection of hair cortex. Despite the outstanding properties given by its structure and composition, hair is prone to damage induced by treatments like bleaching, coloration, waving and straitening. This procedures usually use harsh chemicals that can damage the hair cuticles, thus exposing the cortex to degradation [39,40]. Also, hair exposure to diverse environmental factors or daily hair care routines like combing, washing or application of heat devices can degrade the keratin proteins from hair, weakening the hair fibers [41]. The development of a novel class of hair care formulations and products, which can modulate the hair properties while conferring protection, is therefore foreseen of great importance for the cosmetic industry.

Barba *et al.*, 2010, tested two keratin proteins isolated from wool for hair protection and for the improvement of hair properties [40]. The keratins included an intact keratin intermediate filament protein extract and a low molecular weight keratin peptide derived from intermediate filament proteins, composed

by 6-8 amino acids. The effect of the keratin protein and the peptide was tested on virgin, overbleached and permed hair. When the damaged hair samples were treated with the peptide, the surface contact angle increased to values in the range of the values measured for the untreated virgin hair, indicating a possible restoration of hair fiber surface hydrophobicity promoted by the peptide. The keratin protein also improved the hair surface's characteristics and fibers' hydrophobicity, however at a lower extent. The protein and the peptide were also capable to improve the hair mechanical properties, however at a greater extent for the keratin peptide due to its ability to strongly interact with the hair mainly by disulphide bonds. The change in the mechanical properties was also related with an increase in the fibers' water content promoted by the protein and peptide. This higher hydration degree improved the fibers' ability to be deformed allowing the formation of new disulphide bonds between the cystine groups of different keratin molecules, imparting elasticity to the fiber [40]. This effect of the keratin protein and keratin peptide extracted from the wool on the hair water retention was also demonstrated by the same authors and is described later in this article [42].

Fernandes *et al.*, 2012 [43], studied the effect of a keratin-based peptide (KP) as a strengthening agent for relaxed damaged African hair. This peptide has two cysteine residues and is based on a fragment of hair keratin type II cuticular Hb5 protein. The straightening of the African hair was promoted by the disruption and rearrangement of the disulphide bonds responsible for the hair shape. Two formulations, aqueous and ethanolic, were tested to induce the penetration of the peptide into the hair fiber cortex. Independently of the tested formulation, the treatment with KP was able to restore the fibers' mechanical properties, Young's Modulus and maximum strength, in 28-31% and ≈13%, respectively, when compared with the damaged relaxed hair. In combination with the mechanical properties, the fibers' thermal stability was also assessed where it was demonstrated the repairing effect of KP over the fibers' damage and its capacity to chemically interact with the α-helix structure of keratin. The best results were obtained for the KP incorporated in the aqueous formulations, indicating its potential to be used for the development of new hair care products [43].

In another work from Fernandes *et al.*, 2012 [44], two peptides containing cysteine residues, KP and SPB (pulmonary associated surfactant protein B), were tested as agents for the improvement of the strength and integrity of overbleached caucasian hair. The incorporation of the protein disulphide isomerase (PDI) into the peptide formulations was assessed since this protein is capable to stimulate the peptide penetration into the hair cortex and catalyze the peptide grafting into the hair fibers through the formation of disulphide bonds **(Figure II.3)**. The presence of PDI in the formulation increased the uptake of the KP peptide but decreased the uptake of SPB. Despite the lower uptake of the SPB peptide promoted

by the PDI, this peptide presented a higher affinity towards hair than the KP peptide. Regardless of the penetration degree, the two peptides were able to recover the mechanical properties of the overbleached hair, supporting their potential to be included into hair care cosmetic formulations [44].

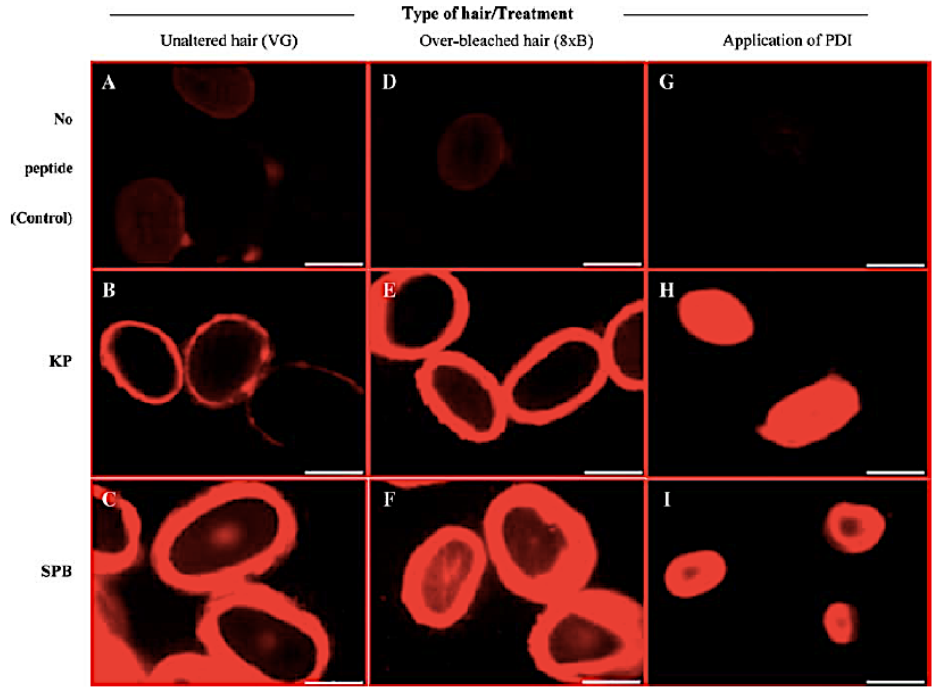


Figure II.3 - Fluorescence images of human hair cross sections from unaltered human hair (A, B and C), overbleached hair (D, E and F) and overbleached hair with PDI (G, H and I). KP and SPB were used to treat all fibers. Bars indicate 50 μm (adapted from Fernandes *et al.*, 2012 [44]).

To improve the binding efficiency of keratin to the hair, Basit *et al.,* 2018 [45] used keratin proteins extracted from human hair. They tested the effect of K31 protein on the thickness and strength of chemically damaged hair (overbleached). This protein is a type I acidic keratin and is described to be involved in the maintenance of hair tensile strength. After the treatment of overbleached hair with the K31 protein, it was verified an increase in 49% in the fibers' thickness and an improvement in the mechanical properties, with an increase in 86% and 135% in fiber's Young's modulus and tensile strength, respectively. These results could indicate that K31 protein can be integrated into the damaged hair by filling the gaps and into the hair intermediate filaments by making disulphide bonds with the damaged keratin [45].

A new wheat-based formulation developed by Shah, in US 6,156,295 [39], was applied in different types of hair to strength and repair damaged hair fibers. The formulation consisted of an aqueous mixture of hydrolyzed wheat protein, wheat amino acids and panthenol. The beneficial effects of this formulation

were associated with the synergistic action between the amount of hydrolyzed wheat proteins, wheat oligosaccharides and wheat amino acids. Besides the strengthening of the hair fibers, this formulation was able to improve the hair moisture retention capacity, helping to prevent further damaged cause by excessive use of cosmetic procedures. The role of the wheat protein and wheat oligosaccharides as moisture regulators also affected other fiber mechanical properties like elasticity, principally in bleached and permed hair [39].

Silk proteins and silk amino acids had been studied by some authors to be included in formulations to improve hair strength. Ross, in US 9,023,404 B2 [41], developed a formulation for hair and skin application containing silk amino acids/protein complexes, seed oils, poly and monosaccharides and botanicals. When applied over hair, this invention was capable to improve hair condition and hair strength. The water-soluble silk protein deposited over the hair fiber and formed a smooth and durable film, providing protection against daily hair care routines and against environmental damage [41].

Water soluble silk proteins and peptides are also described by Fahnestock, in US 7,060,260 B2 [46], as agents capable to form a durable and protective film over the hair fibers, improving some hair properties like smoothness and strength. The formulation was also capable to protect the hair from damage caused by grooming and styling, shampooing, exposure to ultraviolet light and adverse weather conditions, and from reactive chemicals able to denature the hair keratin proteins like coloring, bleaching and straightening agents [46].

A formulation capable to protect hair from UV light was developed by Cannel, in US 6,013,250 [38], where hydrolyzed proteins rich in anionic amino acids, particularly, sulphur containing amino acids, were conjugated with divalent cationic compounds and vitamins to protect the hair fiber. For this study, the protein hydrolysates were obtained from different sources like keratin, milk, collagen, soya or wheat. The hydrolyzed proteins were able to shield the hair against the damage imparted by UV light or harsh chemicals. The ratio between the divalent cationic compounds and the anionic amino acids present on the hydrolyzed proteins determined the ability of the formulation to form an efficient protective protein layer around the hair. The vitamin included in this invention was also capable to protect the hair from photo damage, as it can inhibit the oxidation of hair while absorbing the UV light in a region between the 290 and 420 nm. Besides UV light protection, this formulation also improved hair mechanical properties, smoothness, shine, manageability, moisture and softness [38].

The combination of corneocyte proteins/peptides and transglutaminase was developed by Green *et al.*, in US 5,525,336 [47], to form a protective layer around the hair fibers. Corneocyte proteins may include different proteins like involucrin, loricrin, cornifin, trichohyalin, sciellin, profilaggrin and keratolinin. The

transglutaminase was included into the formulation to act as a crosslinking agent capable to form isopeptides bonds between the corneocyte proteins/peptides and the proteins of the hair surface. The formulation formed a layer over the hair and was able to protect the hair from physical, environmental or chemical damage [47].

A protein from the human eye, was explored by Ribeiro *et al.*, 2013 [48], to be included into a hair cosmetic formulation as an agent for the strengthening of damaged hair. The human eye γD-crystallin belongs to a superfamily characterized structurally by Greek key motifs, which are described to be involved in the coating of diverse structures. The ability of the protein to form a thin-layer around the hair fiber and act as a protective agent was evaluated. For this, two proteins were tested: the wild-type crystallin and a mutant crystallin with three extra cysteine to promote protein aggregation and binding to the hair. Both proteins were able to bind to the hair and to form films, which were uniform for the wild-type and with small aggregates near the cuticles' edges for the mutant crystallin. The two crystallins proteins were able to penetrate into the hair cortex **(Figure II.4),** which was determinant for the recovery and improvement of the hair mechanical properties, measured in terms of Young's modulus and tensile strength.

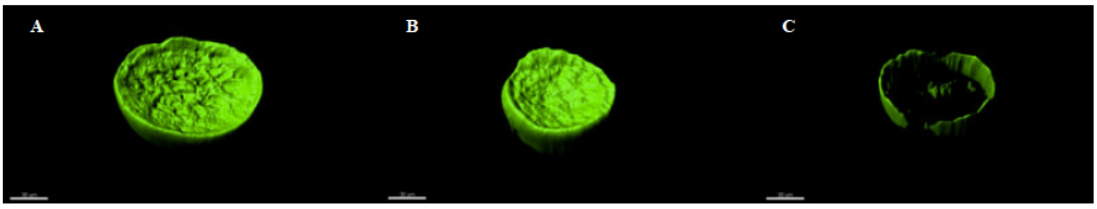


Figure II.4 - Confocal microscopy of human hair cross sections. A: overbleached hair treated with mutant crystallin labelled with fluorescein 5(6)-isothiocyanate (FITC); B: overbleached hair treated with wild type crystallin labelled with FITC; C: unaltered Asian virgin hair (adapted from by Ribeiro *et al.*, 2013 [48]).

A formulation composed by soy proteins was developed by Schultz *et al.*, in US 2005/0008604 A1[49], for hair care cosmetic applications. The soy proteins were initially treated with reducing agents to improve the protein reactivity. These proteins were then reacted with a carboxylic acid anhydride to impart the proteins with film-forming properties. The modified soy proteins were able to form a protective film around the hair fibers after 60 minutes of incubation, and were able to improve hair's mechanical properties, retain the moisture and improve hair conditioning [49].

Synthetic peptides identical to human hair were reported by Azizova *et al.*, in US 2012/0052034 A1 [50], as substitutes to the peptides obtained through the hydrolysis of human hair or sheep wool for cosmetic applications. These peptides sequences were identical to the fragments of KRT35 and KRT85 proteins,

expressed in the hair-forming matrix of the cortex and cuticles, and had molecular weight between 500-700 Da so they are able to penetrate into the hair shaft. To increase the peptides substantivity to hair, cysteine and arginine residues were selected as terminal amino acids. The cysteine is largely present in human hair and participate in disulfide crosslinks, having a high impact in the binding of proteins to the hair; the arginine is also an aminoacid present in the keratin protein and can improve the hair capacity to retain moisture due to its high hydrophilicity. Also, other amino acids were included into the sequence based in their capacity to interact with the hair proteins: tyrosine for hydrogen bonds; glycine, valine and leucine for hydrophobic bonds; and lysine, histidine, aspartate or glutamate for saline bonds. After application on hair, the selected peptides were able to improve hair strength and repair the keratin fibers, with a positive effects on hair conditioning [50].

2.2.1.2 Proteins and peptides to change hair shape and volume

Haircare industry has developed different products capable to modify hair characteristics, with the hair straightening and hair curling being the most popular processes [51]. The chemicals used for hair shaping have a high toxicity to the environment and human health and cause extreme damage to the hair fibers. Excessive or repeated hair chemical treatments negatively affect the top-layer of the hair cuticle, reducing hair hydrophobicity and decreasing the hair cysteine content. These processes results in the loss of keratin structure and decrease of hair mechanical properties, with the hair becoming more dry, brittle and with less color and shine [52].

Salt linkages, hydrogen bonds and disulphide bonds are primarily responsible for hair shape [53]. As the natural shape of hair greatly depends on the orientation of disulphide bonds, their rupture and reorientation is necessary to obtain a long-term effect of the new hair shape [52]. For this, the hair disulfide bonds are reduced through the application of a sulfur-containing agent, along with the application of mechanical stress. Afterwards, new disulfide bonds are allowed to form with the help of an oxidizing agent, in a new orientation, thus giving the hair a new shape [52]. The alteration of hair shape can also be obtained by the use of heat, however only a temporary hair style is obtained since there is no permanent alteration in the disulfide bonds orientation. The heat styled hair will return to its natural shape after a short-term as a result of perspiration, exposure to high humidity and especially after shampooing [54].

Considering the current strategies used for the alteration of hair shape, it is important to develop new cosmetic products capable to permanently change the shape of hair without damaging the hair fibers.

Ideally, the new products should protect the hair style against humidity and wind and impart good feel and conditioning to the styled hair, while the shape of the hair is retained [55].

Hawkins *et al.*, in US 9011828 B2 [52], reported hair straightening compositions comprising transglutaminase, polylysine, sodium metabisulfite and a pH stabilizer. The polylysine was included in the formulation to take advantage of its cationic character and amino acidic sequence enriched in L-lysine residues, to form a surface barrier film around human hair. The polylysine film acts as a vapor and moisture barrier while protecting the straightened hair from proteolytic damage, and from damage caused by environmental factors. This composition was able to straight permanently the hair through alterations in its protein structure, while the surface barrier film provided some mechanical hold and protection from ambient humidity. The hair was maintained straightened for at least 12 shampooing cycles, and the hair fibers presented significantly less damage when compared to fibers modified with known heat and chemical treatments [52].

Emmanuel Dimotakis *et al.*, in US 2013/0309190 A1 [56], developed hair cosmetic styling compositions with maleic acid copolymers, polyamino acids (like polylysine), aminated polysaccharides (like chitosan), a neutralizing agent and water. Maleic acid copolymers are known in cosmetics industry as "fixing agents", making it possible to maintain the hair in a specific shape, while the polyamine is responsible for the cuticle sealing, increasing the hair resistance to humidity and to color fading. An improvement in the hair fibers straightening was observed when the formulation was applied during the styling of the hair [56].

Marion Detert *et al.*, in EP 1878423 A2 [57], described a cosmetic preparation for hair styling, composed by protein hydrolysates from cashmere wool, and at least one fixing polymer. The cashmere wool hydrolysates were used to enhance the properties of the fixing polymers in the cosmetic hair styling preparations. The reported formulation, is considered mild to the skin and hair, and leads to an improvement in the hair structure and in the hair's physical and optical properties. After the application of the formulation, the hair presented a smooth texture with increased strength and long-term maintenance [57].

Grollier *et al.*, in 4,673,568 [58] reported a cosmetic formulation capable to improve hair shape-retention. The formulation was composed by a water-dispersible cationic surfactant, a water-soluble quaternized protein and a cationic silicone polymer. When the components were combined in the hair-care cosmetic formulation, simultaneous deposition of the components on the hair was observed, increasing hair properties like shape-retention, disentangling, softness and shine. The quaternized proteins included in

this formulation were able to improve hair disentangling and softness without the disadvantage of making hair heavier and imparting an oily appearance [58].

Pigiet, in US 5,028,419 [59], applied a thioredoxin-derived dithiol peptide in combination with a sulfite or bisulfite compound for hair styling, waving or straightening. The thioredoxin-derived dithiol peptides were obtained by the cleavage of intact thioredoxins and contain a pair of cysteine residues which form a redox-active disulfide. The presence of this redox-active disulfide enhanced the reductive properties of sulfite or bisulfite compounds, and lead to the reduction of the time necessary to wave the hair and the application of lower amounts of sulfite and bisulfite compounds during hair treatments [59].

Cauwet-Martin *et al.*, in US 6,039,962 [60], described a new cosmetic composition with improved cosmetic properties particularly developed to hold the hairstyle for longer times. The invention comprise an aqueous dispersion composed by lipid ceramide compounds and a peptide with at least one fatty chain. The ceramides were included into the cosmetic composition to protect hair against environmental agents and mechanical or chemical treatments. The presence of the modified peptide improved the ceramides binding to the hair fibers, contributed to the formulation stability and also improved the ability of the formulation to hold a specific hairstyle [60].

A formulation for the permanent shaping of hair containing a modified cationic surfactant was described by Thorsten Cassier, in WO 2008081348 A2 [61]. The surfactant can be modified with protein derivates or protein hydrolysates obtained from wheat, casein, collagen, keratin, and silk. The formulation proved to permanently change the hair shape, to improve the hair fastness to humidity, to deliver unique elasticity and strength to the shaped hair, and to improve the uniformity of the shaped hair with a less disordered appearance [61].

A keratin concentrate composition was explored by Savaides *et al.*, in US 2014/0261518 A1 [54], to be applied over hair providing long lasting curl reduction and styling results for the keratin fibers, with only one application. The active compounds englobe a keratin mixture composed by a keratin concentrate with low and high molecular weight hydrolyzed keratins, and a keratin sealer comprising at least one oxidizing agent. The low molecular weight keratins penetrated into the hair and their effect was more notorious in hair internal areas like the cortex and matrix. The high molecular weight keratins interacted with the external areas of the hair (cuticle and cell membrane complex) and were responsible for the formation of a strong film over the hair surface, improving the styling effects [54].

Yoshioka and Kamimura, in US 4,279,996 [62], also used keratin hydrolysates for hair cosmetic applications. They developed a formulation for the permanent waving of the hair using water-soluble keratin hydrolysates. These hydrolysates had a minimum of two mercapto groups per molecule and an

average molecular weight between 2.000 and 20.000 Da. When the keratin hydrolysate was incorporated into a formulation with ammonium thioglycolate, polyoxyethyletiesorbitan monooleate, EDTA, a polypeptide derived from collagen, and purified water, the resulting waved hair was maintained for about 2 months under normal conditions. After application of the water-soluble keratin hydrolysate, the hair was shaped, and the mercapto groups of the keratin molecules were oxidized to form disulfide bounds between the keratins. The formation of several disulfide bounds, converted the water-soluble keratin hydrolysate into a water insoluble polymer film deposited over the hair, promoting the curling of the hair. The conversion into a water-insoluble polymer makes this formulation highly resistant to wash, increasing the durability of the curling method [62].

A synergistic effect between hydrolyzed silk protein and a water soluble or emulsifiable silicone-based compound was reported by Edman *et al.*, in US 4,798,722 [53] for permanent hair waving. This new permanent waving composition imparted the hair with increased curl retention, as well as improved hair manageability, feel and appearance. The inventors used silk due to the high percentage of strong hydrogen bonding amino acids like glycine, alanine, serine and tyrosine. Due to the small size of the silk hydrolysate, the peptides penetrated the cuticle of undamaged hair and reached the cortex area of the fiber. The incorporation of a silicone-based compound provided the hair with lubricity for increased slip and easy combing action. The formulation was able to provide a long lasting and increased curl retention ability, and improved hair luster, gloss, sheen and manageability [53].

Cruz *et al.*, 2017 [51], studied the capacity of eight engineered small peptides with different cysteine content and hydrophobic character, to act as a substitutes to the harsh chemicals used to straight the hair. These peptides present the ability to interact with keratin as they were based on the sequences of human keratins and keratin-associated proteins. The peptides (PepA, PepB, PepC, PepD, PepE, PepF, PepG and KP) were able to penetrate the hair fibers of African and overbleached Caucasian hair, reaching the cortex and interacting with the hair proteins. The 8 peptides were able to improve the tensile strength and the elasticity of overbleached Caucasian hair, most likely due to the re-establishment of hydrogen and disulphide bonds lost during the chemical treatment. Moreover, the peptides were able to efficiently straight the African hair by the creation of new disulphide bonds between the peptide and the hair proteins (**Figure II.5**). The authors also demonstrated that the action of the peptides was dependent on the reactivity of each cysteine and its accessibility to establish new bounds, on the residues in the vicinity of the cysteines, on the charge–charge interactions and on the location of the cysteines in the peptide structure [51].

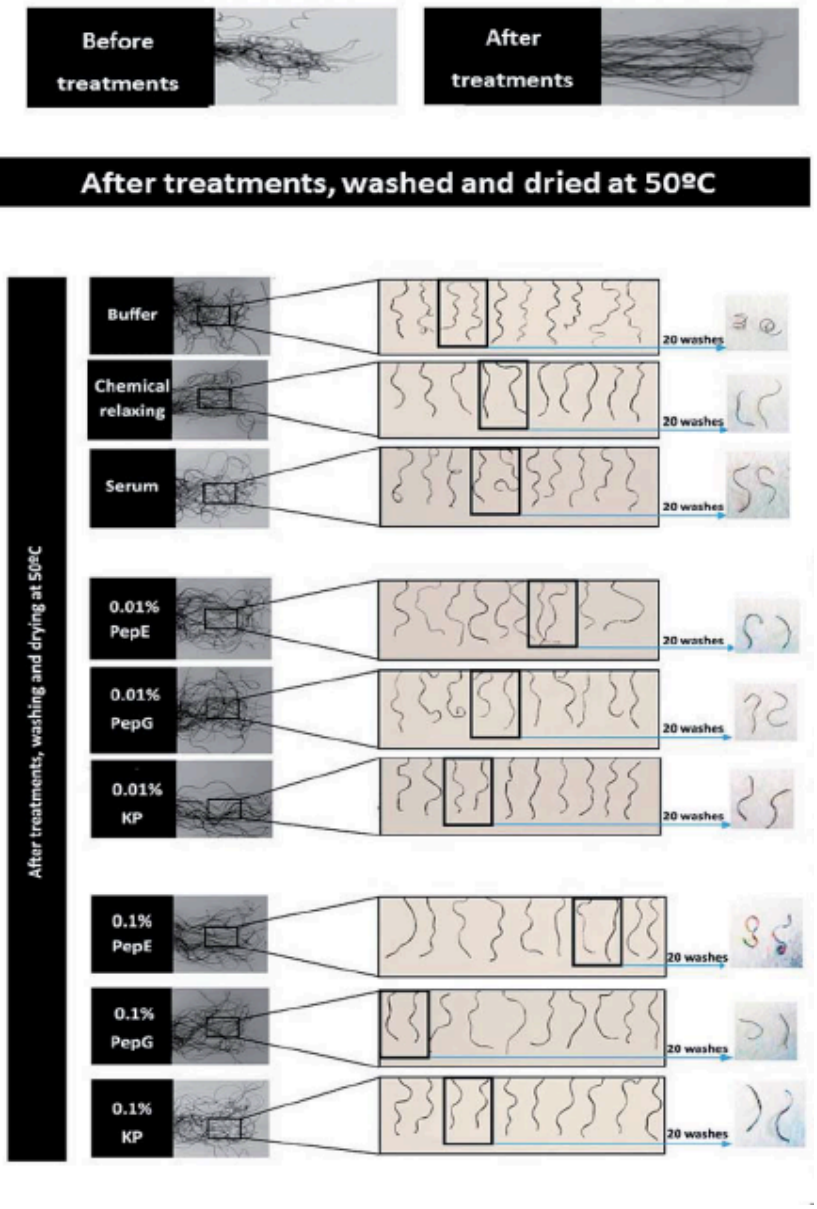


Figure II.5 - Curly hair tresses before and after peptide treatments; after treatments, the hair tresses were washed and dried at 50 °C. The images at the top show the samples treated without peptides (buffer, chemical and base serum formulation treatments); the images in the middle present the samples treated with the serum formulations containing peptides (PepE, PepG, KP) at 0.01% (w/v); the bottom images present the samples treated with the serum formulations containing peptides (PepE, PepG, KP) at 0.1% (w/v);

Considering the effect of humidity on hair shape and volume, new formulations based on the KP peptide were developed by Cruz *et al.*, 2017 [63]. The KP peptide is based on the sequence of a cuticular keratin from human hair, and was used to enhance the hair moisture resistance under different humidity conditions **(Figure II.6)**.



Figure II.6 - Images of Caucasian hair tresses at the beginning of the test (0 h) and after 6 h at 90% relative humidity (RH). The values indicated correspond to the volume change, in percentage.

Water molecules can change the physical properties of the hair fiber by breaking non-covalent bonds, reducing the fibers' tensile properties (elasticity and stress at break), and changing the static charge and electrical resistance of hair. When the KP peptide was incorporated into a water-based climate formulation, a low increase of the hair tresses volume, at low (40%) and high (90%) humidity conditions, was observed. These results were improved when the peptide was applied to the hair in an ethanol-based formulation. The ability of the ethanolic formulation to swell the hair fiber, promoted the penetration of the peptide into the hair cortex, improving the results for hair moisture resistance [63].

2.2.1.3 Proteins and peptides to change hair color

Hair coloring agents may be divided into three categories: permanent, semi-permanent and temporary. The permanent hair dyes are generally oxidative dyes that provide hair color that lasts between four to six weeks. The oxidative hair dyes compositions are formed by oxidative dyes and an oxidizing agent such as hydrogen peroxide, and involves the formation of oligomeric or polymeric compounds in and/or on the hair fiber. Despite the long-lasting color provided by the oxidative hair dyes, the presence of oxidizing agents damages the hair, affecting the overall properties of the hair fibers. The semi-permanent dyes are less aggressive to the hair but they only provide color for about six to twelve shampoos. Temporary hair dyes are coloring agents that are applied to the hair surface and are removed relatively easy after one shampoo [64,65]. The major problem with the current non-oxidative hair dyes is that they lack the required durability necessary for long-lasting effects. For this reason, the development of new hair coloring agents that provide the durability of the permanent hair dyes without damaging the hair, is of outmost importance for hair cosmetic research and industry [65].

One of the strategies currently used in the development of new coloration agents is the exploration of peptides, with the ability to bind to the hair, conjugated with coloring agents. Huang *et al.*, in US 7,220,405 B2 [64], described the use peptides sequences with high affinity towards hair to improve the hair coloration durability. These peptides can be conjugated with a coloring agent to color the hair, or with a conditioner agent to improve the hair conditioning [64].

Peptide-based reagents for hair application, obtained by coupling a dyed-hair-binding peptide to active agents, are described by Benson *et al.,* in US 8,273,337 B2 [66]. In this technology, peptides with strong affinity to dyed hair were engineered to deliver active agents to the surface of dyed hair or to be used as a peptide-based sealants, improving or enhancing traditional colorants and its durability on the hair [66].

Lang *et al.,* in US. Pat. No. 5,192,332 [67] described a cosmetic temporary coloring composition that comprises a cosmetically acceptable support and, as an active ingredient, at least one chemically modified protein derivative containing dyes grafted into the protein chain. The chemically modified proteins were from animal or vegetable source, and they were used to color keratinous fibers, in particular, human hair. This coloring composition presented good cosmetic properties: (i) improved solubility in common cosmetic solvents, (ii) formation of a flexible protective film over the hair and (iii) easy removal of hair coloration by washing [67].

Hair coloring agents consisting of an anti-keratin antibody covalently attached to a dye or pigment were described by Igarashi *et al.*, in US 5,597,386 [68]. The functionalized antibody has the ability to bind to the hair, improving the hair coloration and the resistance of the color to fading. The coloring agent may

be a coloring substance such as an inorganic pigment, a water-soluble or an insoluble coloring matter, or a composite of the coloring substance. Since it provides a good and strong coloring capability specific to hair, the hair dye of the present invention did not stain the skin neither caused skin irritation. In conjugation with the coloration capacity, the tactile properties were also improved [68]. A similar study was performed by Terada *et al.*, in JP 2002363026 where single-chain antibodies, preferably anti-keratin, where coupled to dyes, ligands, and cosmetic agents for hair care compositions [66].

Antibodies with affinity to the surface layer of the hair were also studied by Kizawa *et al.*, in JP 09003100. The antibodies were coupled to colored latex particles and were used to color the hair. The application of the modified antibodies improved the hair coloration as well the duration of the color. Despite the good results reported, the use of these antibodies was hindered by their cost and difficulties associated with its production [66].

Renimel *et al.*, in WO 1995000115 A1 [69] have identified a promising peptide sequence for hair lightening. The depigmenting composition was composed by a peptide chain that comprises at one of its two ends a lysine group (Lys), and at its other end an alanine group (Ala). The depigmenting activity was related with the inhibition of tyrosinase, the enzyme involved in the synthesis of melanin, the pigment responsible for the hair coloration. In this way, when the tyrosinase was inhibited, the production of melanin was reduced or suppressed, changing the hair coloration. When incorporated into a cosmetic formulation and applied on the scalp for several days, the peptide was able to produce a lightening effect on hair [69].

The alteration of the tyrosinase activity as a coloring method was also explored by Rao, in US 2004/0043463 A1 [70]. This invention describes the administration of a fusion protein comprising an enzyme or an enzyme inhibitor linked to at least one signal peptide. The present invention succeeded in regulating the intracellular activity of melanocyte tyrosinase by delivering the fusion protein through topical application. When the objective was the increase of the pigment production, the fusion protein act primarily by increasing the activity of the mammalian melanocyte tyrosinase, resulting in the production of pigment in the melanocytes of hair follicles. When the fusion protein comprise an enzyme inhibitor, an opposite behavior was verified, with a decrease in the pigment production and consequent lightening of the hair [70].

The invention reported by O'Brien *et al.,* in WO 2007/126641 Al [71] relates to diblock and triblock peptide-based body surface coloring reagents comprising body surface-binding peptides and pigment-binding peptides for personal care. This invention can be used to specifically attach pigments to different body surfaces, being capable to color specifically hair, skin and nails [71].

An optimization of the hair dyeing process using proteins or protein hydrolysates with high arginine content combined with a peroxide and various oxidation dye precursors, is described by Milbrada, in US 3,861,868 [72]. When this composition was applied on hair in a pH range between 9-10, an increase in the hair dyes stability was observed. The increase in the dye stability was associated with the pH and with the presence of arginine residues that enhance the penetration of the dye precursors into the hair fibers [72].

2.2.1.4 Proteins and peptides to improve hair smoothness/gloss/moisture

Hair manageability, glossiness and softness are normally provided by sebum. However, daily hair care routines such as combing, waving, straightening, coloring, and bleaching, can remove the protective lipid layer of the hair turning the fibers prone to damage. Hair conditioners are usually used to decrease friction between the hair fibers, detangle the hair, minimize frizz and improve hair combing. Conditioners also add positive charges to the hair fibers, neutralizing the negative charge of the hair surface induced by hair brushing, thus increasing the hair manageability. They tend to deposit in the cuticles' edges, reducing fiber hydrophilicity and fibers friction, while increasing hair smoothness by promoting cuticles adhesion to the hair shaft. Although the improvement and protection given by the conditioners, other cosmetic formulations based on natural products like proteins and peptides, are needed to improve or avoid the loss of smoothness, moisture and gloss caused by daily care routines [73,74].

Barba *et al.*, 2010 [42], explored wool proteins and peptides to improve the water sorption properties of the hair fibers, using for this purpose an intact keratin intermediate filament protein and a low molecular weight keratin peptide derived from intermediate filament proteins. The wool derived protein and peptide, were applied over virgin and overbleached hair, and significant results were obtained for the overbleached hair. The treatment of overbleached hair with the keratin protein and keratin peptide restored the integrity of chemically damaged hair, inducing a decrease in the fiber diffusion coefficient which in turn decreased the hair fibers' permeability. After treatment, the overbleached hair fibers recovered the ability to retain water, presenting retention values similar to the values recorded for the virgin hair [42].

A method for repairing or restoring damaged hair was developed by Hirata, in US 6,521,219 B1 [75]. In this invention, amino acids and peptides were applied to the hair and its penetration into the hair fiber was promoted by an alteration between a hydrated and compressed state. The amino acids (glycine, alanine, arginine, proline, and serine) and peptides (hydrolyzed collagen, hydrolyzed keratin and other hydrolyzed animal proteins), when in the hair cortex, were able to compensate the low protein content of the cortex of damaged fibers, restoring the hair structure. Also, the capacity of the amino acids and

peptides to retain water in conjugation with the cuticle's reorientation and alignment into a certain direction by the compression of the hair shaft, improved some hair properties like hair smoothness, resilience and shine [75].

Keratin hydrolysates were prepared by Villa *et al.,* 2013 [76], to be incorporated into hair care cosmetic formulations. The keratin hydrolysates prepared from feathers using microbial peptidases from *Bacillus subtilis*, were able to penetrate efficiently into the hair fibers subjected to different cosmetic treatments (bleaching, coloration, and relaxation). After treatment, an improvement in the hair hydration degree, brightness and softness was observed for all the samples, particularly when the hair fibers were dried using a straightener. The improvement was also related with the deposition on the keratin fragments in the cuticle edges, contributing to the sealing of the cuticles. The heating step promoted the incorporation of the hydrolysates into the hair fibers and the complete sealing of the cuticles [76].

Mancon *et al.*, 2012 [77], studied the potential of shampoos with proteins obtained from moringa, peas, wild soybean and combined with anionic and non-ionic surfactants, to improve hair gloss and hair conditioning. When tested on cold waved hair, the formulation containing the moringa proteins presented a better conditioning effect, while a better sensory performance was obtained when the pea proteins were applied over bleached hair. Generally, an improvement in the gloss index was obtained for all of the proteins tested regardless of the type of hair, confirming the ability of the plant based proteins to be used in hair cosmetic formulations [77].

A milk protein hydrolysate prepared by enzymatic hydrolysis was reported by Tomita, in US 5,314,873 [78], as a new formulation for the improvement of hair properties. The milk protein hydrolysate was composed by peptides with a molecular weight lower than 1.000 Da and free amino acids. The milk hydrolysate or the fractions prepared from the hydrolysate mixture, when applied on hair, were able to improve hair conditioning and hair smoothness, and to recover the hair from damage and dryness [78].

A cosmetic composition to be applied over hair and improve its sensory properties, and comprising protein hydrolysates, extracts from the threads of the mussels and sericin and/or sericin hydrolysates was developed by Picano *et al.*, in US 2007/0041925 A1 [79]. The protein hydrolysates present in the formulation were obtained from silk, pashmina, cashmere and merino wool. When applied together as a cosmetic formulation, the active ingredients presented a synergistic effect and the hair acquired a natural and healthy appearance, becoming more shining and smoother. An increase in the hair moisture degree, hair fullness and suppleness, was also verified, which had a significant impact in the capacity of the hair to be styled [79].

Fatty acids can be also included in hair cosmetic formulations when conjugated with silk protein-derived peptides with a size between 300-600 Da, forming a salt of an acylated compound. The proteins contained at least 40% of branched fatty acids with 20 to 32 carbon atoms, being this ratio important to guarantee a sufficient adsorption into the hair fibers. This formulation was developed by Oshika *et al.*, in US 5,747,015 [80] and the hair treated with the compounds became free from any tackiness or greasiness. An excellent conditioning effect was also verified, even in dry hair and after several shampooing steps, resulting in an improvement of hair moisture, smoothness, softness and gloss [80].

Table II.3 - Effect of proteins/peptides included into cosmetic formulations to improve hair properties.

Author/ reference	Year	Protein/Peptide Source	Protein/Peptide	Effect on Hair Properties
Milbrada [72]	1975	Animals (ex: herring, salmon, fowl, rainbow/sea trout)	Protein/polypeptide/protein hydrolysates with high arginine content (ex: clupein, fontinin, iridin, gallin)	Increase dye stability
Yoshioka [62]	1981	Animal (ex: Wool, feather, hair, horn, hoof)	Keratin hydrolysates with two mercapto groups per molecule	Enable cold-waving modulation Decrease damage to the fibers
Grollier [58].	1987	Animal	Water-soluble peptides carrying quaternary ammonium groups (ex: chemically modified collagen hydrolysates)	Improve hair shape-retention Improve hair disentangling and softness
Edman [53]	1989	Silk worm cocoons	Hydrolyzed silk protein	Enable cold-waving modulation - permanent Increased curl retention ability Long-lasting hair style Improved hair manageability Hair with soft and silky feel
Pigiet [59]	1991	*E. coli*, yeasts, animals	Thioredoxin-derived dithiol peptides	Improve hair styling Decrease the time necessary to shape hair
Lang [67]	1993	Animal/ vegetable (ex: keratin/soya derived peptide)	Peptide–dye	Color
Tomita [78]	1994	Milk	Protein hydrolysate and amino acids	Improve hair conditioning Improve hair smoothness Recover damaged from hair fibers
Renimel [69]	1995	Chemical synthesizes	Peptide chain with Lys and Ala group in different terminal position	Lighten the hair
Green [47]	1996	Natural source or recombinant protein	Corneocyte proteins/peptides and transglutaminase	Hair protection against damages

Iganashi [68]	1997	Immunizing animals	Anti-keratin antibody–dye	Strong color capability Resistant to color fading Improve hair tactile properties
Oshika [80]	1998	Silk	Silk protein-derived peptides	Improve hair conditioning and moisture Improve hair smoothness and softness Improve hair gloss
Cannel [38]	2000	Plants, seeds, animal bonds or connective tissues	Hydrolyzed proteins with sulphur containing amino acids	Improve hair strength Hair protection against chemicals and UV light Increase hair smoothness and moisture Increase hair shine
Cauwet-Martin [60]	2000	Animal/ vegetable (ex: keratin, elastin, wheat, maize)	Peptide with a fatty chain	Improve hair style holding Improve hair smoothness and softness Protection Improve hair strength
Shah [39]	2000	Wheat	Hydrolyzed proteins and amino acids	Improve hair strength Repair the fibers' damage Increase hair moisture
Hirata [75]	2003	-	Amino acids (ex: glycine, alanine, proline) or peptides (ex: hydrolyzed collagen, hydrolyzed keratin)	Restore hair structure Improve hair smoothness and shine Improve hair resilience
Rao [70]	2004	-	Fusion protein–tyrosinase/tyrosinase inhibitor	Enhancing pigment production
Schultz [49]	2005	Soy	Soy protein	Long-lasting hair protection Improve hair moisture retention Improve hair conditioning
Fahnestock [46]	2006	Spider *Nephila clavipes*	Water-soluble spider silk protein and peptides	Improve hair strength Improve hair smoothness
O'Brien [71]	2007	Recombinant bacteria/fungi	Diblock and triblock peptide-based–pigments	Increase the dye binding Decrease the coloration time

Picano [79]	2007	Silk, pashmina and cashmere and merino wool	Protein hydrolysates and sericin hydrolysates	Maintain hair health Restore hair damage Improve hair shine and smoothness Improve hair moisture
Detert [57]	2008	Cashmere wool	Protein hydrolysate	Improve hair styling and long-term maintenance Improve hair physical properties Improve hair optical properties Improve hair smoothness Increase hair strength
Cassier [61]	2008	Animal/vegetable (ex: keratin, collagen, silk, elastin, wheat)	Cationic modified protein/protein hydrolysates	Long-lasting hair style Improve hair uniformity Improve hair fastness to humidity Increase hair elasticity and strength Improve hair conditioning
Barba [42]	2010	Wool	Keratin intermediate filament protein and low MW keratin peptide	Restore the integrity of bleached damaged fibers Improve fiber permeability Improve hair moisture
Barba [40],	2010	Wool	Keratin intermediate filament protein/peptide	Restore hair surface Increase hair hydrophobicity Improve hair mechanical properties
Hawkins [52]	2011	Animal/plant or microbial source	Transglutaminase and polylysine	Shape: straightening Hold hair shape for longer time Decrease hair damage Hair fiber protection
Azizova [50]	2012	Chemically synthesize	Human hair-based peptides	Increase hair strength Increase hair manageability Increase hair conditioning
Fernandes [43]	2012	Chemically synthesize	Hair keratin type II cuticular Hb5-based peptide (KP)	Increase hair strength Improve hair mechanical properties Increase thermal stability

Mancon [77]	2012	Vegetable (from *Moringa oleifera*, *Pisum sativum* and *Glycine soja*)	Protein	Improve hair conditioning Improve hair smoothness Improve hair gloss
Fernandes [44]	2012	Chemically synthesize	Hair keratin type II cuticular Hb5-based peptide and a peptide based on pulmonary associated surfactant protein B	Improve hair integrity Improve hair strength
Ribeiro [48]	2013	Recombinant bacteria *Escherichia coli*	γD-crystallin wild-type and mutant	Improve hair mechanical properties
Villa [76]	2013	Chicken feathers	Keratin hydrolysates	Improve hair hydration Improve hair softness Improve hair brightness
Dimotakis [56]	2013	-	Polyamines (ex: polylysine, polyaspartic acid)	Improve hair style maintenance Increase hair resistance to humidity Increase hair resistance to color fading Increase strengthening Hair fiber protection
Savaides [54]	2014	-	Keratin protein and keratin hydrolysates	Long-lasting hair style Composition non-damaging to hair Improve hair shine and softness Improve hair volume
Ross [41],	2015	-	Silk protein and amino acids	Improve hair strength Improve hair conditioning Improve hair smoothness
Cruz [51]	2017	Chemically synthesize	Peptides based on hair keratin and keratin associated peptides	Improve hair elasticity and tensile strength Hair straightening Decrease damage to the fibers
Cruz [63]	2017	Chemically synthesize	Hair keratin type II cuticular Hb5-based peptide (KP)	Control hair volume Increase hair resistance to moisture Improve hair resilience and pliability
Basit [45]	2018	Human hair	Keratin proteins (K31 protein)	Improve hair strength Improve hair mechanical properties Increase hair thickness

2.3 Conclusion

The constant desire of people to change their appearance lead to an increase in the application of cosmetic procedures, which results in the degradation of hair fibers quality. New compositions to avoid or repair the damage caused have been developed by the hair cosmetic formulators and hair cosmetic industry. In these formulations, proteins play an important role as active ingredients. In this review, it was demonstrated that the proteins can be applied in the form of whole protein or as hydrolysates and peptides. The effect of the proteins and peptides on the hair fibers depended on their chemical and physicochemical properties such as molecular weight, charge, substantivity or hydrophobicity. They can be used to change or improve hair properties like tensile strength, color, shape, conditioning, smoothening, texture, gloss and volume.

Although the benefits given by the protein-based cosmetic formulations described in this review, new studies are needed to increase and explore the full potential of proteins. The application of the proteins as nanosystems or as fused proteins, open new perspectives in the design of advanced hair cosmetics products. Since proteins can be extracted from natural sources or be tailored to harbor specific functionalities, new safer and eco-friendly formulations can be developed responding to the current and future consumers' needs.

Chapter III

Fusion Proteins with Chromogenic and Keratin Binding Modules

Chapter III – Fusion Proteins with Chromogenic and Keratin Binding Modules

Abstract

The present research relates to a fusion protein comprising a chromogenic blue ultramarine protein (UM) bound to a keratin-based peptide (KP). The KP-UM fusion protein explores UM chromogenic nature together with KP affinity towards hair. For the first time, a fusion protein with a chromogenic nature is explored as a hair coloring agent. The KP-UM protein colored overbleached hair, being the color dependent on the formulation polarity. The protein was able to bind to the hair cuticle and even to penetrate throughout the hair fiber. Molecular dynamics studies demonstrated that the interaction between the KP-UM protein and the hair was mediated by the KP sequence. All the formulations recovered the mechanical properties of overbleached hair and KP-UM proved to be safe when tested in human keratinocytes. Although based on a chromogenic non-fluorescent protein, the KP-UM protein presented a photoswitch phenomenon, changing between non-fluorescent and fluorescent states depending on the wavelength selected for excitation. KP-UM protein shows the potential to be incorporated in new ecofriendly cosmetic formulations for hair coloration, decreasing the use of traditional dyes and reducing its environmental impact.

This chapter is based on the following scientific publication:

Ana Tinoco, Egipto Antunes, Madalena Martins, Filipa Gonçalves, Andreia C. Gomes, Carla Silva, Artur Cavaco-Paulo and Artur Ribeiro, Fusion Proteins with Chromogenic and Keratin Binding Modules, *Scientific Reports*, 2019, 9(1):1–13.

3.1 Introduction

Peptides and proteins have attracted great attention in cosmetic industry for the development of new cosmetic formulations. Particularly, fusion proteins can be used on the development of advanced cosmetic products. Combining more than one function in the same protein sequence, we can explore these proteins for several applications, which represents a huge advantage comparing to the current cosmetic products [81]. Fusion proteins are already used in the cosmetic industry, mainly for skincare. A formulation composed by human serum albumin fused with cytokine peptides was developed for improving skin appearance, smoothing wrinkles and whitening skin [82]. Also, a skin-penetrating peptide was fused with a physiologically active protein to promote collagen synthesis and improve skin conditions [83].

Chromogenic proteins are colored proteins that could, for example, be obtained from reef building corals of the class Anthozoa or the sea anemone, *Actinia equine*, or simply synthetically produced from green fluorescent protein (GFP) [84]. The basic structure of this chromogenic proteins is identical to the one of GFP, and is composed by eleven antiparallel β-strands forming a β-barrel that surrounds and protects the α-helix structure of the chromophore. The environment of the chromophore is of high importance, since it can affect the protein spectral properties by influencing chromophore position, protonation state, and electron delocalization [85,86].

One example of chromogenic protein is the monomeric and non-fluorescent Ultramarine (UM) protein, derived from Rtms5 which presents an intense dark blue color. Rtms5 protein was isolated from the reef building coral *Montipora efflorescens* and 5 different genetic mutations were introduced to obtain the monomeric UM protein: V44A, S125R, F162R, L127T and H146N [87]. In this work, we explored UM chromogenic properties to color and protect overbleached hair.

Human hair is a complex fiber divided into cuticle, cortex, and medulla; and its composed of dead cells mainly filled with keratin and other constituents like water, lipids, pigments and trace elements [88]. Hair, besides conferring protection, is also a symbol of beauty and wealth with significant socio-economic impact [89]. Cosmetic industry has developed several processes and products to improve hair's health, style, feel, shine and color. Although a desired look can be obtained, some of these products and processes can be aggressive to the hair, leading to the degradation of hair's structure and decrease of hair's mechanical properties [88]. The most popular hair cosmetic treatments used are bleaching and permanent waving, where hair color and shape can be respectively modified. Hair coloring is an extremely successful hair care treatment which can be classified as permanent, semi-permanent or temporary coloring depending on the type of dye and methodology used [89,90].

Different disadvantages are associated with the use of dyes in hair coloration. It can damage the hair cuticle, the cortex or even the medulla, affecting the mechanical properties of hair; and can cause allergic reactions and even skin irritation. Also, the introduction of dye-containing effluents into the wastewater treatment systems are considered a factor of environmental pollution, not only because of their color, but also because many of the dyes and related compounds (i.e. dyes breakdown products, pigments, dye intermediates, auxiliary chemicals and heavy metals) are toxic, carcinogenic or mutagenic to many life forms. Adequate treatments are necessary to these effluents in order to decrease the lifetime of the dyes in the environment and minimize its negative side-effects [90].

Some solutions were already described to avoid hair fiber degradation, color fade overtime and to increase dye affinity towards hair. For example, carbon nanotubes can be chemically or physically modified by some agents like polymers and attached to hair dyes so the affinity of these to the hair fiber is increased [91]. Peptides with high affinity to hair and resistant to shampoo washing were already described. These peptides could be used as linkers between the hair and the color agent, increasing the binding efficiency of the dye to the hair fibers and maintaining the color for longer time [92].

Concerns about possible health risks and detrimental environment effects are a major driving force for the substitution of synthetic organic compounds for hair dying by natural colorants. Also, the increasing popularity of a more natural lifestyle also stimulate the development and research on natural compounds for dying and other possible applications [93].

In this work, UM chromogenic protein was conjugated with a keratin-based peptide (KP) to improve its affinity towards human hair [51]. The ability of the KP-UM construct to color overbleached Asian hair and recover hair's mechanical properties was evaluated. The effect of different cosmetic formulations on hair coloration with the KP-UM protein was also assessed in terms of color intensity, penetration degree and leaching of protein from the hair fiber.

3.2 Experimental Section

3.2.1 Materials

Asian black hair samples were provided by International Hair Importers & Products Inc. (Glendale, New York, USA). KP-UM gene was synthesized by GenScript and cloned in the pET-28a(+) plasmid. The amino acidic sequence of KP-UM protein is presented in **Table III.1**. Nickel Magnetic Beads for protein purification were available from GenScript. Molecular weight GRS Protein Marker Blue standards and culture medium were purchased from Grisp, Portugal. All other reagents were acquired from Sigma-Aldrich, Madrid-Spain.

Table III.1 - KP-UM protein sequence and predicted molecular weight. Characters in bold correspond to the KP sequence and the $(GA)_5$ linker is underlined.

KP-UM Sequence	Mw (kDa)
GGVCGPSPPCITTGAGAGAGAGAMSVIATQMTYKVYMSGTVNGHYFE VEGDGKGRPYEGEQTAKLTVTKGGPLPFAWDILSPQCQYGSIPFTKYPEDI PDYVKQSFPEGFTWERIMNFEDGAVCTVSNDSSIQGNCFTYHVKFRGTNF PPNGPVMQKKTQGWEPNSERLFARGGMLIGNNRMALKLEGGGHYLCEFK TTYKAKKPVKMPGYHYVDRKLDVTNHNKDYTSVEQCEISIARKPVVA	26.73

Note: the protein final size considering the extra sequence (His-tag) from pet28a(+) vector is 29.05 kDa.

3.2.2 Expression and purification of KP-UM protein

Escherichia coli BL21(DE3) containing the pET-28a(+)::KP-UM vector was used for KP-UM expression in Terrific Broth – Auto Induction Medium (TB-AIM). A single colony harboring the plasmid was inoculated into LB medium supplemented with kanamycin (kan) and grown overnight at 37 °C. A calculated volume of the pre-inoculum was inoculated into TB-AIM_{kan} and the culture was grown for 24 h at 37 °C, 200 rpm. Cells were harvested by centrifugation at 7000 g, at 4 °C, for 5 min. The cells were resuspended in phosphate buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) with 10 mM of imidazole and protease inhibitor and were lysed by sonication (40 % amplitude, 3.0 s on plus 9.0 s off for a total of 10 min on) in a sonicator vibracell™ SONICS. The suspension was centrifuged at 10.000 g, for 40 min, at 4 °C, to separate the soluble fraction. The protein present in the soluble fraction was purified using Nickel magnetic beads with specificity to the His-tag sequence present in the N-terminal of the protein. KP-UM purity was assessed by SDS-PAGE and the purified protein solution was dialyzed against distilled water.

3.2.3 Characterization of KP-UM protein

3.2.3.1 SDS-PAGE analysis

Protein size and purity were analyzed by SDS-PAGE. The lyophilized proteins were solubilized in ultra-pure water, mix with loading buffer and loaded on 12 % SDS-PAGE and stained with Coomassie Blue.

3.2.3.2 MALDI-TOF mass spectrometry

Mass/charge of KP-UM was verified by MALDI-TOF using sinapic acid as the matrix (≥99.5 %). The mass spectra were obtained using an Ultra-flex MALDI-TOF mass spectrophotometer (Bruker Daltonics GmbH) equipped with a 337 nm nitrogen laser. A double layer deposition was used to analyze KP-UM protein. For this, a saturated solution of sinapic acid in ethanol was deposited in the ground steel plate and let dry. The KP-UM protein, previously dissolved in TA30 (30% acetonitrile/70% TFA), was mixed (1:1) with

a saturated solution of sinapic acid in TA30. A volume of 2 µL was spotted into the ground steel target plate (Bruker part nº 209519) and analyzed using the reflective positive mode [94].

3.2.3.3 Circular dichroism (CD) spectroscopy

The effect of temperature in the structure of KP-UM protein was studied by CD spectroscopy using a Jasco J-1500 spectropolarimeter equipped with a temperature controller, and a path-length cell of 1 mm. Four temperatures, 25, 37, 50 and 75 °C, were tested and the same protein concentration was used for all the conditions. The spectra were obtained over the wavelength interval of 185–260 nm at a scan speed of 20 nm/min and bandwidth of 1 nm. Each condition was analyzed in triplicate and the average was used to obtain the final spectra [94].

3.2.4 Bleaching process

The bleaching of black Asian hair was performed according Tinoco *et al.*, 2018 [95]. The hair tresses were washed before and after treatment with a classic commercial shampoo (Pantene Basic).

3.2.5 Hair treatments with KP-UM protein

Coloring treatments with the KP-UM protein were applied to the overbleached (OB) Asian hair. For the treatments, 0.2 g of OB Asian hair was incubated with 10 mL of KP-UM formulation (2 mg/mL), for 30 min, at room temperature, and dried for 20 min at 60 °C. This process was repeated three times. Three formulations were tested: 0.1 M phosphate buffer, pH 7, (0% EtOH formulation); 0.1 M phosphate buffer, pH 7, with 1.5% (v/v) propylene glycol, 0.5% (v/v) benzyl alcohol and 1% (v/v) ethanol (1% EtOH formulation); 0.1 M phosphate buffer, pH 7, with 1.5% (v/v) propylene glycol, 0.5% (v/v) benzyl alcohol and 10% (v/v) ethanol (10% EtOH formulation). Hair samples incubated with the formulations without the KP-UM protein were used as controls. All samples were thoroughly washed in tap water with shampoo and dried at room temperature after treatment to remove loosely bound KP-UM protein.

3.2.6 Color strength determination

Color differences in the hair samples colored with the KP-UM protein, were determined using a reflectance-measuring apparatus (Spectraflash 600; Datacolor) according to the CIELAB color difference concept. The influence of the treatment on hair color was assessed in terms of K/S values and chromaticity coordinates. K/S is the Kubelka-Munk relationship, where K is an adsorption coefficient and S is a scattering coefficient. The data were the sum of all K/S values obtained at the wavelength range 360–700 nm. All analysis were performed in triplicate at 3 different places on the hair samples and the data presented are the mean values of these measurements [96].

3.2.7 Bright-field, fluorescence and confocal microscopy

Individual hair fibers were embedded in epoxy resin and transversal cuts (20 μm) were prepared using a microtome (Microtome Leitz, Oberkochen, Germany). Transversal cuts of OB Asian hair with and without KP-UM protein treatment were analyzed by bright-field and fluorescence microscopy using a LEICA DM 500B fluorescence microscope (Leica, Berlin, Germany). Samples were also analyzed in a Confocal Scanning Laser Microscope (Olympus BX61, Model FluoView 1000). No dye was used in this analysis since KP-UM protein showed to be fluorescent. Images were acquired with the program FV10-Ver4.1.1.5 (Olympus) integrated with Line Series Analysis.

3.2.8 Evaluation of hair fibers' mechanical properties

The effect of KP-UM protein on the mechanical properties of OB Asian hair was assessed following guidelines outlined in ASTM D1145-95 for fiber tensile testing. Tensile tests were performed using a Hounsfield dynamometer H100KS Model and a set of 30 hair fibers with low variability in terms of hair thickness for each condition was selected. Each hair was individually mounted in the tensile jig by means of a paper template with a fixed gauge length of 20 mm and placed in an excicator prior to analysis. A load range of 25 N and a speed of 1.5 mm/min were defined as settings for the tensile strength test [95]. For each hair, applied load against extension were recorded and, using an average mean diameter of 70 μm, data were converted to stress (load/unit area) versus strain (% extension). All measurements were made in the middle part of the hair fibers.

3.2.9 Molecular dynamics simulations

The molecular dynamics simulations were performed with the Gromacs 4.0.7 software package and the GROMOS 54A7 united-atom force-field [97,98]. Periodic boundary conditions were applied, with the LINCS [99] and the SETTLE [100] algorithms constraining the proteins and water bonds, respectively. The time step used was 2 fs, the water was represented by the single point charge model [101] and the temperature of 300 K was maintained by the V-rescale thermostat [102]. The Parrinello-Rahman barostat [103,104] (with isotropic coupling) was used to maintain the pressure at 1atm. The neighbor list was updated every 10 steps, the twin-range method (0.8 and 1.4 nm for short- and long-range cutoffs) was used for treat the electrostatic interactions. The dielectric constant of 54 was used for reaction field correction [105].

The molecular model of hair keratin protofibril used was previously developed in our group by Egipto Antunes, and showed to be useful in the study of some compounds effects in the hair fiber [4,106]. The KP-UM model was obtained through structure homology of UM with the KP peptide and the $(GA)_5$ linker

amino-acid sequence with the X-ray model of the Rtms5 protein (PDB code: 3vk1) using the SWISS-MODEL web service (https://swissmodel.expasy.org).

The software VMD (Visual Molecular Dynamics) [107]was used to render the simulations snapshots and videos.

3.2.10 Simulations protocol

All the simulated systems were composed by proteins (KP-UM or KP-UM and keratin protofibril) and solvent (water or formulations composed of water (97 or 88% (v/v)), ethanol (1 or 10% (v/v)), benzyl alcohol (0.5% (v/v)) and propylene glycol (1.5% (v/v)). The systems were first energy minimized applying the steepest descent method for 2000 steps. Later three short preparatory simulations of 500 ps, were ran to equilibrate the systems. In these runs positions restrains, with a harmonic force of 10^3 kJ/mol.nm^2, were applied to all heavy atoms of non-solvent molecules for the first two runs, and only applied to protein alfa-carbons in the third run. The first equilibration simulation was performed in the NVT ensemble (number of particles, volume and temperature constant). The second, third and the production runs were simulated in the NPT ensemble (number of particles, pressure and temperature constant). After equilibration simulations, were performed the final production simulations, generally without positions restrains, by around 50 ns. All systems were simulated in triplicate. Due to the big size of the systems composed by KP-UM and the keratin protofibril, two of the three replicas were ran with positions restrains in the alfa-carbons of the keratin, allowing the simulation of smaller systems boxes. To test if the application of the positions restrains in these systems influence the interaction between the KP-UM and the keratin, one big system without restrains was also simulated.

The hydrogen bonds between KP-UM and the keratin protofibrils were determined using g_hbond tool from GROMACS, with average hydrogen bonds determined for the last 10 ns of interaction between the proteins.

Steered simulations were performed to calculate the binding energy between the KP-UM and the keratin fiber in the different formulations (0, 1 and 10% EtOH). The simulation protocol was the same, with the exception that in the production run (around 5ns of simulation) was applied a harmonic force of 1000 kJ.mol^{-1}.nm^{-2} to the center of mass of KP-UM alpha carbons, and the alpha carbons of keratin fiber were restrained. This leads to the complete separation of the KP-UM from the keratin. The resulting trajectories allowed the generation of different configurations along the Y coordinate (in which the distance between the two proteinaceous structures increases) to perform umbrella sampling simulations [108] and later calculate the potential of mean force (PMF) of the crossing process using free weighted histogram analysis (g_wham tool of GROMACS) [108–110]. Each umbrella configuration was simulated by 2 ns, with the

pulled KP-UM restrained. The position of the KP-UM differs around 0.05 nm from the neighboring configurations. The obtained PMF variation graph allowed the calculation of binding energy, which corresponds to the energy at the final state minus the energy at the initial state.

3.2.11 Statistical analysis

Data are presented as average standard deviation (SD), n=3. Statistical comparisons were performed by one-way ANOVA with GraphPad Prism 5.0 software (La Jolla, CA, USA). Tukey's post hoc test was used to compare all the results between them, and a Dunnet's test was used to compare the results with a control. A p-value ≤ 0.05 was considered to be statistically significant.

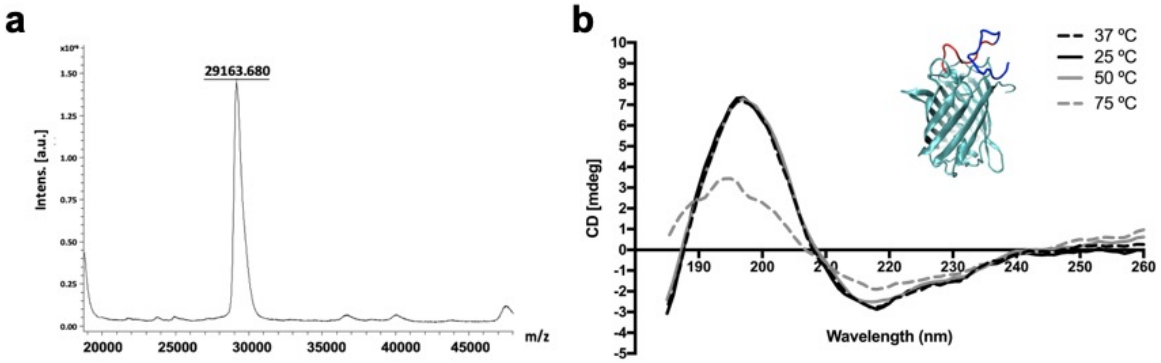
3.3 Results

3.3.1 Purity and size of KP-UM protein

The amino acidic sequence of UM protein was conjugated with a keratin-based peptide (KP) using a linker composed by 5 repetitions of glycine and alanine ($(GA)_5$). KP-UM protein was characterized regarding purity and molecular weight after expression and purification. SDS-PAGE confirmed the high purity of KP-UM protein, presenting a single band with a molecular weight close to the theoretical (KP-UM plus the extra amino acidic sequence (His-tag) from the pet28a(+) vector) – 29.05 kDa (data not shown). MALDI-TOF mass spectrometry (**Figure III.1 a**) also confirmed the purity, the monodisperse character and the size of the KP-UM protein.

3.3.2 Structural analysis by CD spectroscopy

To further study the effect of KP peptide and $(GA)_5$ linker on Ultramarine (UM) structure [87] and understand the role of temperature on the secondary structure of KP-UM protein, the far-UV CD spectra of this protein under different temperatures was monitored. KP-UM secondary structure was studied between 185 and 260 nm, since the π* and π to π* transitions of α-helices and β-sheets normally occur in the UV region [111]. For this study, different temperatures were studied (**Figure III.1 b** and **Figure III.1 c)**: 25 °C correspond to the average air temperature in hairdressers salons, 37 °C correspond to the body temperature and 50 and 75 °C were selected taking in consideration the procedures performed on hair styling where higher temperatures are applied.



c

Temperature (ºC)	α-helix	ß-strands	Turns	Unordered
25	0.127	0.438	0.093	0.342
37	0.129	0.438	0.094	0.338
50	0.128	0.439	0.093	0.339
75	0.084	0.411	0.123	0.383

Figure III.1 - a) MALDI-TOF mass spectra of KP-UM protein obtained in reflective positive mode using sinapic acid as matrix; b) Circular dichroism spectra of KP-UM at different temperatures (37, 25, 50 and 75 °C): a close-up of KP-UM secondary structure is highlighted where cyan represents the UM protein, dark blue the KP sequence and red the $(GA)_5$ linker; c) Effect of temperature on KP-UM secondary structure determined by CD.

KP-UM protein was constructed considering a GFP-like chromogenic blue protein termed Ultramarine (UM), which is derived from the naturally occurring fluorescent protein Rtms5. GFP-like proteins belong to the proteins with β-barrel topology, where the proteins resemble in an 11 stranded β-sheets wrapped around a single central helix, in the middle of which is the chromophore [112]. Analyzing the shape of KP-UM spectra in **Figure III.1 b**, it was observed a maximum and minimum peak at 197 and 220 nm and a similarity between the spectra at 25, 37 and 50 °C. When the temperature increased to 75°C, a decrease in the spectra intensity and a 2 nm shift of the 197 nm peak to 195 nm, was observed. To better quantify the structural transition at different temperatures, the far-UV CD spectra of KP-UM protein were further de-convoluted using the software available from the DICROWEB website. The results of secondary structure content of KP-UM protein incubated at different temperatures are listed in **Figure III.1 c**. The values for helix 1 and helix 2 as well as strand 1 and strand 2 were added to obtain total α-helix and total β-strands content. Almost no differences were verified on the proportions of KP-UM

secondary structures when the protein was incubated at 25, 37 and 50°C. However, when the temperature was increased to 75°C, a decrease in the α-helix and β-strands content was observed, with a concomitant increase in the content of turns and unordered structures.

3.3.3 KP-UM binding to overbleached Asian hair

To evaluate the KP-UM protein capacity to bind and to color OB Asian hair, different KP-UM solutions were prepared and the binding efficiency and the color coordinates of colored hair were determined. KP-UM was dissolved in 0.1 M phosphate buffer, pH 7, or in 0.1 M phosphate buffer, pH 7, with 1.5% (v/v) propylene glycol, 0.5% (v/v) benzyl alcohol and 1 or 10% (v/v) of ethanol. The protein solutions were labelled as 0, 1 and 10% EtOH. These formulations were selected to stimulate protein interaction with the hair fiber by promoting hair swelling and exposing the hair shaft to the protein solution [113].

When preparing the KP-UM solutions differences in the color depending on the formulation where KP-UM was dissolved, were observed. To study the effect of formulations on color, the absorbance spectrum of KP-UM in each formulation was plotted and analyzed **(Figure III.2 a).** A control of KP-UM dissolved in water was also performed. For all the formulations, two absorption peaks around 525 and 585 nm were verified, corresponding to the *trans* and *cis* conformation, respectively.

Comparing the spectra of KP-UM protein in the different formulations, it was observed a shift of the absorption peaks for lower wavelengths as the percentage of ethanol increased. For the KP-UM dissolved in water or phosphate buffer, the maximum absorption peaks were at 525 and 585 nm. When the protein was dissolved in the formulation with 1% ethanol, there was a shift of the less energetic peak from 585 to 575 nm; while there was a shift of both peaks to lower wavelengths (520 and 575 nm) when KP-UM was dissolved in the formulation with 10% ethanol. Also, the ratio between the two absorption peaks showed to be sensitive to different factors, like the solvent polarity. The differences in the ratio were verified on the obtained data, where a more intense peak at 585 nm was observed when the protein was dissolved on water or in the formulation with 0% ethanol. When ethanol was added, it was verified an alteration in the ratio of these two absorption peaks, with an increase in lower wavelength peak intensity.

Although the UM protein is classified as a chromogenic and non-fluorescence protein, the KP-UM fluorescence spectrum was determined (**Figure III.2 b** and **Figure III.3).** Initially, the KP-UM protein was excited at λ_{Abs}^{max} observed in the absorbance spectrum**:** 525 and 585 nm for KP-UM aqueous solution.

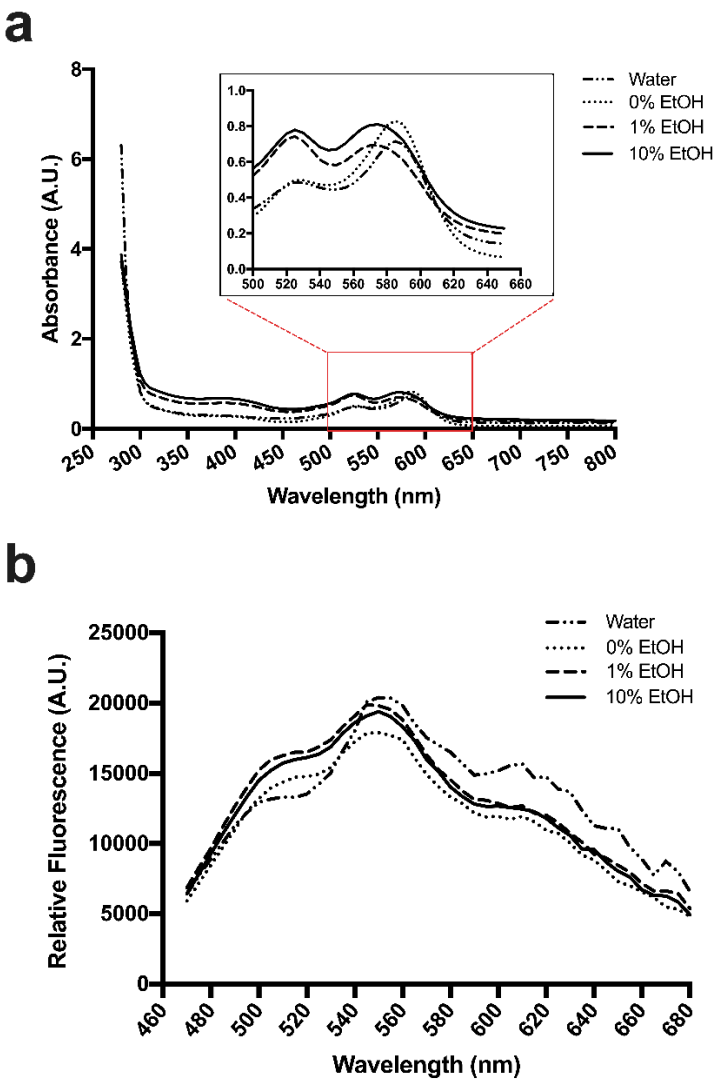


Figure III.2 - a) KP-UM absorbance spectra with a close up of the absorbance peaks in the region between 500 nm and 650 nm and b) KP-UM emission spectra when excited at 440 nm. KP-UM protein was prepared in different solutions: formulation with 10% ethanol (10% EtOH), formulation with 1% ethanol (1% EtOH), phosphate buffer pH 7 (0% EtOH) and water.

However, when KP-UM was excited at these two wavelengths, no bands were observed on the emission spectrum **(Figure III.3).** Taking in consideration previews works performed with other GFP-like proteins and with Rtms5 mutants [114], we tested different excitation wavelengths. When the protein was excited at 440 nm a broad emission spectrum was obtained and, although without any defined peak, three projections λ_{Em}^{max} around 510, 550 and 610 nm were observed **(Figure III.2 b)**.

To better characterize the KP-UM protein, the effect of the pH on the absorbance spectrum was studied between pH 3 and 11. We found that the color of KP-UM protein was pH-dependent, being this color

change reversible (**Figure III.4 a).** KP-UM color can change from yellow to intense purple by increasing the pH from 3 to 11, returning to yellow by a simply decrease of pH until 3. To understand these alterations in color, each solution was studied relatively to its absorbance spectrum (**Figure III.4 b).**

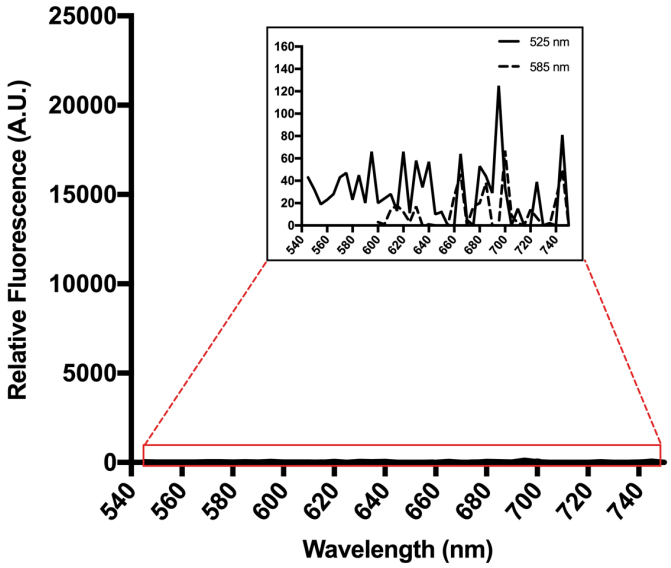


Figure III.3 - Emission spectra of KP-UM when excited at 525 nm and 585 nm.

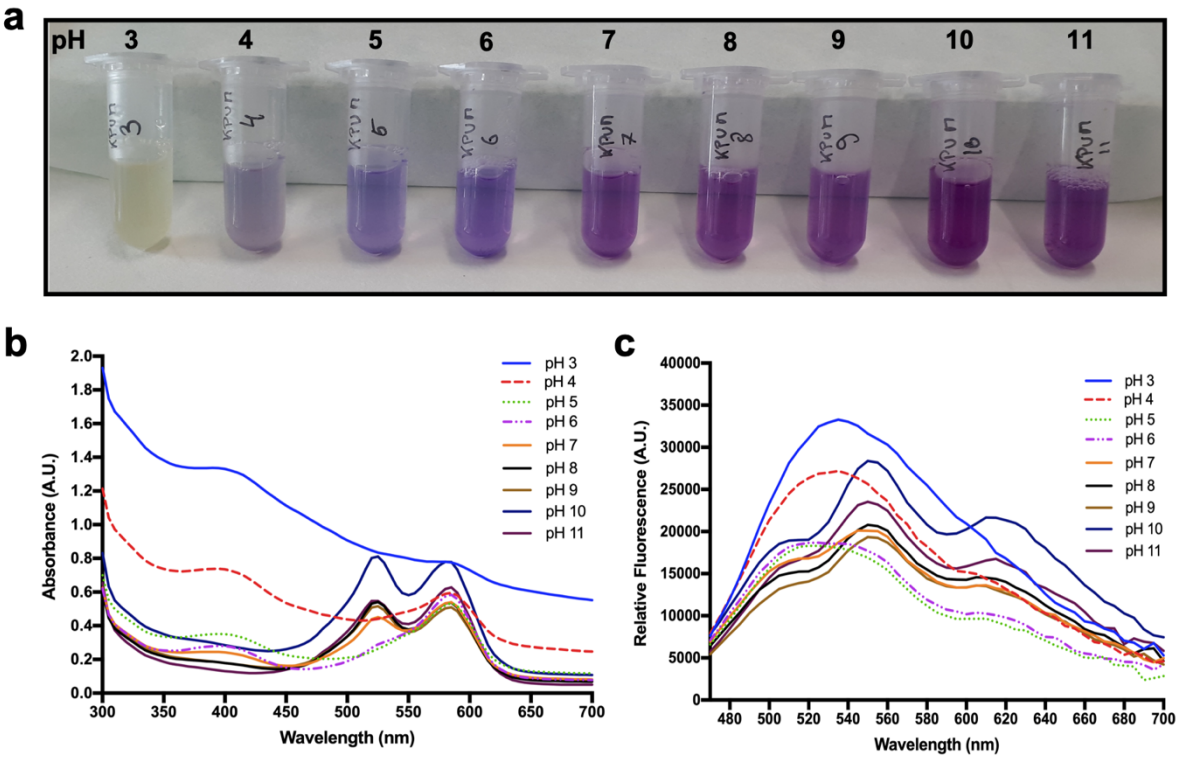


Figure III.4 - Effect of pH on a) the visible color of KP-UM protein on 0.1 M phosphate solution; b) absorption spectra of KP-UM protein; c) fluorescence spectra of KP-UM protein.

The spectra for the KP-UM solutions between the pH 3-5 presents only one peak at 585 nm, indicating that the *cis* conformation is favored for pH between 3 and 5. The *trans* conformation start to appear for pH 6, becoming more pronounced with the pH increasing for the KP-UM solutions. The effect of pH on KP-UM fluorescent spectra was also studied **(Figure III.4 c)** and, generally, two different profiles were obtained. A broad spectrum with a maximum around 530 nm was observed for the solutions with a pH between 3-6. For the other pH values, the spectra presented three peaks around 510, 550 and 610 nm, which became more pronounced for the highest pH values (10 and 11).

Overbleached Asian hair was colored with the KP-UM protein dissolved in the three formulations. The hair strands were washed with commercial shampoo, 24 h after treatments, to remove roughly bound proteins **(Figure III.5).** All hair strands treated with the KP-UM protein acquired a purple/pink coloration supporting the ability of KP-UM to bind and colorize the hair. The hair color obtained after KP-UM treatment was dependent on the formulation where the protein was dissolved.

The binding efficiency of KP-UM was determined by quantifying the amount of protein on solution after hair incubation. A binding efficiency of 43.11±0.58, 59.50±0.30 and 52.79±1.44% for the 0,1 and 10% EtOH formulations, was verified.

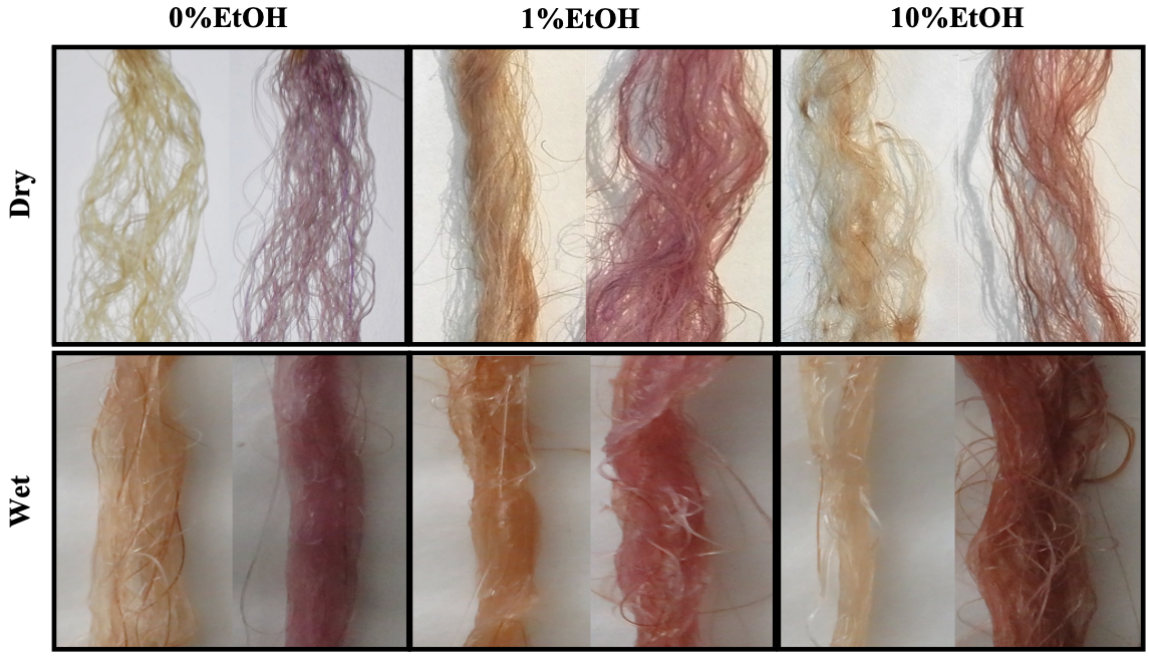


Figure III.5 - Overbleached Asian hair colored with KP-UM protein (right) and the respective controls (left). The coloration process consisted in: 0.2 g of OB Asian hair incubated with 10 mL of KP-UM formulations, for 30 min, at room temperature, and then dried for 20 min at 60 °C; this process was repeated three times.

3.3.4 Hair Coloration degree

To evaluate the effect of formulations on hair color, CIELAB color measurements were performed to determine color coordinates, brightness, color differences and color strength by means of K/S determination.

Color strength of hair strands treated with KP-UM was evaluated taking into consideration the checksum of K/S values between 360 and 700 nm **(Figure III.6 a)**. The checksum of K/S values of overbleached Asian hair treated with each formulation, untreated virgin and overbleached Asian hair were also considered. The incubation of overbleached Asian hair with the three formulations, without the KP-UM protein, resulted in a decrease in the checksum of K/S values, when compared with the untreated overbleached hair. Significant differences in the K/S checksum values were observed when the hair was incubated with the KP-UM protein. The increase in the K/S checksum values were dependent on the amount of ethanol present on the formulation, with the highest results being observed for the hair colored with KP-UM in the formulation with 10% ethanol.

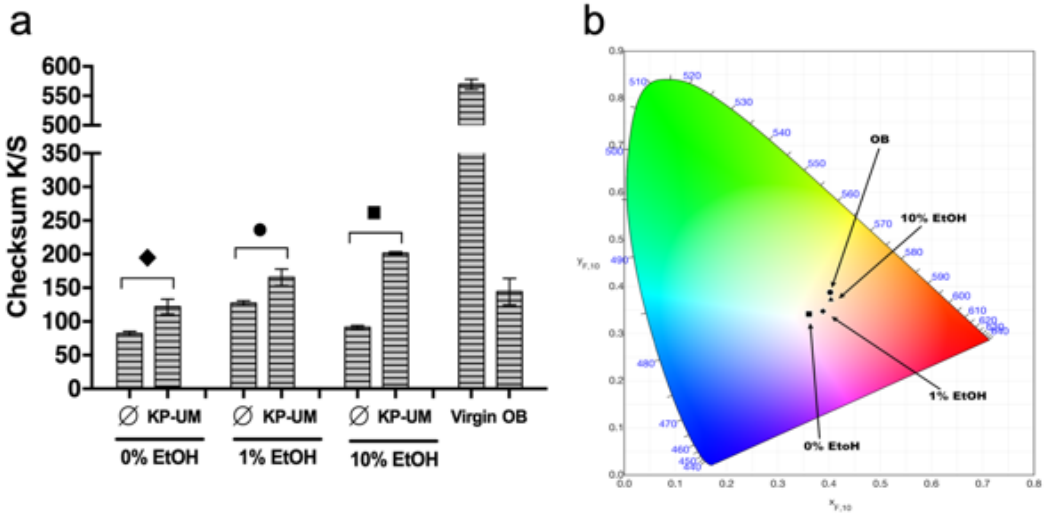


Figure III.6 - a) K/S values (checksum: 360 -700 nm) of hair samples obtained after incubation with KP-UM: 0% EtOH - overbleached hair incubated with 2 mg/mL KP-UM in 0.1 M phosphate buffer, pH 7; 1% EtOH - overbleached hair incubated with 2 mg/mL KP-UM 1% ethanol formulation; 10% EtOH - overbleached hair treated with 2 mg/mL KP-UM in a 10% ethanol formulation. Values are the mean of SD of three independent measurements. Statistically significant differences from the respective control are indicated as: * p-value ≤0.05, ● p-value ≤0.01; ♦p-value ≤0.001; ■ p-value ≤0.0001; b) CIE chromaticity diagram and chromaticity coordinates $D_{65/10}$ ($X_{F,10}$, $Y_{F,10}$) of: ● - overbleached Asian hair; ■ - overbleached hair colored with KP-UM in 0.1 M phosphate buffer, pH 7 (0% EtOH); ◆ - overbleached hair colored with KP-UM in formulation with 1% EtOH; ▲ - overbleached hair colored with KP-UM in formulation with 10% EtOH.

Figure III.6 b shows the corresponding chromaticity points $D_{65/10}$ ($X_{F,10}$, $Y_{F,10}$) of the overbleached Asian hair with and without the KP-UM protein in the Commission International de l'Eclairage (CIE) diagram. This diagram maps color in terms of hue and saturation and the boundary of the CIE diagram is defined by the saturated hues that the human eye can detect (from 380 to 780 nm)[115]. The addition of ethanol into the KP-UM formulation lead to a color change from the purple zone of the diagram (0% EtOH) to pink (1% ethanol) and even red for the highest ethanol percentage (10% EtOH). As expected, the overbleached hair is located closer to the area corresponding to the yellow color.

To better understand the differences observed in the color of hair, and if these could be also related with the localization and penetration degree of the KP-UM protein into the hair fiber, transversal cuts of colored hair were analyzed by optical microscopy (**Figure III.7 – Panel A)** and by confocal microscopy **(Figure III.7 – Panel B).** Independently of the formulation, the KP-UM protein was able to bind to the hair and penetrate into the hair cortex. Despite that in the optical microscopy images (**Figure III.7 - Panel A)** the KP-UM seems to be more concentrated near the cuticle, the higher sensitivity of fluorescence microscopy show that the protein was able to bind and penetrate into all the areas of the hair cortex (**Figure III.7 - Panel B).** KP-UM penetration profiles were determined taking in consideration the KP-UM relative intensity through a fiber's cross section. Penetration profiles represented on **Figure III.7 - Panel C**, give an indication how the KP-UM protein distributes along the hair and if there is some tendency to accumulate in a specific area of the hair fiber. It was observed for all the samples, an intensity higher than the overbleached hair (No KP-UM) throughout the fiber cross section, mainly in the first 10-15 μm of the hair fiber.

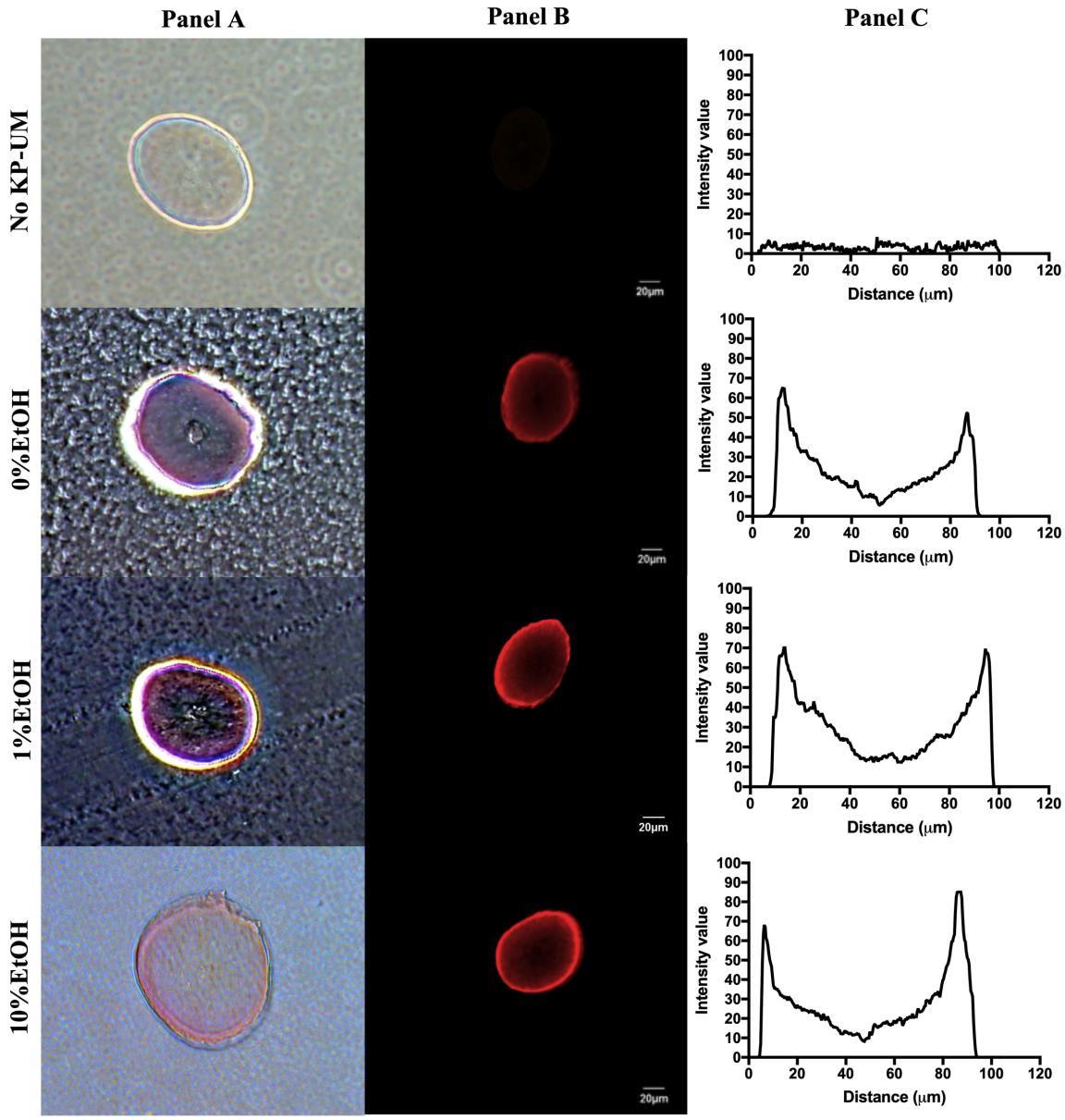


Figure III.7 - Optical microscopy (Panel A) and confocal microscopy (Panel B) of transversal cuts of overbleached Asian hair without treatment (No KP-UM) or treated with 2 mg/mL KP-UM dissolved in: 0.1 M phosphate buffer (0% EtOH), 1% ethanol formulation (1% EtOH), and 10% ethanol formulation (10% EtOH). On Panel C are represented the KP-UM penetration profiles on Asian hair's cross sections.

3.3.5 Recovery of the mechanical properties of overbleached hair colored with KP-UM protein

Systematic exposition of hair to chemical treatments affects irreversibly hair's characteristics like smoothness, shining and even its mechanical properties. The mechanical resistance of overbleached Asian hair before and after treatment with the KP-UM was studied to determine the effect of the protein in hairs' mechanical properties in terms of Young's modulus and tensile strength **(Figure III.8).** Considering the 8 overbleaching cycles applied to the Asian hair, a loss of stiffness of about 21.5% and a

loss of tensile strength of about 23.1% was verified, comparatively to the virgin Asian hair. These results agreed with the data previously reported by Tinoco *et al*, 2018 [95].

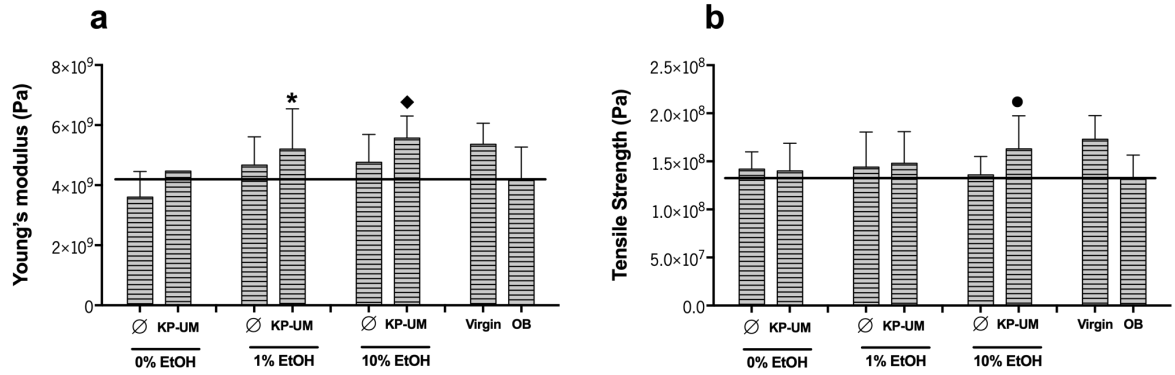


Figure III.8 - Mechanical resistance parameters: tensile strength (a) and Young's modulus (b) of overbleached Asian hair (OB) before and after treatment with 2 mg/mL of KP-UM protein resuspended in formulations with 0, 1 and 10% ethanol. Virgin Asian hair was used as control of the effect of bleaching and KP-UM coloration. Values are the mean ± SD of twenty independent measurements. Statistically significant differences from the respective control are indicated as: * p-value ≤0.05, ● p-value ≤0.01; ◆ p-value ≤0.001.

The percentages of recovery of the hair samples colored with the KP-UM protein were determined comparatively to the respective controls and are shown on **Table III.2**. These percentages were calculated relatively to the hair samples incubated with the respective formulations without the KP-UM protein. Analyzing **Figure III.8** and the data from **Table III.2**., it is possible to observe that KP-UM protein in the three formulations has the capacity to recover the hair's mechanical properties. The condition that most increased the hair's Young's modulus was the KP-UM protein in the formulation with 0% EtOH (24.17%), while the condition which most contributed to an increase of the tensile strength was the KP-UM in the formulation with 10% EtOH (19.85%). Generally, considering the tensile strength and the Young's modulus, the best condition was the KP-UM in the formulation with 10% EtOH.

Table III.2 - Recovery percentage (%R) of overbleached Asian hair mechanical properties measured in terms of Young's modulus and tensile strength after treatment with KP-UM protein. The hair samples were compared with the respective control.

	% Recovery	
Ethanol (%)	Young's modulus	Tensile Strength
0	24.17	1.43
1	11.35	2.78
10	17.02	19.85

3.3.6 Leaching of KP-UM protein during washing

The leaching of KP-UM from colored hair during washing was evaluated for a total of 20 washing cycles, consisting each cycle of an incubation with a shampoo solution followed by a washing with water. At the end of the 20 washing cycles, 1.79 and 9.4% of KP-UM was leached from the colored hair for the 1 and 10% EtOH formulation, respectively. For the 0% EtOH formulation, no leaching was verified even after 20 washing cycles. Even though the amount of KP-UM leached at the end of the washing cycles was below 10% of the initial amount of protein bound to the hair for the 3 formulations, there were some differences between the 3 conditions. An increase on the amount of KP-UM leaching from the colored hair when increasing the amount of ethanol on the formulation was observed at the end of the washing cycles.

3.3.7 In vitro evaluation of KP-UM protein potential cytotoxicity

The development of new hair care cosmetic formulations needs to be supplemented with safety studies, being crucial that the new formulations do not cause any type of cytotoxicity to the users. In 2003, the 7th amendment of the European Union's Cosmetics Directive (76/768/EEC) was approved, which stipulated the end of animal testing for cosmetic products in European Union. Since 2009, it was also prohibited the animal testing of cosmetic ingredients (Cosmetic Regulation (EC) 1223/2009). Continuous efforts have been done to find alternative approaches capable to replace animal tests by others that give the same information about possible compound's toxicity [116].

The potential cytotoxic effect of cosmetic products can be evaluated *in vitro* using appropriate cell lines. Considering the guidelines for testing of new cosmetic products, the NCTC-2544 human keratinocytes cell line was chosen to evaluate the potential cytotoxicity of KP-UM protein. The results of potential cytotoxicity, evaluated in terms of metabolic viability after 24 and 48 h of incubation with the KP-UM protein, are presented in **Figure III.9**.

No signs of evident cytotoxicity were verified at the end of 24 h of incubation, for any of the concentrations of protein tested. At the end of 48 h of incubation there was a trend on cell viability which was dependent on protein concentration, with a decrease on cell viability when increasing the concentration of KP-UM protein. This protein could be considered safe to be incorporated in cosmetic formulations since the cell viability remained always higher than 85%.

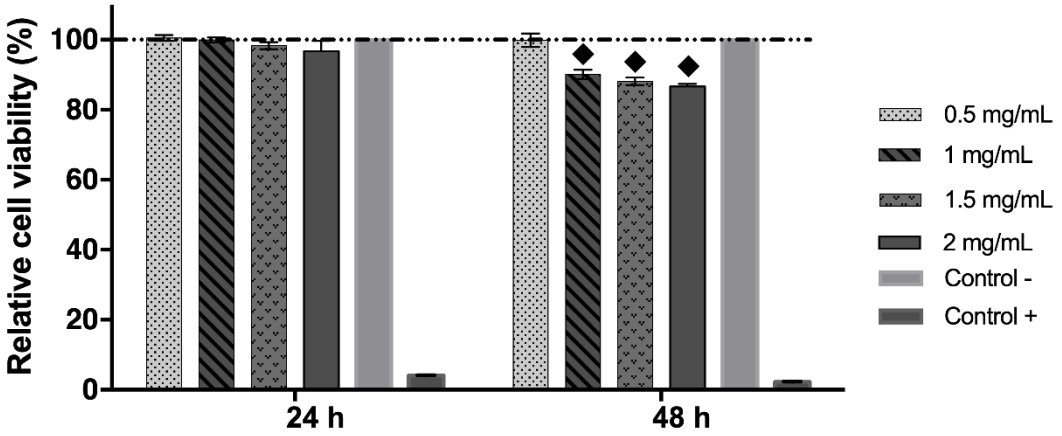


Figure III.9 - Relative viability of NCTC-2544 keratinocyte cell line evaluated with the MTT assay, after 24 and 48 h incubation with 0.5, 1.0, 1.5 and 2.0 mg/mL of KP-UM protein. Cells incubated with culture medium only were used as life control (Control -) and cells incubated with 30% DMSO as death control (Control +). Data were determined in relation to the life control. Results are the mean ± SD of triplicate of three independent experiments. Statistically significant differences from the control are indicated as follows: ● p-value ≤0.01; ◆ p-value ≤0.001.

3.3.8 Molecular dynamics of KP-UM binding to keratin filaments

The alignment of the structures of the UM protein with and without the KP peptide and the $(GA)_5$ linker, were obtained after 50 ns in water simulations **(Figure III.10).** It is visible an almost complete superposition of both beta barrels pointing that the insertion of the KP peptide and the linker sequence do not have significant impact in the UM conformation. These data support the results obtained by CD (**Figure III.1 b** and **Figure III.1 c**). A simulation of KP-UM protein in water and in the 10% EtOH formulation, was also performed to study the effect of formulation on KP-UM structure. The alignment of both structures **(Figure III.10)** show that the formulation does not affect the structure of the protein as no significant differences between the two structures are visible.

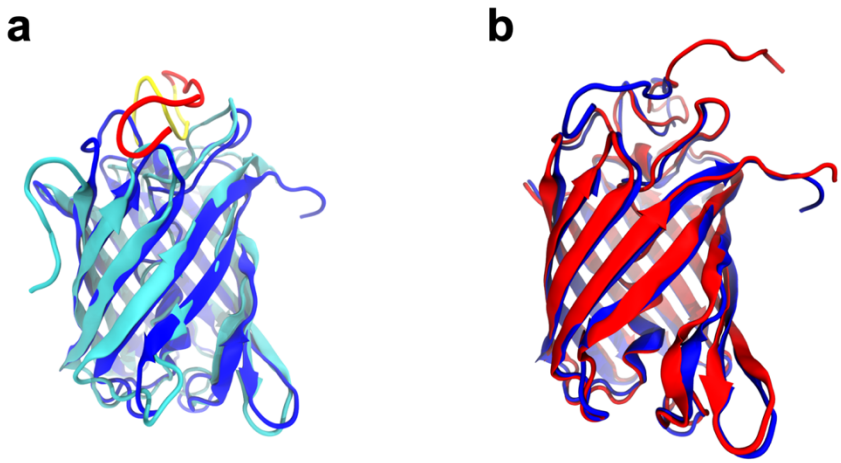


Figure III.10 - a) Structural comparison between UM (cyan) protein and the KP-UM protein (blue - UM protein; yellow - $(GA)_5$ linker; red - KP sequence) from a lateral (point of view; b) Structural comparison between the KP-UM conformation in water (blue) and in 10% EtOH formulation (red).

In order to study the role of KP and the effect of ethanol from the formulation in the interaction of KP-UM with the keratin filaments, molecular dynamics in different conditions were performed. **In Figure III.11**, are visible the snapshots for 0, 1, 5 and 50 ns of the simulation with the KP-UM protein and a keratin protofibril. The visual inspection of the simulations clearly shows that the beginning of the interaction between these two proteins is always mediated by the KP sequence added to the UM N' terminal. Visually, it is also possible to notice that higher concentrations of ethanol difficult the interaction on the KP sequence with the keratin protofibril.

The average number of hydrogen bonds between KP-UM and keratin protofibril was determined for the last 10 ns of simulation **Figure III.12**. The increase of ethanol decreased the number of hydrogen bonds measured for the interaction: 3.8±2.47, 3.22±1.24 and 1.58±0.742 for 0% EtOH, 1% EtOH and 10% EtOH, respectively. The determination of the binding energies between the KP-UM protein and the keratin filaments were also determined at the end of 50 ns of simulation shown to be inconclusive. The interaction between the KP-UM and the keratin is mainly governed by disulfide bonds involving the cysteine residues present in KP sequence of KP-UM and in the keratin [51]. However, this S-S bonds cannot be simulated using the current force fields and the determination of the binding energies does not represent all the interactions. Despite that ethanol seems to decrease the formation hydrogen bonding, ethanol is known to enhance swelling of keratin and to promote the fixation cysteine containing peptides as previous reported [51].

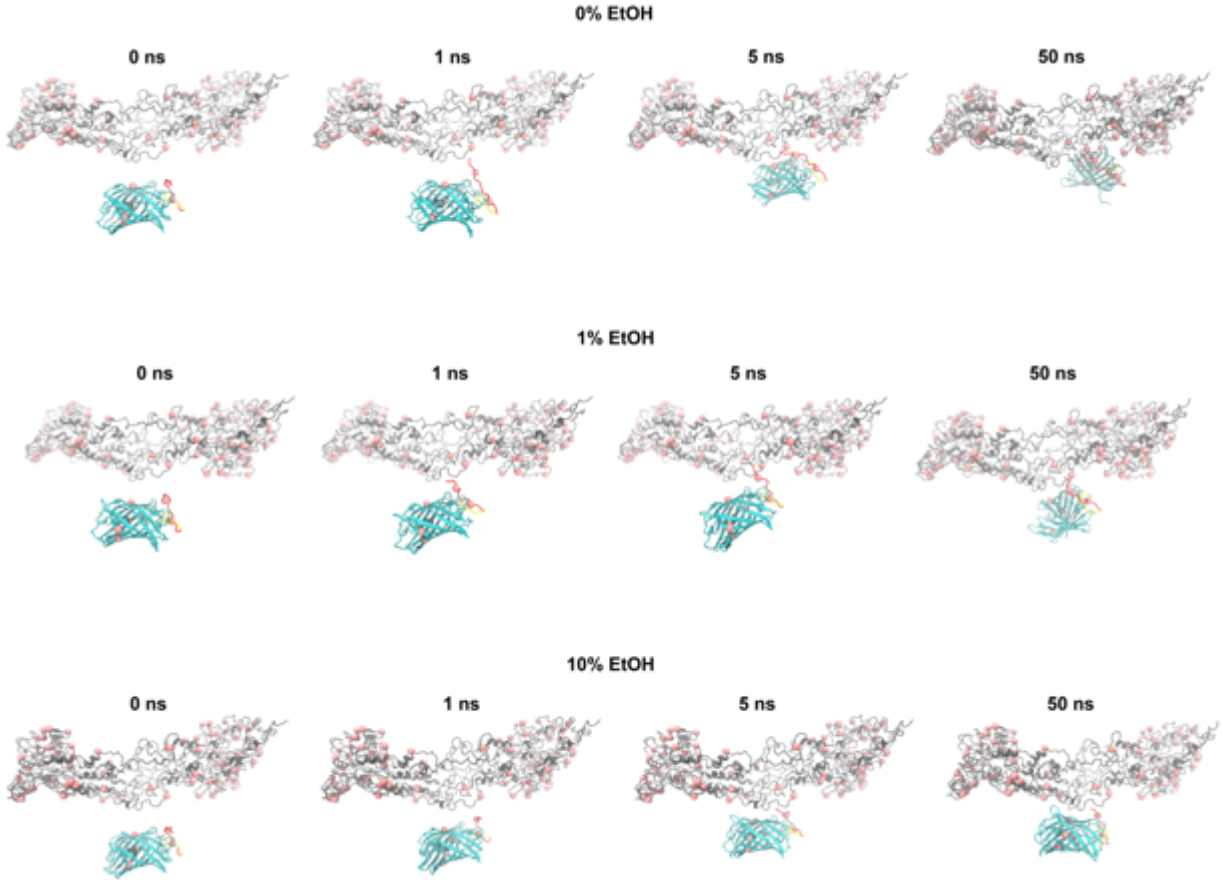


Figure III.11 - Simulation snapshots of 50 ns of the interaction between KP-UM (cyan) and keratin protofibril (grey). The red spheres represent cysteine residues capable to form disulfide bonds.

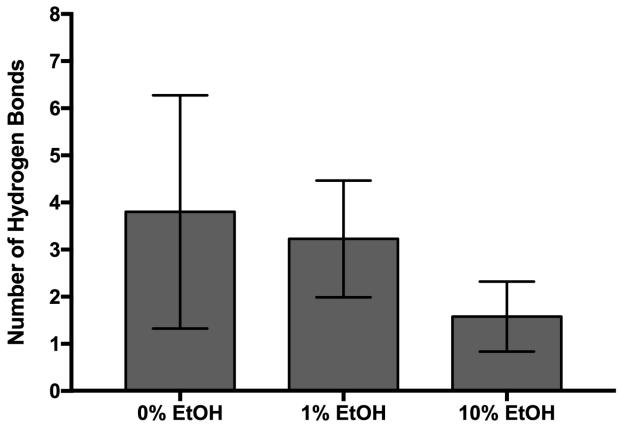


Figure III.12 - Average number of hydrogen bonds between KP-UM protein and the keratin protofibril, determined in the last 10 ns of simulation.

3.4 Discussion

A fusion protein, with the capacity to color and improve hair's mechanical properties, was designed and obtained considering the sequence of the ultramarine chromogenic protein (UM) and a sequence from a keratin-based peptide (KP). A linker constituted by 5 repetitions of glycine and alanine $(GA)_5$, was included to increase the structural mobility of UM protein and KP peptide.

To study the effect of temperature on the secondary structure of KP-UM protein, four temperatures (25, 37, 50 and 75°C) were tested. A structure with a high content of β-sheets, proved by the characteristic maximum and minimum peak at 197 and 220 nm [117], was verified for all the temperatures. Although, at 75°C, it was observed an alteration in protein structure proved by the decrease in α-helix and β-strands content and increase in the unordered content. This change in protein structure could be due to partial protein unfolding caused by higher temperatures. Considering a future application in hairdressing salons, the structural similarities observed at 25, 37 and 50 °C **(Figure III.1 b** and **Figure III.1 c),** allowed us to select the room temperature for the binding assays of KP-UM protein to OB Asian hair.

When KP-UM protein was dissolved in the different formulations, two absorption peaks at 525 and 585 nm were observed on the proteins' absorbance spectrum **(Figure III.2 a).** These two absorption peaks, corresponding to different protonation states of KP-UM chromophore, were predictable since this protein is derived from a GFP-like protein. The *cis* conformation corresponds to the deprotonated state (anionic – 585 nm) while the *trans* conformation corresponds to the protonated state (neutral – 525 nm) [117,118]. Comparing the spectra of KP-UM protein in the different formulations **(Figure III.2 a)** a shift for lower wavelengths in the absorption peaks was observed as the percentage of ethanol increased. This shift for lower wavelengths with a decrease of solution polarity and solvation promoted by water could be associated with a solvatochroism effect. Solvatochroism is the change of color of a molecule as a function of solvent polarity and dielectric properties. Since more polar solvents (0% EtOH > 1% EtOH > 10% EtOH) gave rise to a red-shift, the KP-UM protein is classified as positively solvatochromic. It was also observed that the peak of the anionic form (585 nm) was more dependent on the solvent since it changed the maximum absorption peak in 10 nm, while the neutral form only changed 5 nm when in the presence of 10% ethanol. This behavior was already described for other GFP-like proteins, where only the anionic peak changed with the solvent ionic strength while the neutral peak remain almost unchanged [119]. Also, differences in the ratio between the two absorption peaks were verified, and this equilibrium seems to be ruled by a hydrogen bond network that allows the proton transfer between the chromophore and neighboring side chains [120]. Analyzing the effect of pH on KP-UM absorbance spectra **(Figure III.4 b),** it was verified that an increase in pH benefit the conversion of *cis* into *trans* conformation, both of them

showing similar absorbance values for pH values higher than 8. Moreover, the data suggests that the neutral form (525 nm, *trans* and protonated conformation) of the chromophore is favored at higher pH (pH 8-11), while the anionic form (585 nm, *cis* and deprotonated conformation) dominates at lower pH (pH 3-6). This finding was unexpected since acidic pH should favor the protonated state and more basic pH should favor the deprotonated state. In this way, it was observed a reverse chromophore protonation where acidic pH favored the deprotonated state. This mechanism was already observed for mKeima, a red fluorescent protein derived from the GFP family [121].

Although the KP-UM protein was based on the sequence of UM protein, a monomeric and non-fluorescent chromogenic protein [87], the protein showed some fluorescence. In this work, the UM sequence was fused with a keratin-binding peptide (KP) [43] and a $(GA)_5$ linker to promote the binding of the protein to the hair cuticle and to the cortex improving the coloration process. Despite the UM protein is described as chromogenic and non-fluorescent, we found that both, UM and KP-UM proteins, had the ability to convert between non-fluorescent and fluorescent states by irradiation with a specific wavelength of light, a phenomenon often referred to as photoswitching [122,123]. This spectral property was first identified in the *Anemonia culcata* chromoprotein and its mutants [124]. When the KP-UM was excited at λ_{Abs}^{max} – 525 and 585 nm – no significant emission was observed (**Figure III.3**). However, when the protein was excited at 440 nm a broad emission spectrum was obtained **(Figure III.2 b).** The photoswitching behavior of UM and KP-UM proteins is currently being studied in more detail and will be subject of a future publication. However, taking in consideration the photoswitching studies performed in GFP or GFP-like proteins, we believe the key event in the switching process is a cis-trans isomerization of the chromophore. This phenomenon is accompanied by changes in the immediate chromophore environment and by distinct protonation equilibria of the chromophore [124].

Despite the KP-UM protein was able to color the OB Asian hair in all tested conditions (**Figure III.5**), the hair color and the amount of protein bound to the hair were dependent on the formulation. We were expecting a higher binding efficiency for the formulation with 10% ethanol, related with a higher degree of hair swelling promoted by the ethanol. However, the best result was observed for the formulation with 1% ethanol. This could be related with the swelling of hair shafts and with the protein conformation (exposure of KP peptide to the hair surface) in the formulation with 1% ethanol.

To evaluate the capacity of KP-UM to color hair, CIELAB color measurements (**Figure III.6**) were performed on hair samples treated with the formulations and on hair samples treated with the formulations and the KP-UM protein. A decrease was observed in the checksum of K/S values for the OB hair incubated with the three formulations without the KP-UM protein. This reduction on the K/S values could be related with

the loss of some melanin from the cortex of the damaged hair fibers during the incubation with the formulations. For the OB hair treated with the protein, the highest K/S checksum obtained was for the hair colored with KP-UM in the 10% EtOH formulation. This increase could be related with the effect of ethanol concentration on the color of KP-UM protein or with the swelling of the hair fiber which promotes the binding and penetration of KP-UM into the hair cortex [113]. Comparing the CIELAB differences of all the samples treated with the KP-UM protein with the overbleached Asian hair (**Figure III.6 b)**, a darker, reddish and less yellow color was verified, due to the addition of the chromogenic protein. This diagram corroborates the color differences observed for the hair treated with the KPUM protein in the different formulations.

The protein localization and penetration degree into the hair fiber was also assessed by optical (**Figure III.7 – Panel A**) and by confocal microscopy (**Figure III.7 – Panel B**). The higher intensity observed in all the samples compared to the No KP-UM sample demonstrates the capacity of KP-UM protein to bind to other areas of the hair besides the hair cuticle. Nevertheless, there was a preferential binding of KP-UM in the first 10-15 μm of the hair fiber, corresponding to the cuticle and initial part of the cortex [125].

All the formulations tested have the capacity to improve the mechanical properties of hair, namely the Young's modulus and the tensile strength (**Table III.2**). The differences observed between the different conditions (KP-UM in the formulations with 0, 1 and 10% EtOH) could be related with the swelling of the fiber promoted by the ethanol; the amount of KP-UM protein bound; the degree of penetration into the hair cortex; the preferential localization of the protein in the hair fiber and with potential mechanisms of stabilization [51] of the KP-UM with the keratin proteins of hair.

The leaching of KP-UM protein from the hair was evaluated for a total of 20 washing cycles. It was verified that higher percentages of ethanol in the formulation lead to higher protein leaching from the colored hair. These differences could be associated with the binding of KP-UM protein and with the protein localization in the hair fiber. For example, for the formulation with 10% ethanol, the majority of KP-UM was leached during the first washing cycle, while for the formulation with 1% ethanol, the leaching was only after eleven washing cycles. This might indicate that the protein that was leached during the first washing cycles was predominantly located at the surface of the hair fiber while the remaining protein was trapped inside the inner hair fibers and cannot came out easily to the washing solution [126].

The molecular dynamics of KP-UM interacting with the keratin filaments (**Figure III.11**) showed that the KP sequence seems to act as a hook, which when finds the keratin protofibril promotes the junction of both proteins. In addition, this sequence has one cysteine residue that should be accessible to make disulfide bonds with the many cysteine present in the keratinaceous fiber. For all the simulated systems

the interaction between the KP-UM protein and the keratin protofibril begun through the KP sequence, and after 5 ns the UM barrel adhered completely to the surface of the keratin protofibril. The effect of the different formulations on KP-UM interaction with the keratin filaments showed that higher concentrations of ethanol can difficult the interaction between the proteins. The hindering effect of ethanol could be related with the number of hydrogen bonds formed between the KP-UM protein and the keratin protofibril, since a reduction in the number of hydrogen bonds was observed when increasing the ethanol concentration.

These results support the differences obtained in the leaching assay during washing, where a higher leaching of KP-UM protein was verified with increasing the percentage of ethanol in the formulation. In this way, the higher bounding stability observed when the hair was colored by KP-UM dissolved in the phosphate buffer could be due to the absence of ethanol in this formulation.

3.5 Conclusion

In conclusion, in this study we investigated the capacity of KP-UM protein (Ultramarine protein conjugated with a keratin-based peptide - KP) to simultaneously color overbleached Asian hair and recover hair's mechanical properties. The KP-UM protein was able to color the overbleached Asian hair, being the hair color obtained mainly dependent on the polarity of the formulation where the protein was solubilized. The binding to the hair cuticle and penetration into the cortex supports its ability to color the hair. Additionally, molecular dynamic simulation studies demonstrate that the interaction of KP-UM with hair is mediated by the KP sequence, proving the importance of this peptide on the ability of KP-UM to bind and color the hair. The KP-UM protein in the three formulations (0, 1 and 10% EtOH) was able to induce a significant recovery of overbleached Asian hair tensile strength and stiffness. Also, the KP-UM was strongly bound to the hair since almost no protein was leached after 20 washing cycles, with a maximum of 9.4% of protein leaching for the 10% EtOH formulation. Considering the safety of KP-UM protein when tested in human keratinocytes, we believe that the use of this protein for hair coloration has a great potential.

In this work, we unravel the potential of fusion proteins as complex systems to be incorporated in hair-care products. Chromogenic proteins can be excellent candidates for the design and development of new eco-friendly products for hair coloration. The chromogenic nature of these class of proteins can be also explored in future in textile industry and in the development of new color-based sensors.

Chapter IV

Crystallin Fusion Proteins Improve the Thermal Properties of Hair

Chapter IV – Crystallin Fusion Proteins Improve the Thermal Properties of Hair

Abstract

Styling hair with straightening irons is a popular daily hair routine that significantly damage the hair keratin fiber due to the high temperature applied. In this study, we investigate the effect of two fusion proteins based on the human eye γD-crystallin conjugated with a keratin-based peptide (KP-Cryst Wt and KP-Cryst Mut) on hair exposed to thermal damage. The mutant form was designed to improve protein stability and promote interaction with the hair. Through the study, it was demonstrated the protection of Asian and Caucasian virgin hair's structure by the pretreatments with the KP-Cryst fusion proteins. After hair thermal exposure, a higher water content was quantified by TGA on the hair fibers pretreated with the fusion proteins (about 38% for the KP-Cryst Wt and 44% for the KP-Cryst Mut). Also, negligible alterations in hair fibers' stiffness were observed after iron application, demonstrating the proteins capacity to effectively prevent the conversion of keratin α-helix structure into β-sheets. The results proved the capacity of the fusion proteins to bind to hair and protect it against high temperatures, supporting the development of new formulations based on the KP-Cryst proteins.

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4.1 Introduction

Human hair is a highly structured fiber organized in cuticle, cortex and sometimes medulla. The characteristics of these structures influence and determine the mechanical and optical properties of hair. For example, the stiffness of the central α-helical core of keratin and the high number of disulfide crosslinks, makes hair very resistant to external factors [127].

Regardless of hair's great stability, weather, pollution, chemical treatments, and daily routine can cause several negative morphological and chemical changes in the hair [128,129]. Styling the hair with straightening irons or curling tongs is nowadays a popular daily hair routine [130], however, during these procedures, hair strands are put in contact with excessive heat (usually between 150 and 250 °C) causing the disulfide bonds to break [131]. Both irons and curling tongs act to drive out any remaining water in the hair, promoting the formation of more bonds between hair proteins, helping to set the hair in its new conformation [130]. The popularity of straightening irons and curling tongs has created a large market for hair products associated with heat styling, including heat-protection sprays, straightening balms, curl creams, and heat-protection shampoos/conditioners [130]. These heat-protecting products are able to form film-like structures over the hair fibers, smoothing hair's imperfections and help protect the hair from extreme internal water loss caused by higher temperatures or long exposure time [132].

The ability of proteins to bind to the horny layer of the hair has been explored for the development of new formulations for topical applications [42,48]. Proteins and peptides are considered useful for imparting gloss, softness, conditioning and manageability to the hair and prevent damage to the hair fibers, due to proteins' substantivity and their amphoteric and buffering properties [42,48,60].

Particles composed by keratin and silk fibroin were proven to recover asian hair stiffness and tensile strength while improving hair smoothness for virgin and overbleached hair [95]. Water-soluble silk proteins, when applied on skin or hair, are able to form a durable film that improves skin and hair smoothness while protects against environmental, chemical and grooming associated damage [46]. Milk protein hydrolysates are effective for conditioning of hair and skin and are also capable to prevent and recover the damage existent in both structures. The smoothness and softness of skin and hair are also improved when milk protein hydrolysates are applied [78].

Crystallins are the main proteins of the vertebrate eye lens and can be divided into two major families, α-crystallins and βγ-crystallins. These proteins are able to form very stable and durable structures [133,134]. Human γD-crystallin is the third most abundantly expressed γ-crystallin in the lens and contains 173 amino acids [133]. X-ray crystallographic studies showed that this monomeric protein consists of two highly homologous domains, each composed of two tightly packed β-sheet Greek key

motifs that are organized as two four-stranded antiparallel β-sheets, with a total of 16 β-strands [135,136]. This fold, with additional contributions from domain interactions, provides this protein an unusual high level of thermodynamic stability [137].

Previously, it was demonstrated the capacity of wild-type and mutant crystallins to bind to overbleached asian hair and improve/recover its mechanical properties. Moreover, none of the proteins displayed significant toxicity when tested in human fibroblasts [48]. In this work, we took advantage of the properties previously observed with the crystallin thermodynamic stability, to develop a new formulation for hair application, formulations capable to protect the keratin fibers against styling procedures using high temperatures. The new fused proteins combine the thermodynamic stability of γD-crystallin with the ability of a keratin-based peptide (KP) to bind to hair. During the design, two crystallins were used, the wild-type (Wt) crystallin and a mutant (Mut) crystallin. The mutant protein was constructed by the substitution of three arginine residues by three cysteine, on the wild-type crystallin sequence, that might improve the stability of proteins by the formation of covalent inner polypeptidic crosslinks [138]. Also, the higher cysteine content of mutant crystallin promotes the protein interaction with hair keratin that is naturally reach in cysteine residues [48]. The KP peptide is based on the sequence of hair keratin and keratin-associated proteins, rich in cysteine residues. This cysteine residues enable the formation of intra and intermolecular disulphide bonds between the KP and the hair proteins, improving even more the capacity of crystallins to bind to the hair [51].

In this work, a full characterization of the protective effect of KP-Cryst formulations on hair was performed by evaluating the protein ability to bind and penetrate through the hair fiber, and by measuring the effect of KP-Cryst fusion proteins on hair's mechanical properties and water content before and after iron application.

4.2 Materials and Methods

4.2.1 Materials

Natural Asian and Caucasian Brown hair samples were provided by International Hair Importers & Products Inc. (Glendale, New York USA). The genes coding for KP-Cryst Wt and KP-Cryst Mut fusion proteins were synthesized by GenScript (New Jersey, USA) and cloned in pET-28a(+) plasmid. The amino acidic sequences of KP-Cryst Wt and KP-Cryst Mut proteins are presented in **Table IV.1**. Nickel Magnetic Beads for His-Tag Protein Purification were available from GenScript, Molecular weight GRS Protein Marker Blue standards and culture medium were purchased from Grisp, Portugal. All other reagents used were of analytical grade, acquired from MerckSigma, Spain, and used as received.

Table IV.1 - Amino acidic sequences of KP-Cryst Wt and KP-Cryst Mut proteins. Characters in bold correspond to the keratin-based peptide (KP) and the characters underlined correspond to the glycine-alanine linker $(GA)_5$. The double underlined characters correspond to the substitutions of arginine residues by cysteine in the mutant form.

Protein	Sequence	Mw (kDa)[1]
KP-Cryst Wt	**GGVCGPSPPCITT**GAGAGAGAGAMGKITLYEDRGFQGRHY ECSSDHPNLQPYLSRCNSARVDSGCWMLYEQPNYSGLQY FLRRGDYADHQQWMGLSDSVRSCRLIPHSGSHRIRLYERE DYRGQMIEFTEDCSCLQDRFRFNEIHSLNVLEGSWVLYELS NYRGRQYLLMPGDYRRYQDWGATNARVGSLRRVIDF	24.84
KP-Cryst Mut	**GGVCGPSPPCITT**GAGAGAGAGAMGKITLYEDRGFQG**C**HY ECSSDHPNLQPYLSRCNSA**C**VDSGCWMLYEQPNYSGLQY FLRRGDYADHQQWMGLSDSV**C**SCRLIPHSGSHRIRLYERE DYRGQMIEFTEDCSCLQDRFRFNEIHSLNVLEGSWVLYELS NYRGRQYLLMPGDYRRYQDWGATNARVGSLRRVIDFS	24.68

[1]protein final size considering the protein and the extra sequence (His-tag) from pet28a(+) vector.

4.2.2 Expression and purification of KP-Cryst Wt and KP-Cryst Mut fusion proteins

Escherichia coli BL21(DE3) containing the pET-28a(+):KP-Cryst Wt and the pET-28a(+):KP-Cryst Mut vectors were used, respectively, for KP-Cryst Wt and KP-Cryst Mut expression in Terrific Broth – Auto Induction Medium (TB-AIM). A single colony harboring the plasmid was inoculated into LB medium supplemented with kanamycin (kan) and grown overnight at 37 °C. A calculated volume of the pre-inoculum was inoculated into TB-AIM_{kan} and the culture was grown for 24 h at 37 °C, 200 rpm. Cells were harvested by centrifugation at 7000 g, at 4 °C, for 5 min. The cells were resuspended in phosphate buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) with 10 mM of imidazole and a protease inhibitor and were lysed by sonication (40 % amplitude, 3.0 s on plus 9.0 s off for a total of 10 min on) in a sonicator vibra-cell™ SONICS. The suspension was centrifuged at 10.000 g, for 40 min, at 4 °C, to separate the soluble fraction. The protein present in the soluble fraction was purified using Nickel magnetic beads with specificity to the His-tag sequence present in the N-terminal of the proteins. Proteins purity was assessed by SDS-PAGE and the purified proteins solutions were dialyzed against distilled water for 4 days [94].

4.2.3 Characterization of KP-Cryst Wt and KP-Cryst Mut fusion proteins

4.2.3.1 SDS-PAGE analysis

Protein size and purity were analyzed by SDS-PAGE. The lyophilized proteins were solubilized in ultra-pure water, loaded on SDS-PAGE gel and stained with Coomassie Blue.

4.2.3.2 MALDI-TOF mass spectrometry

Mass/charge of KP-Cryst Wt and KP-Cryst Mut proteins was verified by MALDI-TOF using sinapic acid as the matrix (≥99.5%). The mass spectra were obtained using an Ultra-flex MALDI-TOF mass spectrophotometer (Bruker Daltonics GmbH) equipped with a 337 nm nitrogen laser. KP-Crys Wt and KP-Crys Mut proteins were detected using a double layer deposition, with a saturated solution of sinapic acid in ethanol deposited in the ground steel plate and dry. A solution of TA30 (30% acetonitrile/70% TFA) was selected to dissolve both proteins, and the resulting solution mixed in a ratio of 1:1 with a saturated solution of sinapic acid also in TA30. A sample composed by 2 μL was spotted into the ground steel target plate (Bruker part n° 209519) and analyzed using the reflective positive mode [139].

4.2.3.3 Fourier-Transform Infrared spectroscopy (FTIR)

FTIR spectra were acquired using KBr discs made with 10 bar pressure, at room temperature, and after 16 scans with a resolution of 32 cm^{-1} from 4000 to 600 cm^{-1} (NICOLET-AVATAR 360 FTIR spectrometer). OriginPro 8.5 software (OriginLab Corporation, MA, USA) was selected to analyze the FTIR spectra by Gaussian deconvolution of Amide I ban region (in the range of 1600 and 1700 cm^{-1}) and conclude about the proteins secondary structure. For the Amide I deconvolution, the following procedure was performed: a linear baseline was fitted; the second derivative spectrum between 1600 and 1700 cm^{-1} was calculated and the secondary structure content determined considering the ratio between the areas of the assigned peak and the total area of Amide I range. Using Gaussian function, three fitting modes were tested. First, the baseline was held fixed and fitted whereas intensity and bandwidth were allowed to vary. Then, the baseline and the bandwidth were fixed and fitted again, and finally, the baseline and center peaks were fixed and fitted once more. In the end, the frequencies determined for the deconvoluted peaks were appointed to the respective protein secondary structure: β-sheet, β-turns, random coil and α-helix [95].

4.2.3.4 Circular Dichroism (CD) spectroscopy

The effect of temperature in the structural state of KP-Cryst Wt and KP-Cryst Mut proteins was studied by CD spectroscopy using a Jasco J-1500 spectropolarimeter equipped with a temperature controller, and a path-length cell of 1 mm. The proteins were dissolved in 5 mM potassium phosphate buffer and a concentration of 10 or 20 μM was used for all the conditions. Two different temperatures were tested in this assay: 37 and 90 °C. The spectra were obtained over the wavelength interval of 180–260 nm at a

scan speed of 20 nm/min and bandwidth of 1 nm [140]. Relatively to the thermal scans, the monitorization of CD signal at 218 and 195 nm was performed for both proteins from 25 to 90 °C, with a gradient of 3 °C/min.

4.2.4 Application of KP-Cryst fusion proteins

4.2.4.1 Hair treatment with the KP-Cryst fusion proteins

KP-Cryst Wt and KP-Cryst Mut fusion proteins were applied to Asian and Caucasian Brown virgin hair. The hair strands were washed with a classic commercial shampoo (Pantene® Pro-V Classic) before application. For the treatments, 400 mg of each hair type were incubated with the KP-Cryst Wt and KP-Cryst Mut proteins dissolved in a buffer composed by 25 mM HEPES and 120 mM NaCl, at pH5 and pH9. Prior treatment, the hair strands were pre-conditioned with 1 mL of buffer, for 15 min at room temperature, and then dried with the hairdryer. Then, 1 mL of protein solution (1 mg/mL) was applied to the hair strands, incubated for 15 min, and dried with hairdryer. This step was repeated 5 times, with a total 5 mg of protein applied in each hair strand. Hair strands only incubated with the buffer were treated as controls. All samples, 24 h after treatment, were thoroughly washed in tap water with the same commercial shampoo and dried with hairdryer.

4.2.4.2 Substantivity test

An amount of 20 mg of treated and untreated hair fibers were incubated with 2mL of 1.04×10^{-4} M Rhodamine B aqueous solution, pH 4.1, in a bath at 50 °C, for 30 min. After incubation, the fibers were rinsed 5 times with deionized water to remove loosely attached dye molecules, and were dried under a nitrogen atmosphere [141]. Individual hair fibers were photographed with a fluorescence microscope (Olympus BX51, Massachusetts USA; excitation=535/550 nm, emission=645/675 nm) for the determination of the apparent diffusion coefficient by image analysis using image processing program ImageJ 1.46 r.

4.2.4.3 Differential scanning calorimetry (DSC)

For the DSC characterization, 2 mg of each hair samples (with and without the KP-Cryst fusion proteins) were analyzed using DSC instruments (DSC 6000, Perkin Elmer). Thermal studies of KP-Cryst Wt and KP-Cryst Mut proteins were conducted using a power compensated differential scanning calorimetry instrument and aluminum pans (max. pressure: 1 bar), at a temperature range from 50 to 265 °C (heating rate: 5 °C/min). The DSC instrument calibration was performed using high-purity indium and zinc and all samples were measured in duplicate, with the mean value and standard deviations calculated and presented [95].

4.2.4.4 Mechanical properties

The effect of KP-Cryst Wt and KP-Cryst Mut proteins on the mechanical properties of hair was assessed by the differences in the Young's modulus before and after treatment with the proteins. The hair mechanical properties were determined following guidelines outlined in ASTM D1145-95 for fiber tensile testing. Tensile tests were performed using a Hounsfield dynamometer H100KS Model and a set of 25 hair fibers with low variability was selected for each condition. Each hair was individually mounted in the tensile jig by means of a paper template with a fixed gauge length of 20 mm and placed in an excicator prior to the analysis. A load range of 25 N and a speed of 1.5 mm/min were defined as settings for the tensile strength test. For each hair, applied load against extension were recorded and, using an average mean diameter of 70 μm, the data were converted to stress (load/unit area) versus strain (% extension). All measurements were made in the middle part of the hair fiber [95].

4.2.5 FITC linkage to crystallins

To study the adhesion/penetration profiles of KP-Cryst Wt and KP-Cryst Mut fusion proteins into Asian and Caucasian virgin hairs, fluorescein 5(6)-isothiocyanate (FITC) was linked to both proteins. The proteins were dissolved in 0.1 M sodium carbonate buffer, pH 9, at 2 mg/mL. A volume of 5 mL from these solutions was incubated with 250 μL of a 1 mg/mL FITC in DMSO solution, at 4 °C. Unbound FITC was separated from the conjugate (KP-Cryst-FITC) by dialysis against water, at 4 °C. Labelled crystallins were used in the hair treatments as described earlier in section 4.2.4.1, in a proportion of 1:3 (KP-Cryst--FITC:KP-Cryst).

4.2.5.1 Confocal microscopy

Individual hair fibers treated with the KP-Cryst-FITC conjugates were embedded in epoxy resin and transversal cuts (20 μm) were prepared using a microtome (Microtome Leitz, Oberkochen, Germany). Transversal cuts of Asian and Caucasian hairs, with and without the KP-Cryst-FITC conjugates, were analyzed by Confocal Scanning Laser Microscope (Olympus BX61, Model FluoView 1000). Acquisition for all the hair samples was done using the same settings (filter, exposure time and brightness). Images were acquired with the program FV10-Ver4.1.1.5 (Olympus) integrated with Line Series Analysis. The detection was obtained with a laser excitation line at 488 nm and emissions filters BA 505–605. Images were acquired with the program FV10-Ver4.1.1.5 (Olympus) integrated with Line Series Analysis.

4.2.6 Protection against thermal damage

4.2.6.1 Heat application to hair strands using a flat iron

To evaluate the protective effect of KP-Cryst proteins against heat, hair strands with and without the KP-Cryst proteins were exposed to 200 °C for 5 s using a hair iron (Ricki Parodi®). This process was repeated three times, with 1 min interval between applications.

4.2.6.2 Mechanical properties

The protective effect of KP-Cryst Wt and KP-Cryst Mut proteins was evaluated by the differences in the Young's modulus before and after iron application. The mechanical properties were determined using the same protocol as described in Section 4.2.4.4.

4.2.6.3 Hair Hydration

The loss of water content from treated and untreated hair strands, before and after iron application, was evaluated using thermal gravimetric analysis (TGA). TGA was performed with a TGA 4000 (Perkin Elmer, Waltham, Massachusetts, USA) using an alumina crucible with sample weights between 8-10 mg. The temperature calibration was done by Curie temperatures of reference materials: alumel, nickel and perkalloy at the same sample scanning rate. The measurements were performed from 25 to 250 °C at 10 °C/min under a nitrogen atmosphere (flow rate: 20 mL/min). The weight loss, in percentage, and its derivative were represented as function of temperature. The data were acquired using Pyris software (version 13).

4.2.7 Statistical analysis

Data are presented as average standard deviation (SD), n=3. Statistical comparisons were performed by one-way ANOVA with GraphPad Prism 5.0 software (La Jolla, California, USA). Tukey's post hoc test was used to compare all the results between them, and a Dunnet's test was used to compare the results with a specific control. A p-value ≤0.05 was considered to be statistically significant.

4.3 Results and Discussion

4.3.1 Characterization of KP-Cryst Wt and KP-Cryst Mut fusion proteins

Cosmetic industry is constantly seeking for advanced and cleaner solutions for the development of new hair care products. The approach here presented takes advantage of the ability of γD-crystallin to form film-like structures on the hair [48]. Exploring the great thermodynamic stability of this protein, we aim to develop new formulations to improve hair mechanical performance while protecting the hair fiber from damage resultant from the exposition to high temperatures.

Two crystallins were used in this work, the wild-type, with the same sequence as the human eye γD-crystallin, and a mutant form, obtained by the substitution of three arginine residues (R) at positions 15, 37 and 80 on crystallin sequence by cysteine residues (C). These positions, pointing towards three 'cardinal' positions, were selected considering previous works with mutant crystallins and amyloid-forming proteins [48,142].

The two crystallins were fused with a keratin-binding peptide (KP) [51] and a $(GA)_5$ linker [143] to promote the binding of the proteins to the hair cuticle and cortex and impart the constructs with structural mobility, respectively. The $(GA)_5$ linker, composed by five repetitions of Glycine-Alanine, was included since spacers rich in glycine are described to be stable against proteolytic digestion and to impart flexibility to the proteins, being capable to bind several domains without compromising their function [143].

After expression and purification, the KP-Cryst Wt and KP-Cryst Mut fusion proteins were characterized regarding purity and molecular weight. The high purity of both proteins was confirmed by SDS-PAGE and MALDI-TOF (**Figure IV.1**). On the SDS-PAGE gel **(Figure IV.1 (A))**, it is possible to observe that both KP-Cryst Wt and KP-Cryst Mut proteins present well-defined bands with molecular weight close to the theoretical, 24.84 and 24.68 kDa, respectively. MALDI-TOF mass spectrometry **(Figure IV.1 (B))** also confirmed the monodisperse character and the molecular weight of KP-Cryst Wt and KP-Cryst Mut proteins.

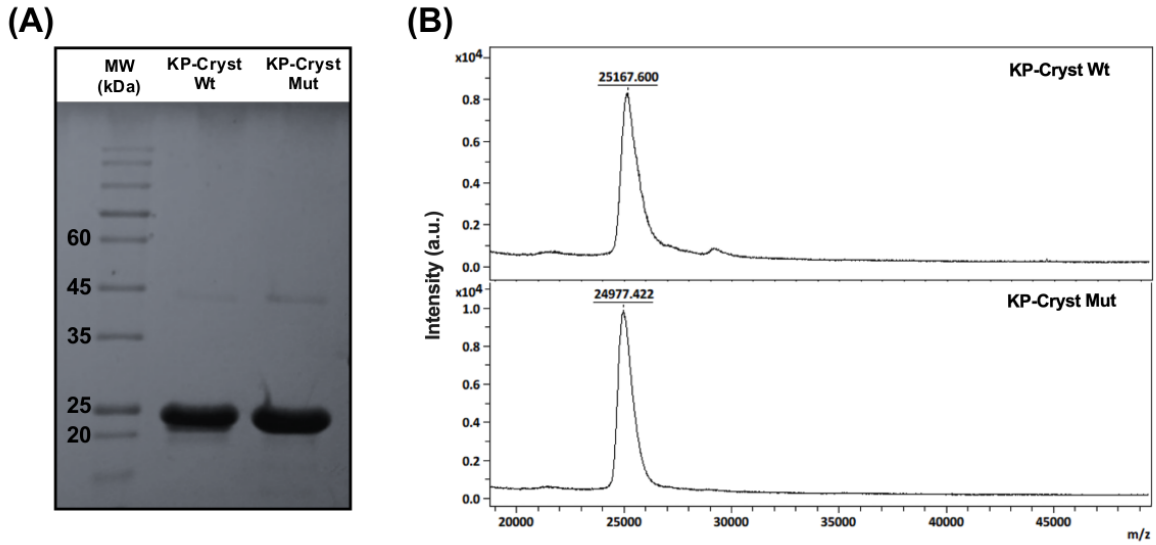


Figure IV.1 - (A) SDS-PAGE electrophoresis of KP-Cryst Wt and KP-Cryst Mut fusion proteins and GRS Protein Marker Plus molecular weight; (B) MALDI-TOF mass spectra of KP-Cryst Wt and KP-Cryst Mut proteins obtained in reflective positive mode using sinapic acid as matrix.

To evaluate the effect of arginine substitutions by cysteine residues on the structure of KP-Cryst Mut variant, the FTIR spectra of KP-Cryst Wt and KP-Cryst Mut proteins were acquired. The structural

information given by FTIR is predominantly derived from analysis of the so-called amide band, particularly the amide I band (1600–1700 cm^{-1}). Deconvolution and differentiation of the amide band makes possible to distinguish between the individual component types. The deconvolution of Amide I allowed to analyze the corresponding structural assignments [144]. The components centered between 1658-1650 cm^{-1} were assigned to α-helix structures; between 1640–1620 cm^{-1} and 1695–1690 cm^{-1} were assigned to β-sheet; and bands between 1680-1660 cm^{-1} were assigned to β-turns [145]. The percentages corresponding to the different types of secondary structure, obtained by analysis after amide I band deconvolution, are summarized in **Table IV.2**.

Table IV.2 - Secondary structural assignments on KP-Cryst Wt and KP-Cryst Mut fusion proteins.

Secondary Structure Amide I	KP-Cryst-Wt	KP-Cryst-Mut
	%	%
β-sheet	46.29	52.72
β-Turn	22.87	17.63
Random Coil	7.6	17.45
α-helix	23.32	12.2

The substitution of arginine residues by cysteines in the KP-Cryst Mut protein resulted in an increase on the β-sheets content (from 46.29 to 52.72%) and on the amount of random coil (from 7.6 to 17.45%), when compared with the KP-Cryst Wt protein. Concomitantly, there was a reduction in the percentage of α-helix (from 23.32 to 12.2%) and of ß-turns (from 22.87 to 17.63%). Moreover, the unordered structures percentage for the KP-Cryst-Mut protein increased about 10% comparatively to the wild type protein. This increase can be attributed to an alteration in the protein structure promoted by the three mutations. Also, this effect on the protein structure could be observed in aqueous solution, where visible aggregates of KP-Cryst Mut protein have formed over time. This behavior was also observed by Ribeiro *et al.* 2013 for the Cryst Mut protein [48].

The high percentage of β-sheets on both proteins is in accordance with the literature, where is described that each domain of γ-crystallins is composed of intercalated double β-sheet Greek key motifs [136,146], a characteristic structural feature of the βγ-crystallin superfamily [147].

The structure of macromolecules, particularly proteins, is known to be sensitive to its environment, temperature and pH. The KP-Cryst fusion proteins were analyzed by circular dichroism (CD) to determine if the substitution of the arginines influenced the folding of the mutant form when comparing with the wild-type construct and to evaluate the thermal stability of the proteins at several temperatures.

The KP-Cryst fusion proteins were dissolved in 5 mM potassium phosphate buffer to a final concentration of 10 μM and were analyzed by CD. The CD spectra in the far-UV (below 260 nm) was reported to predict the effect of the amino acid substitution in proteins' thermal stability (**Figure IV.2**). In **Figure IV.2**, it is possible to observe a CD spectra typical of β-sheets conformation, with a characteristic negative band near 215 nm and a positive band near 195 nm [148]. Analyzing **Figure IV.2**, it is possible to observe, that the lower ellipticity measured for the mutant form support the effect of mutations on the proteins' structure previously observed by FTIR (**Table IV.2**.).

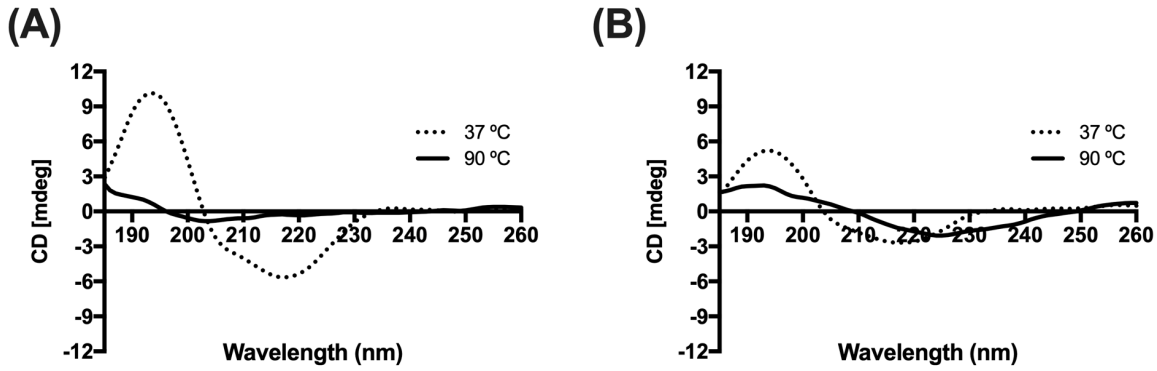


Figure IV.2 - Effect of temperature on the circular dichroism spectra of KP-Cryst Wt (A) and KP-Cryst Mut proteins (B). Experiments were conducted at 37 and 90 °C.

The increasing of temperature until 90 °C lead to a loss of protein conformation in both cases, however it is possible to observe that the spectrum of KP-Cryst Mut still maintain the oscillating profile observed for lower temperatures. Since the ellipticity at 90 °C for the KP-Cryst Mut protein was lower than 3 mdeg, a higher concentration was tested for this protein **(Figure IV.3 (A))**. As expected, higher ellipticity was obtained and the same profile was observed, comparing with the lower concentration, when exposing the protein to the highest temperature tested (90 °C). This result indicates that KP-Cryst Mut is more resistant to higher temperatures than the wild type protein. The increased thermal stability of KP-Cryst Mut protein was also demonstrated by the thermal scans reported at 218 and 195 nm from 25-90 °C **(Figure IV.3 (B))**, where the KP-Cryst Wt protein completely denatures around 80 °C while the KP-Cryst Mut protein still display some of its original structure at 90 °C. Also, comparing the spectrum of both proteins at 37°C, the spectrum of KP-Crys Mut show about half of the band amplitude at 195 and 215 nm, indicating differences in the secondary structure of the protein.

The resistance to higher temperatures and the decrease in band amplitude could be due to the substitution of the three arginine residues by the cysteines in the mutant protein. This increase in protein thermal stability could also be related to the increase in protein hydrophobicity or to the formation of

protein disulfide bridges resulting from the incorporation of cysteine residues in the protein sequence. It was already proved by other authors that mutations where cysteines where included, lead to the formation of disulfide bridges and, consequently, increased the proteins' thermal and structural stability [149].

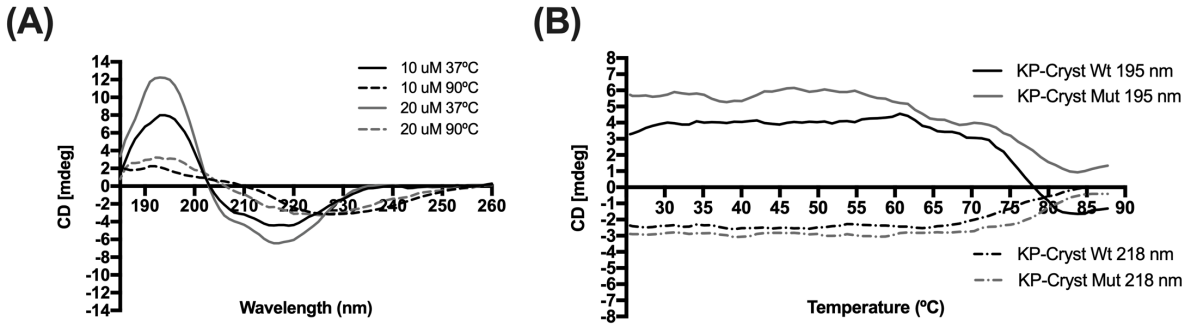


Figure IV.3 - (A) Effect of protein concentration and temperature on the circular dichroism spectra of KP-Cryst Mut proteins. Experiments were conducted at 37 and 90 °C; (B) Thermal unfolding curves obtained for KP-Cryst Wt and KP-Cryst Mut proteins by monitoring CD signal at 218 and 195 nm from 25 to 90°C. All the spectra are background corrected.

4.3.2 Hair treatment with the KP-Cryst Wt and KP-Cryst Mut fusion proteins

To evaluate the binding capacity of KP-Cryst Wt and KP-Cryst Mut to hair, different protein solutions were prepared and applied to Asian and to Caucasian Brown virgin hairs. The proteins were dissolved at a final concentration of 1 mg/mL in 25 mM HEPES and 120 mM NaCl buffer, at pH5 or pH9. This concentration corresponds to the maximum protein solubility without the formation of visible aggregates.

The pH values were selected taking in consideration the isoelectric points of KP-Cryst Wt and KP-Cryst Mut fusion proteins, 7.13 and 6.07, respectively. Both proteins present a positive charge at pH5 and a negative charge at pH 9. Besides the effect of the presence of the KP peptide and the substitutions in the amino acidic sequence of the proteins (three extra cysteine residues for the mutant form), we also evaluated the effect of proteins' charge on their ability to bind to hair fibers.

The ability of KP-Cryst Wt and KP-Cryst Mut proteins to adhere to the hair fibers was studied using Rhodamine B as a probe. Taking in consideration the binding and diffusion of Rhodamine B into the hair, this dye gives information concerning the hair structure and the presence of polymers/proteins on the surface of the fiber [141].

Images of the hair after immersion in a Rhodamine B solution, were acquired using a fluorescence microscope **(Figure IV.4 (A))**. For each condition, 5 hair fibers were analyzed using the software ImageJ 1.50i. The fluorescence intensities were determined in three different regions for each hair fiber, and the average values were considered for the results analysis **(Figure IV.4 (B) and (C))**.

The treatment with the KP-Cryst Wt and KP-Cryst Mut proteins prior to the incubation with Rhodamine B lead to a decrease in fiber's fluorescence intensity **(Figure IV.4)**. The differences observed were significant when compared with the respective controls (Asian and Caucasian hairs treated with the buffers). The decrease in fluorescence was directly related with the presence of the proteins on the hair surface. Crystallins were already described to form film-like structures over the hair protecting the fiber from damage [48]. The reduction in fluorescence intensity might indicate that the KP-Cryst Wt and KP-Cryst Mut proteins are also able to form film-like structures over the hair cuticles, decreasing the binding and penetration degree of Rhodamine B into the hair [141].

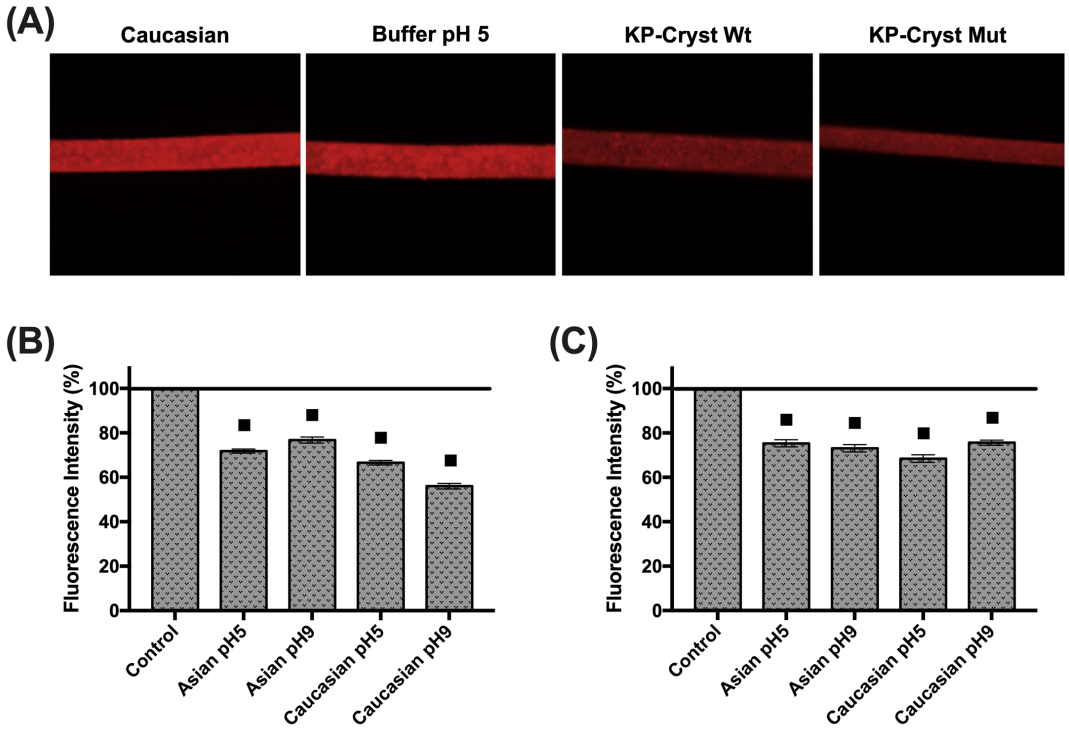


Figure IV.4 - (A) Fluorescence microscopy images of treated and untreated Caucasian Brown hair fibers, after incubation with Rhodamine B solution; (B) Fibers fluorescence intensity decrease of Asian and Caucasian Brown hair after incubation with KP-Cryst Wt protein dissolved in 25 mM HEPES + 120 mM NaCl buffer, at pH 5 or pH 9; (C) Fluorescence intensity of Asian and Caucasian Brown hair after incubation with KP-Cryst Mut protein dissolved in 25 mM HEPES + 120 mM NaCl buffer, at pH 5 or pH 9. Statistically significant differences from the respective control are indicated as: ■ p-value ≤0.0001.

For both proteins there was a decrease in the fluorescence intensity between 25 and 45%, with the best result obtained for the Caucasian Brown hair treated with KP-Cryst Wt at pH9. Comparing the samples treated with KP-Cryst Wt with the samples treated with KP-Cryst Mut, the most significant differences

were observed for the Caucasian Brown hair at pH 9, where a difference of almost 20% was verified between the measured intensities.

Analyzing both types of hair, Asian and Caucasian Brown hair, differences in fluorescence intensities were also observed. Generally, a greater fluorescence decrease was observed for Caucasian Brown hair, when both proteins were separately applied. These differences could be associated with the hair structural variations like the overlapping of the cuticles on hair surface [150]. Compared with the Asian hair, the Caucasian hair has a lower number of cuticle layers with a wider interval between them [150]. These differences in hair structure influence protein's penetration into the hair fiber, resulting in a decrease in fiber fluorescence intensity for the Caucasian Brown hair.

To determine the effect of the pH/protein charge and type of hair on the binding and penetration profiles of KP-Cryst Wt and KP-Cryst Mut, both proteins were conjugated with FITC and applied to Asian and Caucasian Brown virgin hairs (**Figure IV.5**).

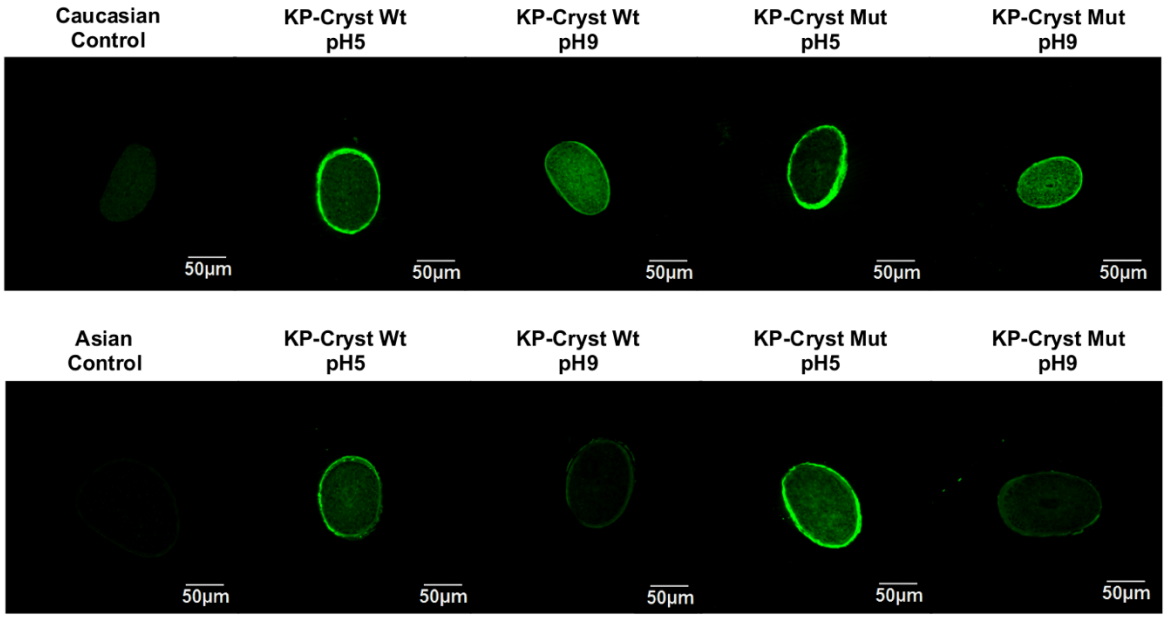


Figure IV.5 - Confocal images of human hair cross sections from untreated Caucasian Brown /Asian virgin hair and Caucasian Brown /Asian virgin hair treated with KP-Cryst Wt and KP-Cryst Mut labelled with FITC, dissolved in 25 mM HEPES + 120 mM NaCl buffer, at pH 5 or pH9.

Depending on the hair type and protein charge, the KP-Cryst Wt and KP-Crys Mut fusion proteins were able to bind to the hair cuticle and even to penetrate into the hair cortex. For the Caucasian Brown hair, the proteins bind preferentially into the hair surface/cuticle at pH5 (proteins with positive charge), while at pH9 (proteins with negative charge) they tend to penetrate into the hair cortex. This effect was

expectable since higher pH values can promote the hair swelling and the raising of the cuticles, facilitating the protein penetration into the fiber cortex [21]. Also, at pH5, both proteins have a positive net charge, which can improve the proteins bounding to the negative hair surface by electrostatic interactions [151]. For the Asian hair, a different tendency was observed. A higher penetration degree and higher protein binding were obtained for pH5, regardless of the protein. This behavior could be related with structural differences between the Asian and Caucasian Brown hairs. The Asian hair has more cuticle layers, wider cuticle cells and thinner cuticular interval than the Caucasian Brown hair [150]. These differences in the cuticle layer of the Asian hair could act as a stronger barrier, hindering the entrance of the proteins into the hair fiber cortex even at higher pH values.

4.3.3 KP-Cryst fusion proteins improve the thermal properties of Asian and Caucasian Brown hairs

When developing a new hair care formulation, it is important to evaluate its effect on the properties of hair. DSC is a technique which can be used to evaluate the hair properties, specially to assess the interactions of the main morphological components of human hair with the components of cosmetic formulations (Barba *et al.*, 2009). All these results can give information, for example, about the effect of new formulations on hair thermal stability. The parameter analyzed by this technique is the keratin α-helix denaturation enthalpy, which is identified by a peak with a temperature between 210 and 250 °C [153]. Peaks around these temperature range were selected to evaluate the effect of KP-Cryst Wt and KP-Cryst Mut fusion proteins on the α-helix keratin denaturation enthalpies of Asian and Caucasian Brown hair.

Analyzing the thermograms obtained by DSC (**Figure IV.6**), two keratin denaturation peaks around 220 and 230 °C were identified. This behavior was already reported by many authors, which state that these peaks correspond to the *ortho-* and *para-* cortical cells of hair, respectively. These cells are structurally different, being the concentration of disulfide linkages smaller on the *ortho-*cells [153]. Also, it is considered that the occurrence of double denaturation endotherms of keratin is attributed to the cystine content and disulfide linkages which, being large enough, make possible to separate both peaks [153]. The enthalpy of both peaks was taken in consideration to determine the keratin α-helix denaturation enthalpies and the values are represented on **Table IV.3**.

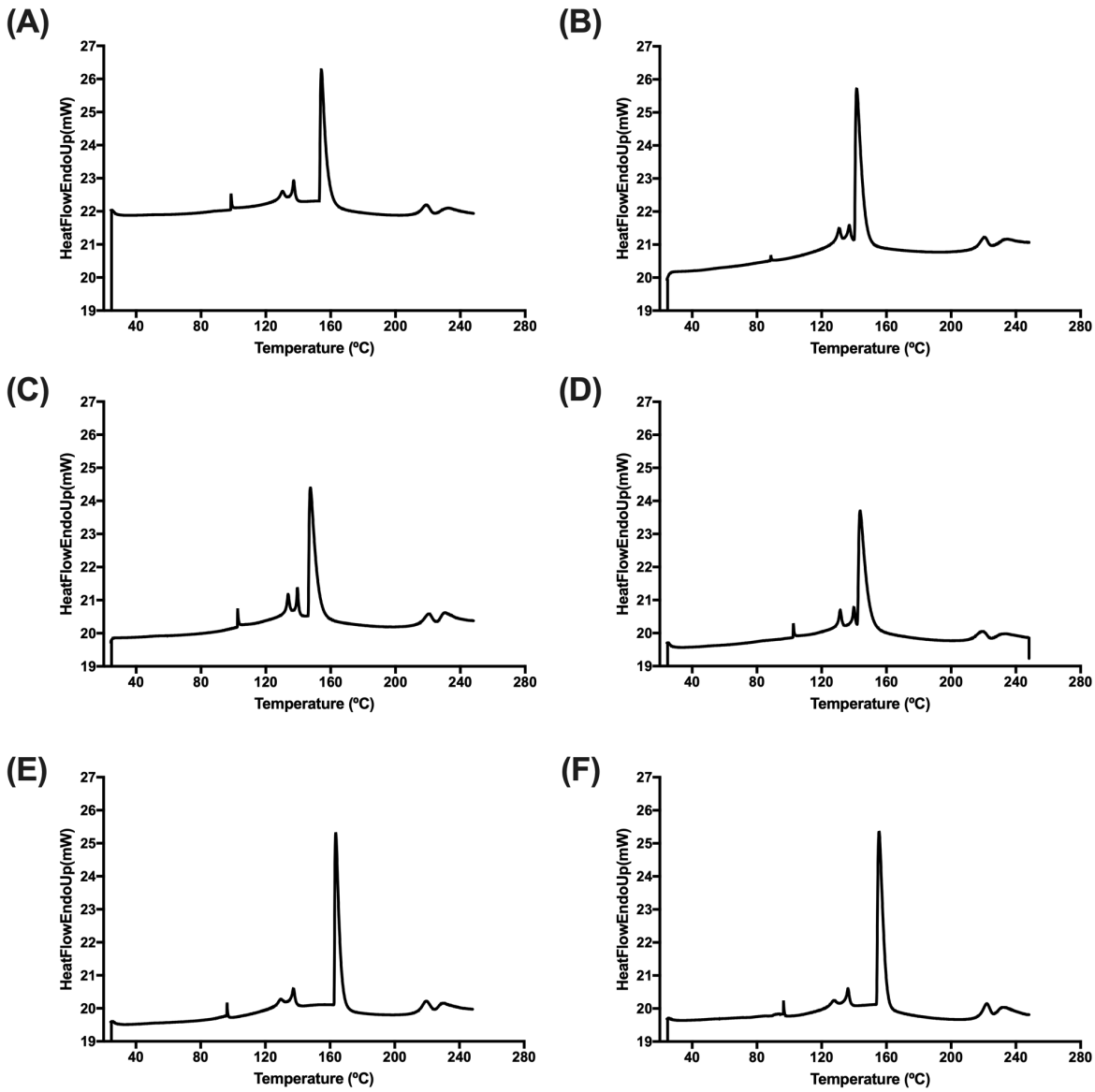


Figure IV.6 - DSC thermograms of Caucasian Brown hair treated with (A) buffer pH5, (B) buffer pH9, (C) KP-Cryst Wt pH5, (D) KP-Cryst Wt pH9, (E) KP-Cryst Mut pH5 and (F) KP-Cryst Mut pH9 proteins.

Analyzing **Table IV.3**, it was verified that both proteins, independently of the pH, are able to increase the keratin α-helix denaturation enthalpy, thus demonstrating that the proteins can interact with and stabilize the hair keratin fibrils. This increase means that more energy is need to promote keratin fibers' degradation, being the proteins able to protect the fibers against thermal damage.

When comparing the two types of hair, higher improvements were observed for the Caucasian Brown hair. These differences could be related with the structural differences between the two types of hair, with the amount of protein on the hair surface, and with the degree of penetration of the KP-Cryst Wt and KP-Cryst Mut proteins into the hair cortex. The higher number and size of the cuticles in the Asian hair might

hinder the protein penetration into the fiber cortex resulting in less amount of protein available to interact with the keratin fibrils.

Comparing both proteins, generally, KP-Cryst Wt protein increases the denaturation enthalpies around 10-20% more than its mutant form. This may be related with the differences in the protein structure, which can disturb their interaction with the keratin filaments, or with the amount of protein present inside the hair fibers.

Table IV.3 - Keratin α-helix denaturation enthalpies of Caucasian Brown and Asian hair treated with KP-Cryst Wt and KP-Cryst Mut formulations, at pH5 and pH9.

Hair	Condition	Denaturation Enthalpy (J/g)
Caucasian Brown	Control pH5	12.08±0.31
	Control pH9	11.09±1.09
	KP-Cryst Wt pH5	16.26±0.44
	KP-Cryst Wt pH9	16.80±1.15
	KP-Cryst Mut pH5	14.02±1.13
	KP-Cryst Mut pH9	14.46±0.46
Asian	Control pH5	14.64±0.51
	Control pH9	13.16±0.18
	KP-Cryst Wt pH5	14.92±2.98
	KP-Cryst Wt pH9	15.64±0.67
	KP-Cryst Mut pH5	14.90±0.50
	KP-Cryst Mut pH9	16.73±0.76

4.3.4 Protective effect of KP-Cryst fusion proteins against heat

Damage resulting from high temperature thermal styling treatments such as hot flat irons, blow dryers and curling irons has become an increasing concern in hair care. During these styling procedures the temperatures can exceed 200°C, imparting significant damage to the hair keratin [154]. Since these styling appliances are known to cause significant damage to hair, there is a need for the development of thermal protective formulations capable to maintain the hair properties.

The protective effect of KP-Cryst Wt and KP-Cryst Mut proteins against high temperatures was evaluated in terms of water content (TGA) and mechanical performance (stress-strain curve), before and after application of high temperatures using a hot flat iron.

Water changes the properties of human keratin fibers and, therefore, plays an important role in cosmetic performance. Hot flat irons that lack heat control can destroy the hair protein structure resulting in changes in hair water content. **Figure IV.7** represents the effect of KP-Cryst Wt and KP-Cryst Mut fusion

proteins on hair's water content before and after iron application. The treatment with the KP-Cryst fusion proteins increased the water content of Caucasian Brown hair in more than 10% when compared with the untreated hair, with the best results being obtained for the treatments at pH5 (KP-Cryst Wt - 14.58±0.58%; KP-Cryst Mut – 19.65±1.21%). This behavior was expected since proteins show a high capacity do bind water molecules, creating a suitable environment for hair [155].

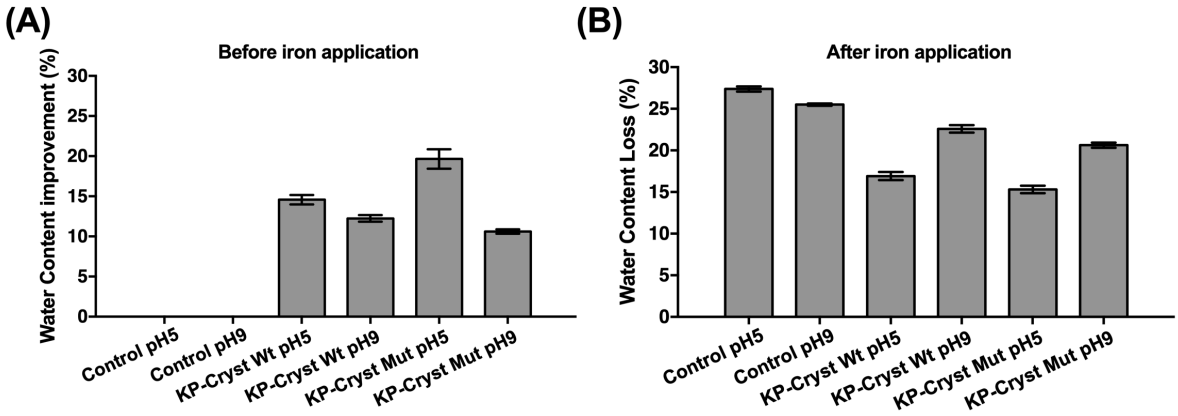


Figure IV.7 - Effect of KP-Cryst Wt and KP-Cryst Mut treatment on the water content of Caucasian Brown hair fibers determined by thermal gravimetric analysis (TGA): (A) hair water content before iron application, with the percentages calculated relatively to the respective control; (B) hair water content reduction after iron application (3x5s), with the percentages calculated relatively to the respective samples before iron application.

After iron application **(Figure IV.7 (B))**, a reduction of around 25% in hair's water content was verified, for both control samples. However, when the hair fibers were pretreated with the proteins, a lower reduction in the fibers' water content was obtained. The results demonstrate that the pretreatment with the KP-Cryst proteins provide a protection against heat, measured in terms of water loss resulting from the heating treatment. The percentage of heating protection was calculated based on the difference in water content reduction between the untreated hair sample and the KP-Cryst Wt or KP-Cryst Mut pretreated hair sample, after iron application. At pH5, the pretreatment with the KP-Cryst Wt and the KP-Cryst Mut proteins provided, respectively, about 38 and 44% of heating protection to the Caucasian Brown hair subjected to 200 °C for 15s (3x5s). Again, the best results were verified for the treatments performed at pH5, where both proteins acquire a positive charge that improves the protein interaction with the hair's negative surface charge by electrostatic interactions [151].

The reduction of fibers' water content after iron application can be attributed to changes in protein conformation induced by extreme heat. It is already reported in the literature that the application of heating to keratin fibers induces a change in the keratin conformation, going from an α-helix structure to β-sheets [154,156]. The undamaged hair displays a well-organized structure based on α-helical coiled coil protein conformation, but, once the protein is exposed to high temperatures, it can unfold and convert into a β-sheet structure. These changes in protein conformation affect the hydrogen bonding structure that stabilizes the α-helical structure and, therefore, may change the water accessibility to the hair [154]. We can deduce that, when KP-Cryst Wt and KP-Cryst Mut are applied to the hair before iron application, the proteins reduce the heat flow from the source to the hair fiber, and subsequently reduce the water loss from the hair. The protection of native keratin structure associated with the application of KP-Cryst Wt and KP-Cryst Mut proteins leads to an improved water retention, contributing to a reduction of the negative impact of heat on the hair fibers without compromising the ability of hair to be styled (data not shown).

In order to assess the protective effect of the KP-Cryst proteins in the integrity of the hair fibers, we determined the mechanical resistance of hair fibers, before and after the application of heat. Hair resistance was determined using the calculated parameters of Young's modulus (stiffness) of Caucasian Brown hair treated with both wild-type or mutant proteins, before and after iron application (3x5s, 200 °C). The protective effect of the proteins could be determined taking in consideration this parameters, since the slightest modification in the chemical composition or in the structure of hair may greatly alter its mechanical properties [48].

After proving the affinity of both proteins towards the Caucasian Brown hair, our objective was to check their ability to improve the mechanical properties of Caucasian Brown hair and protect it against heating procedures that thermally damage hairs' keratinous structure (**Figure IV.8**).

Both proteins, independently of pH, were able to significantly improve the Caucasian Brown hair's Young's modulus (p-value≤0.0001) after treatment (**Figure IV.8**). The application of KP-Cryst Wt protein resulted in an improvement of hair's Young's modulus in a magnitude of 37 and 48% when applied at pH5 and at pH9, respectively. Relatively to the KP-Cryst Mut protein, the improvement was around 45 and 40% when applied at pH5 and at pH9, respectively. The improvement in the Young's modulus after proteins' application could be related with the interaction between these proteins with the keratin fibers, resulting in an increase in fibers' stiffness. Also, the capacity of these proteins to penetrate into the fiber cortex, demonstrated on **Figure IV.5**, seems to be an essential condition for the improvement of hair's mechanical properties. The increase in Young's modulus could also be related with the KP-Cryst Wt and KP-Cryst Mut

structure. Proteins structurally characterized by the Greek key motifs show the ability to coat specific structures, so these proteins might form a thin coating around the hair fibers [48,157], decreasing the hair elasticity and, in consequent, increasing the Young's modulus values. No significant differences were observed between the hair fibers treated with KP-Cryst Wt and KP-Cryst Mut proteins, regardless of the pH tested.

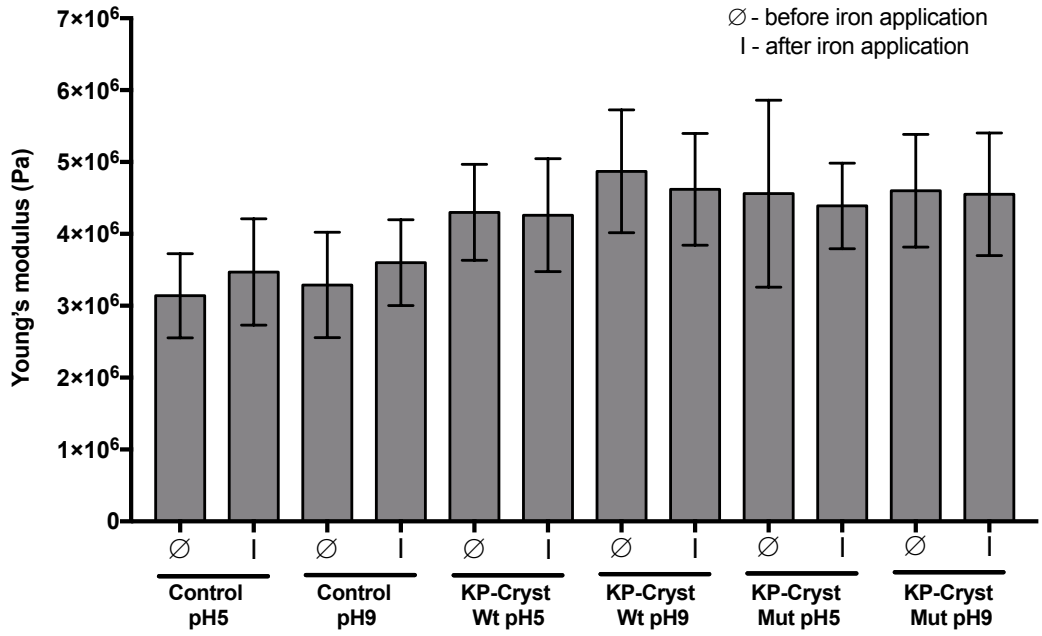


Figure IV.8 - Mechanical resistance parameters: Young's modulus of Caucasian Brown hair before (Ø) and after (I) iron application (3x5s). Values are the mean±SD of 30 independent measurements.

After iron application, a 10% increase in the Young's modulus values was observed for the control samples, comparatively to the values obtained before iron application. This increase could be attributed to the modifications of hair proteins induced by excessive heating, including the conversion of keratin structure from α-helix to β-sheet. Some authors propose that the conversion of α-helix into β-sheet is driven by the disruption of the hydrogen bonding structure that hold together the protein helices, suggesting the unfolding of part of the helical structure and concomitant refolding into β-sheet [154]. This change into a keratin β-sheets structure increases the fibers' crystallinity, resulting in an increase of their stiffness.

When the KP-Cryst Wt and KP-Cryst Mut were applied on the Caucasian Brown hair as a pretreatment, a smaller change in the Young's modulus value was verified after exposition to high temperatures, when compared to the values before iron application. These results support the protective role of KP-Cryst proteins, since the pretreatment with four protein-based formulations effectively prevented the conversion

of keratin α-helix structure into β-sheets, resulting in negligible alteration in the hair fibers' stiffness values [154]. One may assume that the protection against high temperatures can be associated with the structure of these two proteins which are composed by a pair of domains containing two tightly packed Greek key β-sheets motif. This fold, in conjugation with contributions from domain interactions, contributes towards an unusually high thermodynamic stability of crystallin proteins [158].

4.4 Conclusion

The procedures generally used for hair shape modulation have negative effects on hairs' stiffness and physical properties. The development of new formulations to protect hair against the adverse effects of high temperature procedures is needed. In the present study, KP-Cryst Wt and KP-Cryst Mut proteins were successfully expressed, purified and applied on the pretreatment of virgin Asian and Caucasian Brown hair. Both proteins showed the capacity to bind and penetrate into the hair fibers, being their deposition or penetration pattern dependent on the formulation pH and hair type. For the Caucasian hair, the KP-Cryst proteins are localized preferentially at the hair surface at pH5, while at pH9 the proteins tend to penetrate through the inner layers of hair fiber, reaching the cortex. A similar behavior was observed for the Asian hair, however with a lower amount of protein inside the hair fiber, possibly due to the differences in the structure of the hair cuticles of this type of hair. A protective effect of KP-Cryst Wt and KP-Cryst Mut proteins was observed when both proteins were applied as a pretreatment before iron. The pretreatment with the fusion proteins preserved the mechanical properties and reduced the water loss for the Caucasian Brown hair after iron application.

This study provides new insights about the protective effect of KP-Crys Wt and KP-Cryst Mut proteins of hair fibers' when exposed to repeated procedures using high temperature. Both proteins are potential ingredients to be included in new hair thermal protection formulations.

Chapter V

Keratin-based Particles for Protection and Restoration of Hair Properties

Chapter V – Keratin-based Particles for Protection and Restoration of Hair Properties

Abstract

Human hair is an element with unquestionable relevance in society both for women and men. Therefore, it is of great importance to develop new cosmetic products for hair care capable to restore and improve hair's characteristics. Here we explore the potential of keratin-based particles in the protection and recovery of hair mechanical properties and thermal stability. Keratin-based particles were obtained by high pressure homogenization using keratin and silk fibroin. The particles were characterized regarding size, superficial charge and polydispersity index. Their safety to cells was assessed using human skin keratinocytes. Virgin and overbleached Asian hair were treated with eight keratin-based formulations. The effect of particles on hair's mechanical properties was evaluated in terms of stiffness and tensile strength. The impact of treatments in hair thermal performance was studied using differential scanning calorimetry (DSC). Keratin-based particles were capable to recover and/or improve the mechanical properties of virgin and overbleached hair. Virgin hair treated with $K_{80}SF_{20}P$ particles presented an improvement of the mechanical properties around 40%. An increase in keratin α-helix denaturation enthalpy and in surface smoothness for both types of hair was also verified after treatment. These particles demonstrated stability over time and proved to be safe when tested in human keratinocytes. The keratin-based particles here presented have the potential to be incorporated in the development of new and effective hair care cosmetic formulations.

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This chapter is based on the following scientific publication:

Ana Tinoco, José Gonçalves, Carla Silva, Ana Loureiro, Andreia C. Gomes, Artur Cavaco-Paulo and Artur Ribeiro, Keratin-based particles for protection and restoration of hair properties, *International Journal of Cosmetic Science*, 2018, 40:408–419.

5.1 Introduction

Hair is an important component of the body image with great psychological importance for both men and women which we can change and manipulate according to culture and fashion[3]. Nevertheless, successful cosmetic alteration of hair, like coloring and straightening, usually require harsh chemical processes altering the normal structure of the hair shaft. Bleaching is a chemical process commonly used in hair coloration to eliminate or lighten hair natural tone and prepare the hair fiber for dyeing [48]. This process uses strong oxidative agents which destroy the disulphide bonds within the keratin resulting in a decrease of hair fibers' properties [48]. Around 80% of human hair is formed by α-keratin protein located into the cortex, that is responsible for the hair strength, flexibility, durability, and functionality [159]. After bleaching, the hair may appear cloudy, dry, rough, fragile and dull, presenting alterations in its mechanical and surface properties [159].

There is an increasing demand for the development of new hair protective products and hair cosmetic industry is focused in alternative solutions like proteins and protein-based materials. These can be incorporated in formulations of numerous topical applications due to their beneficial effects. Proteins can create a suitable environment for healthy hair because of their ability to bind water with the horny layer skin and its annexes. Proteins are included in the formulations as hair conditioning agents since they are capable to prevent the damage caused to the hair fibers based on their amphoteric and buffering properties [36,74].

Keratin and silk fibroin were used in this work as base ingredients to prepare formulations for hair care cosmetic products. The effect of keratin hydrolysates on hair fibers was already described by many authors [76]. Keratin based peptides increase the hydration, the brightness, the softness, the mechanical and the thermal properties of hair fibers [76]. Silk fibroin is non-toxic, biocompatible and shows an excellent water binding and absorbing capacity which makes it also an excellent candidate to promote a moisturizing effect of hair formulations [160].

The aim of this study was the development of a new set of products for the protection and restoration of hair properties using keratin and silk fibroin as core ingredients. We evaluate the potential of keratin-based particles in the protection and strengthening of virgin and overbleached hair increasing the number of solutions available in hair care market. In this work, we have explored the keratin-based particles has hair restorative formulations rather than the respective bulk solutions, to take advantage of their physical characteristics. Furthermore, using protein-based particles instead of protein solutions and protein hydrolysates, we can explore in future the particles' ability to encapsulate fragrances and bioactive molecules.

The keratin-based particles were obtained by high pressure homogenization (HPH) and a full characterization of the formulations was performed in terms of size, stability, safety and their ability to improve and recover hairs' mechanical and thermal properties (**Figure V.1**).

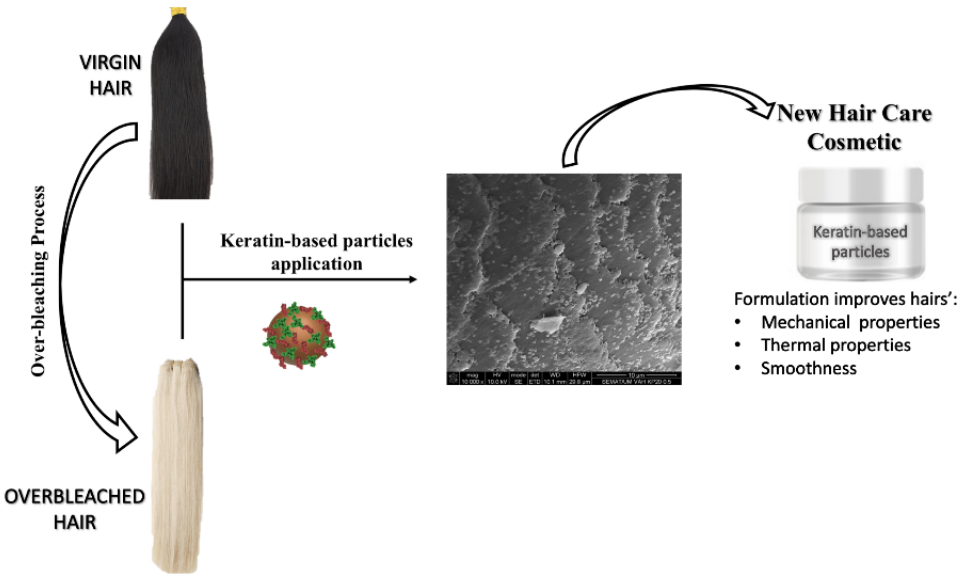


Figure V.1 - Scheme showing the fundamental concept of the described research study. Application of keratin-based particles as innovative hair care cosmetic formulations to improve the smoothness, the mechanical properties and the thermal stability of virgin and overbleached hair.

5.2 Experimental

5.2.1 Materials

Natural Asian black human hair samples were provided by International Hair Importers & Products Inc. (Glendale, New York, USA). The silk fibroin (SF) was extracted from *Bombyx mori* cocoons donated by Dr. Silvia Cappellozza from "Sezione Specializzata per la Bachicoltura" (Padova). The DC (detergent compatible) protein assay kit was obtained from Bio-Rad, Portugal and all other chemicals were supplied by Sigma Aldrich, Spain.

5.2.2 Extraction and purification of keratin

Keratin was extracted from donated human hair obtained from a local barbershop. The hair samples were first washed according to the IAEA/RL/50 1978 recommendations to remove contaminants and lipids. The hair washing consisted of cycles of distilled water and acetone with continuous agitation. After washing, hair samples were kept at 40 °C until completely dried.

The protocol for keratin extraction was adapted from Ayutthaya *et al* [161], where a solution containing 8M urea, 0.2 M SDS and 0.5 M of sodium metabisulfite in a ratio of 10:1 (volume (mL) of solution to

mass (g) of dry hair) was used. The mixture was heated at 100 °C for 30 min and then incubated overnight at 37 °C with constant agitation. The extraction solution was then centrifuged at 5000 rpm for 10 min and the supernatant was filtered to remove hair fragments. The keratin solution was then dialyzed for 5 days against distilled water using a dialysis membrane with a 14 kDa cut-off. The water was renewed two times per day. After dialysis, the keratin solution was quantified by DC method according to manufactures protocol [161].

5.2.3 Extraction and purification of silk fibroin (SF)

About 5 g of *Bombyx mori* cocoons were immersed in 1 L of boiling Na_2CO_3 (0.05 M) solution for 10 minutes. The Na_2CO_3 solution was changed several times until the cocoons get fragmented. The degummed SF was then kept at 40 °C until completely dried. The degummed SF fibers were incubated in a solution of 9.3 M lithium bromide at 60 °C until total solubilization. The soluble SF was then filtered to remove undissolved fibers and dialyzed for 5 days in distilled water using a dialysis membrane with a 14 kDa cut-off. The water was changed two times per day. After dialysis, the SF solution was quantified by DC method according to manufactures protocol.

5.2.4 Synthesis of keratin-based particles

Eight different formulations of keratin-based particles were produced by High Pressure Homogenization (HPH). The final particles were made-up within the following composition:

$Keratin_xSilkFibroin_yPoloxamer407_z$ ($K_xSF_yP_z$),

where x = 80, 90, 95 and 100%; y = 0, 5, 10 and 20%; z = 0 and 5 mg/mL.

The protein solutions were prepared in phosphate buffer saline (PBS 1x) pH 7.4 to a final concentration of 10 mg/mL with 0.5% (v/v) of vegetable oil and Poloxamer 407 (Pol_{407}) (5 mg/mL), when present. The homogenization process occurred at 240 – 580 bar during 10 – 13 min (5 min of open cycles and 8 min of close cycles) for each particle formulation. The emulsions obtained after homogenization cycles were centrifuged at 4000 rpm for 10 min, at 4 °C, in Amicon tubes (Amicon Ultra-15 Millipore with a 100 kDa cut-off) to separate the free protein from particles. The free protein was quantified by DC method and the particle formation efficiency was estimated for each formulation using the formula:

$$\text{Keratin-based particles formation}(\%) = \frac{[C]_i - [C]_f}{[C]_i} x100,$$

where $[C]_i$ and $[C]_f$ is the initial and final concentration of the protein in the aqueous solution, respectively. Each sample was assayed in triplicate.

In the current work, we used the keratin-based particles instead of the keratin and silk fibroin solutions, in order to take advantage of the particles' physical characteristics like size and ζ-potential. These properties could influence the particles' affinity to hair as well as their deposition pattern, which could render different degrees of improvement and restoration of human hair properties.

5.2.4.1 Size, size distribution and surface charge characterization

The emulsions were physicochemical analyzed by means of size distribution and electrophoretic mobility (ζ-potential) at pH 7.4 (PBS buffer) and 25 °C, by photon correlation spectroscopy and by electrophoretic laser Doppler anemometry, respectively, using dynamic light scattering in a Malvern Zetasizer NS. The values for viscosity and refractive index were taken as 0.890 cP and 1.330, respectively. Each sample was measured in triplicate and results are presented as mean value ± standard deviation. Keratin-based particles were kept at 4 °C and measured weekly until month one and then were measured monthly until month three. Nanoparticle tracking analysis (NTA) was used to assess quantitatively the keratin-based particles. The experiments were performed using a NanoSight NS500 instrument (Salisbury, UK). This system includes a charge coupled device camera that allows visualization and tracking Brownian motion of laser-illuminated particles in suspension. The samples were ten times diluted with water and filtered (Millipore filters with pore size of 0.45 μm) and then injected into the system. The measurements were made at room temperature and each video sequence was captured over 60 sec.

5.2.4.2 Particle morphological characterization

The morphology of particles was evaluated by STEM analysis. The diluted emulsions suspensions were dropped on copper grids with a 400 mesh carbon film, 3 mm in diameter. The shape and morphology of the microspheres were observed using a NOVA Nano SEM 200 FEI instrument (50000 and 100000x).

5.2.4.3 Secondary structure analysis

Infrared (FTIR) spectra were acquired at room temperature on a NICOLET-AVATAR 360 FTIR spectrometer using KBr disks made with 10 bar pressure. FTIR spectra were collected after 16 scans with a resolution of 32 cm^{-1} from 4000 to 600 cm^{-1}. For protein, secondary structure Gaussian deconvolution of Amide I band region (wavenumber between 1600 and 1700 cm^{-1}) was analyzed using OriginPro 8.5 software (OriginLab Corporation, MA, USA). In deconvolution analyses, a linear baseline was fitted; the number of components and their peak position were determined according to the second derivative spectrum of this same region; and the secondary structure content was calculated from the areas of the assigned peak as percentage fraction of the total area of the Amide I range. Using Gaussian function, all data were treated manually in three fitting modes: first, the base line was held fixed and fitted while intensity and bandwidth were allowed to vary, then, the base line and bandwidth were fixed and fitted again, and finally, the base

line and center peaks were fixed and fitted once more. The deconvoluted frequencies were then assigned to the respective secondary structure β-sheet, β-turns, random-coil and α-helix.

5.2.5 Cellular viability assays

5.2.5.1 Cell culture maintenance

The NCTC 2544 cell line (human skin keratinocytes) was cultured in DMEM medium, supplemented with 7.5% FBS, 1% Glutamine (2 mM) and 1% (v/v) penicillin/streptomycin solution. Cells were maintained in 75 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere with 5% CO_2. The cell culture medium was renewed twice per week. For subcultures and plating, the adherent cells were detached with a trypsin solution 0.05%.

5.2.5.2 Cell viability assessed by MTS assay

NCTC 2544 cells were seeded at a density of 22500 cells/well on a 48-well tissue culture plate, the day before the experiments. The cells were then exposed to 6 concentrations (0.01, 0.05, 0.1, 0.25, 0.5 and 1 mg/mL) of keratin-based particles. Cells incubated with DMSO (30% of the total volume) and cells without the addition of the compounds were used as controls: control of death and control of life, respectively. After 24 and 48 h of contact, cell metabolic activity was assessed by MTS viability assay, following the manufacturer's standard protocol. The reduction of MTS by viable cells was measured with a 96-well plate reader at 490 nm in a microplate reader SpectraMax Plus (Molecular Devices).

5.2.6 Hair treatments

Black Asian Hair fiber samples (0.1 g) were washed before treatment with a classic commercial shampoo (Pantene). The hair samples were then subjected to eight cycles of bleaching (8xB). Bleaching consisted on the application of 12% H_2O_2 (v/v) in the presence of 0.1 M Na_2CO_3/$NaHCO_3$, pH 9.0 buffer at 50°C for 1 h, in a bath ratio of 1:10. Treatments were applied to the same tresses of hair. Hair samples (virgin and overbleached black Asian hair) were then subjected to the treatment with keratin-based particles. Treatments were performed for 1 h at 37 °C with 0.5 mg/mL of particles in 0.05 M phosphate buffer pH 7.5. Subsequently, all samples were thoroughly washed in tap water with a commercial shampoo and dried.

5.2.6.1 Scanning electron microscopy (SEM)

Hair fibers were randomly taken from the hair tress and mounted onto aluminum stubs using conductive carbon adhesive tape. Hair samples were coated with 80% Au and 20% Pd before SEM (NOVA NanoSEM 200 FEI, Eindhoven, Netherlands) observation at 5.0 kV.

5.2.6.2 Hair fiber tensile test

The effect of keratin-based particles on virgin and overbleached Asian hair was assessed by the differences in mechanical properties following guidelines outlined in ASTM D1145-95 for fiber tensile testing. For this test, a set of hair fibers with low variability in diameter was selected using a light microscope. The measurements were performed using a 250 N dynamometer Hounsfield machine. For each measurement, 30 single hair fibers were randomly taken from the tress. Each hair was individually mounted in the tensile jig by means of a paper template with a fixed gauge length of 20 mm. Before the tensile test, the paper template was cut across. Measurements were performed under controlled conditions (25±0.8 °C; 70±5% humidity) at a rate of 2.5 mm/min with 0.01 N preload force, and stretched until fibers broke. All measurements were made in the middle part of the hair fiber. Results were presented in Young's module and define the relationship between stress and strain, which is related to material strength and elasticity [162].

5.2.6.3 Differential scanning calorimetry (DSC)

Thermal studies of the hair samples were conducted using a power compensated DSC instrument (DSC 6000, Perkin Elmer), and aluminum pans (max. pressure: 1 bar), at a temperature range from 50 to 250 °C (heating rate: 5 °C/min, sample weight: approximately 2 mg). The DSC device was calibrated using high purity indium and zinc. Each test group was measured in triplicate and mean values are reported.

5.2.6.4 Assessment of feel assay

A feel test with volunteers was performed to assess the effect of treatment using the keratin-based particles on the smoothness of virgin and overbleached Asian hair. The cohort included 30 volunteers from different ethnicities, 20 females and 10 males, with ages between 20 and 50 years old. Two test series were performed, one with the virgin Asian hair and other with the overbleached Asian hair. A blind control (untreated hair) was included in each set of hair samples for the fidelity of the results. The feel test was performed as follows: the volunteer contacted first with the references (virgin and overbleached asian hair without treatment) and then with the hair samples treated with the keratin-based particles and with the blind control, in a random order. The samples and the blind control were numbered to guarantee that volunteers did not have any information regarding sample's treatment. Afterwards, the volunteer was asked to give a classification between 0 and 4, where 0 corresponds to no increase on hair smoothness and 4 corresponds to a great improvement on hair smoothness.

5.2.7 Statistical analysis

Data are presented as average standard deviation (SD), n = 3. Statistical comparisons were performed by one-way ANOVA with GraphPad Prism 5.0 software (La Jolla, CA, USA). Tukey's post hoc test was

used to compare all the results between them, and a Dunnet's test was used to compare the results with a specific control. A p-value≤0.05 was considered to be statistically significant.

5.3 Results and Discussion

5.3.1 Particle Formation Efficiency

After synthesis, the efficiency of keratin-based particle formation was determined by the quantification of free protein using DC method – **Table V.1**. The efficiency of particles formation ranged from 90.6 to 97.8%, which clearly demonstrates the high capacity of keratin and silk fibroin to form particles during the HPH process.

Table V.1 - Particle formation efficiency of keratin-based particles obtained by High Pressure Homogenization (HPH). The values were calculated and expressed as mean ± SD (n=3).

Particle Formulation	Particle Formation Efficiency (%)
K_{100}	96.7 ± 2.3
$K_{100}P$	97.8 ± 0.4
$K_{95}SF_5$	91.0 ±1.1
$K_{95}SF_5P$	90.3 ± 1.3
$K_{90}SF_{10}$	95.2 ±3.4
$K_{90}SF_{10}P$	90.6 ± 0.7
$K_{80}SF_{20}$	92.6 ± 2.6
$K_{80}SF_{20}P$	91.3 ± 0.4

5.3.2 Particles Physicochemical Characterization

Particle physical stability was evaluated over 3 months by measurements of size, PDI and ζ-potential when stored at 4 °C. The physical characteristics of all formulations 3 months after synthesis are presented on **Table V.2**. Also, the physical stability over time of $K_{100}P$ is depicted in **Figure V.2**, that correspond to the formulation only with keratin and Poloxamer 407. The physical stability of the other keratin-based particles is present in **Figure V.3**.

All keratin-based particles presented a net negative surface ζ-potential and a narrow size distribution with sizes ranging from 160 nm to 237 nm (**Table V.2**). Yet, considering composition and time, some statistically significant differences were identified. There was a relative decrease on particles size along time, going up to 26% for the $K_{100}P$ formulation. Regarding the ζ-potential, there was a variation from -27.40 mV, at day 1, to -22.00 mV after 3 months of storage at 4°C for this formulation. These changes

in ζ-potential could be associated with a reorganization of the proteins and Pol_{407} at particles' surface, leading to small alterations in the surface charge.

Table V.2 - Size, polydispersity index (PDI) and ζ-potential of keratin-based particles at the end of 3 months of storage at 4°C. The values were calculated and expressed as mean ± SD (n=3).

Formulation	Size (nm)	PDI	ζ-Potential (mV)
K_{100}	210.2±2.4	0.100±0.045	-39.1±1.7
$K_{100}P$	160.5±2.5	0.266±0.007	-22.0±1.0
$K_{95}SF_5$	205.4±4.9	0.167±0.008	-42.9±0.6
$K_{95}SF_5P$	236.4±2.0	0.267±0.006	-11.3±1.2
$K_{90}SF_{10}$	186.8±7.0	0.154±0.007	-39.4±0.4
$K_{90}SF_{10}P$	205.3±8.4	0.218±0.015	-9.0±1.8
$K_{80}SF_{20}$	210.9±7.4	0.135±0.022	-35.0±1.2
$K_{80}SF_{20}P$	226.0±2.3	0.212±0.020	-9.50±1.4

There was also a stabilization of the $K_{100}P$ particles at the end of the first week of storage, with a decrease in PDI value. The same trend was observed for the other keratin-based particles (data not shown). Pol_{407} is a surface-active compound that, due to its amphiphilic structure, exhibits a tendency to accumulate at the interface between the aqueous and organic phase, lowering the surface tension and stabilizing the system [163]. This compound was used to control particle size, size distribution and shape, and to prevent aggregation and coalescence. Except for the $K_{100}P$ particles, there was an increase in size and PDI when the Pol_{407} was added to the formulations with keratin and silk fibroin. It was already described that Pol_{407} propylene oxide chains are not strong enough to be ideally absorbed in silk fibroin particles. Thus the presence of this protein in the formulation could have influenced the effect of Pol_{407} on particle size and PDI [164].

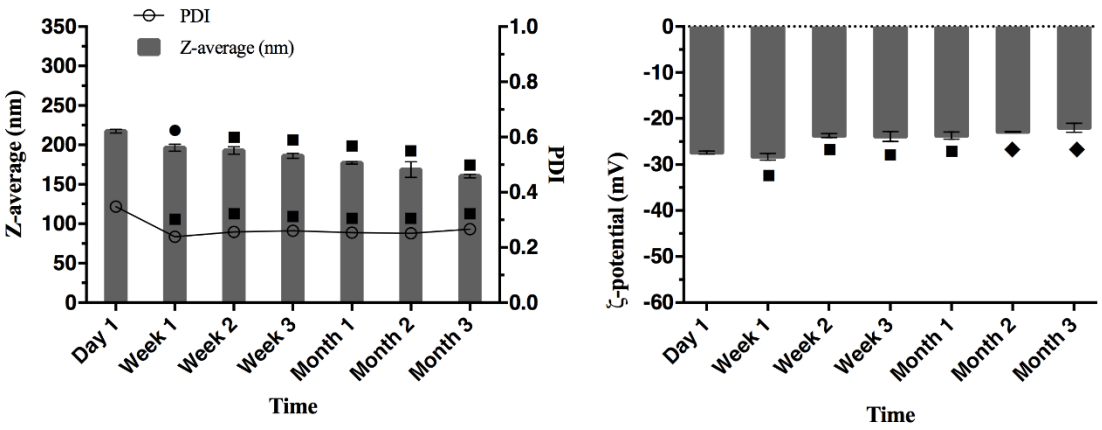


Figure V.2 - Characterization of $K_{100}P$ in PBS, during storage at 4 °C: A) Z-average and polydispersity (PDI); B) surface charge (ζ-potential). The data represents the mean ± SD from three independent experiments. Data were analyzed by one way-ANOVA: * p-value ≤0.05, ● p-value ≤0.01, ♦ p-value ≤0.001, ■ p-value ≤0.0001, when compared to the results obtained at day 1.

Regarding the ζ-potential, a change towards a less negative potential for all the particles was observed. These results were expected since the negative charge of the keratin side chains (RSO_3^-) was masked by the presence of Pol_{407} coating the particles, resulting in an increase in ζ-potential to less negative values [165]. Despite the small changes observed regarding the particles' surface charge, the highly negatively charged formulations are considered as stable.

The shape and the size of the keratin-based particles were also evaluated by STEM and NTA. Analyzing STEM micrographs presented on **Figure V.4,** it was possible to verify the particles' spherical shape and the presence of a less dense layer surrounding the particles. This layer can be attributed to the presence of Pol_{407} which tends to accumulate in the interface between particles and water [163].Using NTA, a size of 210 nm was observed for the $K_{100}P$ particles, considering 90% of population distribution (D90). These results agreed with the size determined by DLS at day one after particle synthesis. The newly produced keratin-based particles presented size properties generally considered suitable for cosmetic applications [166].

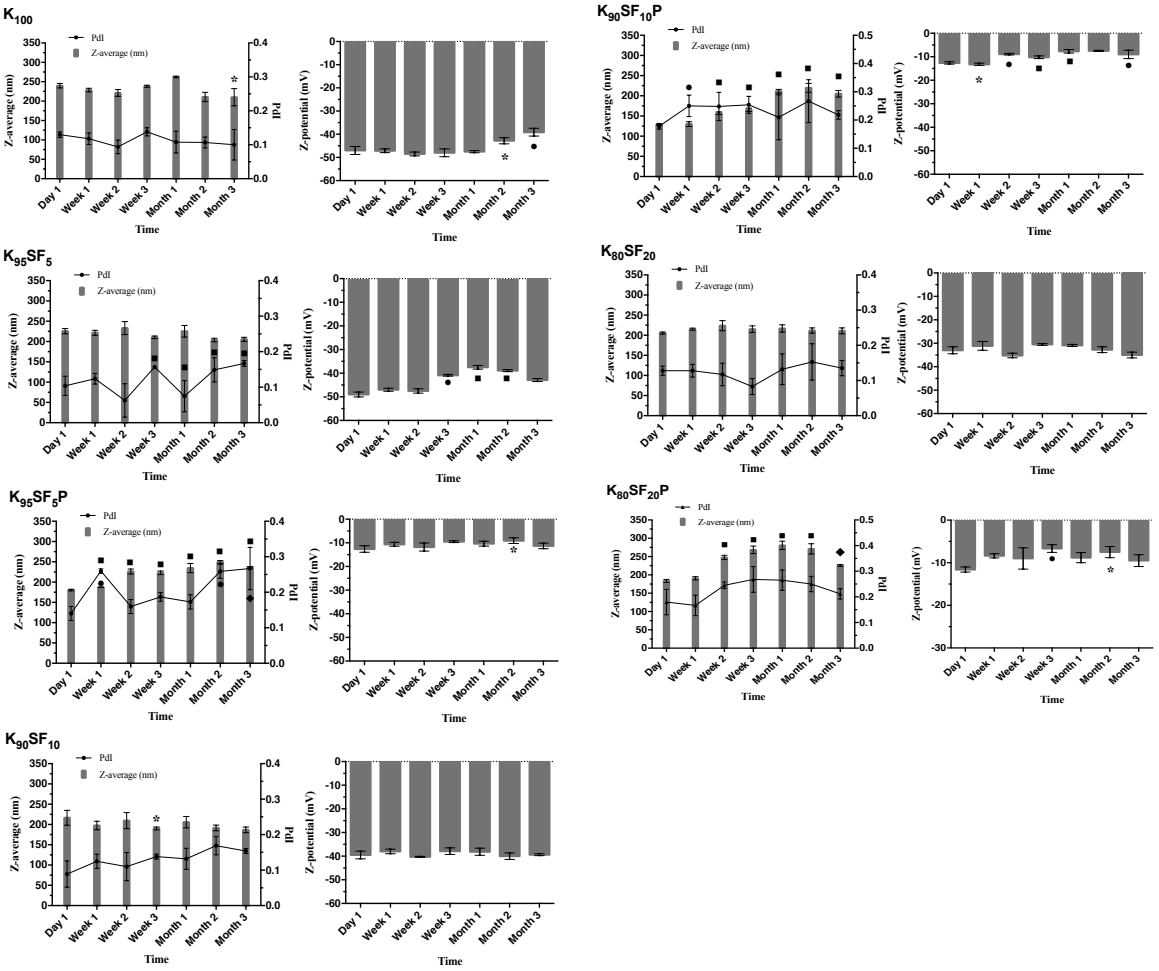


Figure V.3 - Characterization of Keratin-based particles in PBS, during storage at 4° C. The data represents the mean ± SD from three independent experiments. Data were analyzed by one way-ANOVA: * p-value ≤0.05, ● p-value ≤0.01; ♦ p-value ≤0.001; ■ p-value ≤0.0001, when compared to the results obtained at day 1.

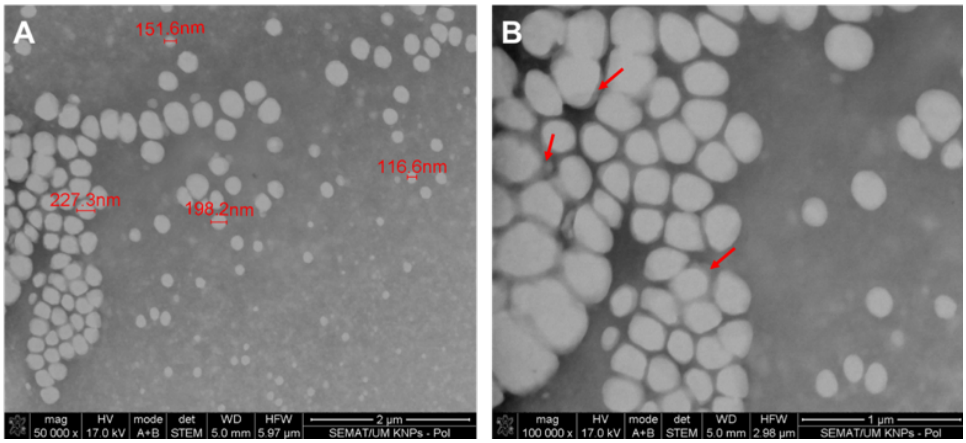


Figure V.4 - STEM micrographs (50000 (A) and 100000x (B)) of $K_{100}P$ particles at day one after synthesis. The presence of Pol_{407} coating the particles is highlighted by arrows.

5.3.3 Effect of homogenization on the secondary structure of particles containing proteins

To evaluate the extent of the emulsification effect on the secondary structure of keratin and silk fibroin, FTIR spectra of formulations were acquired.

To assess the type of secondary structures present, a deconvolution of Amide I (1600-1700 cm^{-1}) region was performed and the results are presented on **Table V.3**. When the proteins were subjected to cycles of HPH, some structural modifications were detected. Comparing the K_{100} particles with the keratin solution, the K_{100} particles showed higher absorbance for the analyzed amides, which is related with the chemical-bonds concentration formed during the emulsification process.

The variations of FTIR spectra could indicate conformational changes on the proteins and the achievement of complex particles. The results presented in **Table V.3** summarize the structural alterations on the proteins throughout the HPH process. The band assignment was performed according to Kong [145] where the deconvolution of Amide I allowed to analyze the corresponding structural assignments. A component centered between approximately 1658 and 1650 cm^{-1} was assigned to α-helix structures. Bands in the regions of 1640–1620 cm^{-1} and 1695–1690 cm^{-1} were assigned to β-sheet and bands flanked by 1680 and 1660 cm^{-1} were assigned to β-turns [145].

Comparing the values before and after the application of HPH to produce the particles, an increase on the percentage of β-strands (sheets and turns) was observed. Concomitantly, there was a reduction in the percentage of α-helix/random coil structures. The increase on β-strands was related with the HPH emulsification process which contributes to an increase of protein's crystallinity, mostly due to the formation of β-strand structures [167–169]. The incorporation of silk fibroin on particles formulation contributed to an increase in the percentage of β-sheets [170,171]. Silk fibroin self-assembly process is predominantly composed by this conformation which increases encapsulation efficiency and enables control of drug release kinetics [170–172]. When compared with the K_{100} particles, the addition of silk fibroin and Poloxamer 407 to the formulations led to a reduction in absorbance on the regions corresponding to the Amides I, II and III (data not shown). These changes evidence the chemical modifications of the formulations observed by addition of other components.

In **Table V.3** is patent the effect of silk fibroin and Pol_{407} on particles conformation triggering the formation of β-strands, in special β-turn, during the HPH process. An increase of these conformations may contribute to more stable particles because β-sheets are envisioned with reverse β-turns conferring stability and the possibility to connect with other β-sheets, improving particle resistance and rigidity [172,173].

Table V.3 - Secondary structural assignments on keratin-based particles obtained by HPH. The results of Amide I deconvolution, expressed in percentages, were analyzed using OriginPro 8.5 software.

	Secondary Structure (%) Amide I deconvolution		
	ß-Sheet	**ß-Turn**	**α-helix/ Random coil**
Keratin Control	27	33.7	39.3
Silk Fibroin Control	28.2	39.2	32.6
K_{100}	29	40.3	30.7
$K_{100}P$	31.0	38.4	29.6
$K_{95}SF_5$	34.8	39.2	25.9
$K_{95}SF_5P$	30.8	42.7	26.4
$K_{90}SF_{10}$	32.4	41.8	25.8
$K_{90}SF_{10}P$	30.1	39.6	30.0
$K_{80}SF_{20}$	33.4	39.4	27.2
$K_{80}SF_{20}P$	34.2	38.7	27.1

5.3.4 *In vitro* evaluation of keratin-based particles potential cytotoxicity

When developing new products that will contact with human skin, it is crucial to understand if those products will potentially display any type of cytotoxicity. Ultimately, the development of new cosmetic products relays on product safety when applied to humans. The use of cell lines is well established for the evaluation of the potential cytotoxicity of cosmetic products [174,175]. Also, in European Union there are provisions prohibiting animal testing of cosmetic products and is well stablished that animal experiments must be replaced by alternative methods that are scientifically accepted. Human keratinocytes were chosen since the keratin-based particles will be applied on hair and would therefore come into contact with the hair scalp, and eventually be adsorbed through it. More specifically, the NCTC-2544 keratinocytes cell line was selected to evaluate the potential cytotoxic effect of keratin-based particles since this cell line is already a reliable method to prevent animal skin tests for cytotoxicity studies and contact of potential allergens [174,175].

The effect of keratin-based particles on the cellular metabolic activity of human keratinocytes, as a means to predict cytotoxicity, was evaluated by the MTS assay. The data on viability of cells after 48 h of incubation with the keratin-based particles is presented in **Figure V.5**.

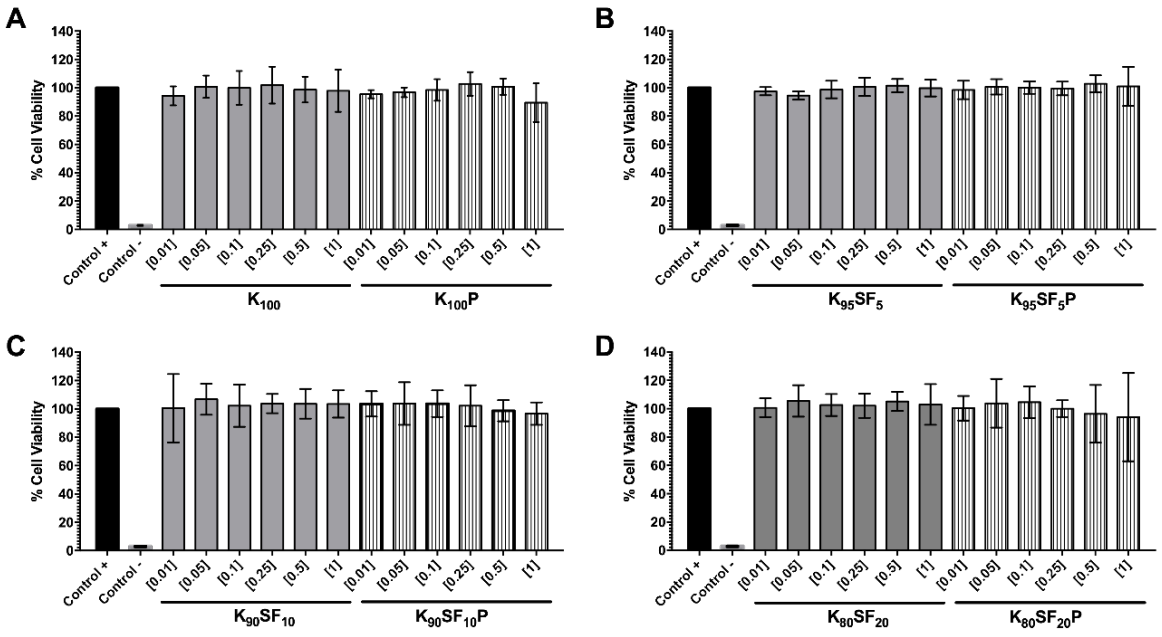


Figure V.5 - Relative viability of NCTC-2544 human keratinocytes (evaluated by the MTS assay) after 48 h incubation with 0.01, 0.05, 0.1, 0.25, 0.5 and 1 mg/mL of K_{100} and $K_{100}P$ (A), $K_{95}SF_5$ and $K_{95}SF_5P$ (B), $K_{90}SF_{10}$ and $K_{90}SF_{10}P$ (C), $K_{80}SF_{20}$ and $K_{80}SF_{20}P$ (D), particles. Cells incubated with culture medium only were used as life control (+) and cells incubated with 30% DMSO as death control (-). Data was calculated in relation to the life control (100%). Results are the mean ± SD of triplicates of three independent experiments.

The cytotoxic effect of the individual components of the particles (keratin, silk fibroin and Pol_{407}) was initially evaluated and no signs of cell toxicity were verified for any of the tested concentrations and time points (data not shown). The same tendency was verified when cells were exposed to increasing concentrations of keratin-based particles.

The lowest viability was observed for the highest concentration of $K_{80}SF_{20}P$ particles (94.1± 31.1 %) which still was not significantly different from the life control. For some particle concentrations (K_{100} [0.05], $K_{100}P$ [0.1], $K_{95}SF_5$ [0.1], $K_{95}SF_5P$ [0.5], $K_{90}SF_{10}$ [0.5] and $K_{80}SF_{20}$ [0.5]), there was a pro-proliferative effect of the keratin-based particles. This proliferative effect of keratin-based materials, like hydrogels, scaffolds and films was already described in cell adhesion and proliferation studies [176,177]. The uniformity of the viability results after 48 h of incubation and comparing with the 24 h incubation (data not shown), show the ability of the keratinocytes to adapt to the presence of the keratin-based particles, which argues for the safety of these particles. Furthermore, the addition of Pol_{407} and silk fibroin to the formulation did not significantly affect the viability of keratinocytes.

5.3.5 Hair treatment with keratin-based particles

After confirmation of the keratin-based particles safety in human keratinocytes, virgin and overbleached (8xB) Asian hair were incubated with 0.5 mg/mL of each formulation for 1 h. This concentration was selected considering the particles' cytotoxicity profiling. Although no significant cytotoxic effect was verified for any of the tested concentrations after 48 h of exposition, 0.5 mg/mL of particles was the highest concentration where no decrease on cellular viability was noted for any of the keratin-based particles tested.

When developing new formulations for hair care, it is important to evaluate the ability of their components to bind hair. After the hair treatment, roughly bound keratin-based particles were washed off and the remaining tightly bound particles (on the hair surface and/or in the cortex) were visualized by SEM (**Figure V.6**).

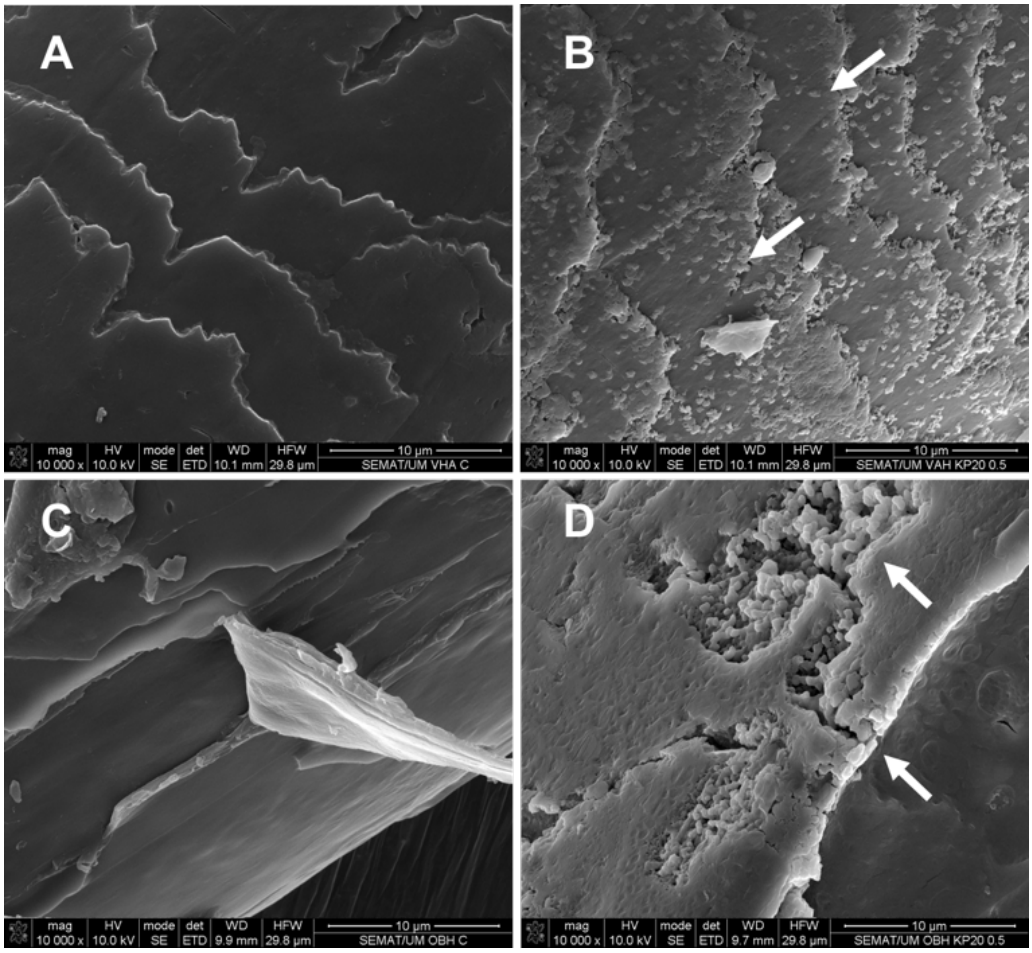


Figure V.6 - SEM micrographs (10000x magnification) of Asian virgin hair (A), Asian virgin hair treated with $K_{80}SF_{20}P$ particles (B), Asian overbleached hair (C), and Asian overbleached hair treated with $K_{80}SF_{20}P$ particles (D). Particles are highlighted by arrows.

Analyzing **Figure V.6**, the effect of the bleaching process on the appearance and structure of hair is notorious. While the virgin hair **(Figure V.6 A)** shows a natural appearance and displays a scale structure with smooth edges, the overbleached hair **(Figure V.6 C)** showed signs of scale erosion and hair degradation which contributed for the weakening of the hair fiber [178].

The deposition of the keratin-based particles on the hair fibers was evident when comparing the micrographs of treated and untreated hair. The deposition and distribution were dependent on the particle formulation and type of hair used (virgin vs overbleached). In **Figure V.6 B** (treated virgin hair) and **Figure V.6 D** (treated overbleached) is presented the binding of $K_{80}SF_{20}P$ particles to hair.

This formulation was able to recover and improve hair mechanical properties to a great extent compared with the other formulations (**Table V.4**), and thus its data is presented here. In the treated virgin hair **(Figure V.6 B)**, the $K_{80}SF_{20}P$ particles tend to be distributed along the hair fiber with a preferential deposition in the inter-layer zone of the cuticles. This behavior is similar to that observed with the hair conditioners. It was already described by many authors that the conditioner tends to accumulate in the area surrounding the cuticle scale edges, filling the damaged area of the cuticles, making it possible to physically 'repair' some damages in the hair fiber [179,180].

For the treated overbleached hair (**Figure V.6 D**), the $K_{80}SF_{20}P$ particles are evenly distributed along the hair fiber with a tendency to form film-like structures over the damage hair. The differences observed on the deposition pattern could be directly related with the overbleaching process which affected the cuticle structure and the surface chemistry. The appearance of cystic acid termination SO_3^- after bleaching resulted in an increase on the net negative charge and surface hydrophilicity of overbleached hair improving the electric attraction to keratin-based particles [181]. The formation of a film-like deposition in the $K_{80}SF_{20}P$ particles on the overbleached hair resulted from a stronger interaction between the particles and the hair fiber.

5.3.6 Recovery of the mechanical and thermal properties of virgin and overbleached Asian hair

To evaluate the extent to which the particles contribute to the mechanical strength and thermal properties of virgin and overbleach hair, we performed the determination of the mechanical resistance of hair samples. Hair resistance was determined using the calculated Young's modulus (stiffness) and tensile strength of virgin and over- bleached Asian hair before and after treatment with 0.5 mg/mL of keratin-based particles (**Figure V.7**).

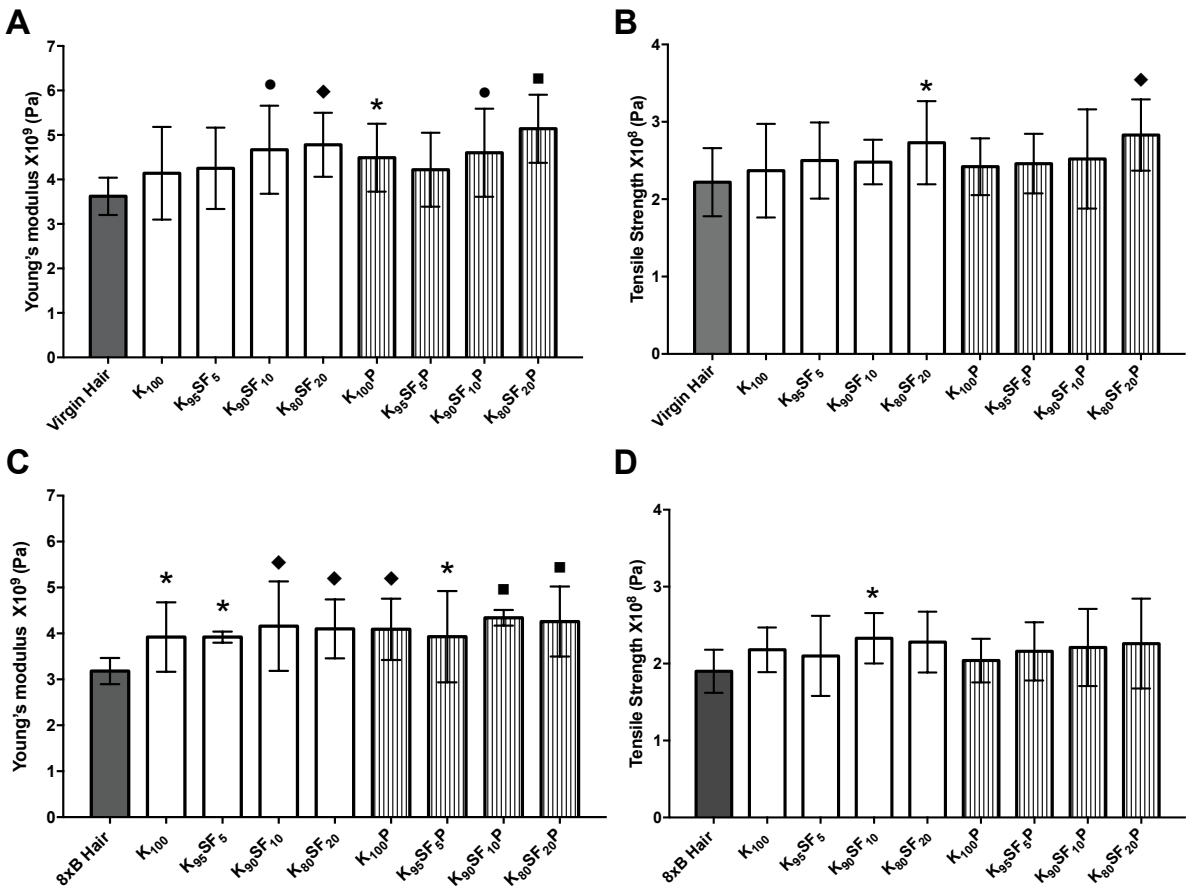


Figure V.7 - Mechanical resistance parameters: (A) and (C) Young's modulus and (B) and (D) tensile strength of virgin and overbleached Asian hair (8xB) before and after treatment with 0.5 mg/mL of keratin-based particles. Values are the mean of SD of twenty independent measurements. Statistically significant differences from the control ((A) and (B): untreated virgin air; (C) and (D): overbleached hair (8x)) are indicated as: * p-value ≤0.05, ● p-value ≤0.01; ♦ p-value ≤0.001; ■ p-value ≤0.0001.

The repeated chemical bleaching treatments affect the appearance and structure of hair which results in the loss of hair resistance. The overbleaching of Asian black hair induced a loss of stiffness in about 14% and a loss of tensile strength in about 17%.

Analyzing **Figure V.7**, it was possible to observe that the application of the keratin-based particles resulted in the recovery (R) and even improvement (I) of overbleached hair mechanical properties to values higher than what was measured for the control (untreated overbleached hair). Regarding virgin hair, all the keratin-based particles were able to improve (I) its mechanical properties. The treatment of overbleached and virgin hair with buffer did not show any significant recovery of the mechanical properties (data not shown), thus attesting the beneficial role of the keratin-based particles.

The Young's modulus analysis indicated that the particles with higher percentage of Silk Fibroin (10 and 20%) significantly improve the stiffness of both virgin and overbleach hair. An increase in tensile strength with increasing percentages of silk fibroin was only significant for $K_{80}SF_{20}$ and $K_{80}SF_{20}P$ formulations, which improve the tensile strength of virgin hair in 23.07 and 27.74% (**Table V.4**), respectively. The tensile strength of overbleach hair was significantly increased in 22.69 % after the application of $K_{90}SF_{10}$ particles (**Table V.4**). Comparing all particle formulations (**Table V.4**), the best formulation in terms of Young's modulus was the $K_{80}SF_{20}P$, which led to an improvement up to 41.85 and 36.14 % for the virgin and overbleach hair, respectively.

Table V.4 - Improvement (I%) and recovery (R%) of hair mechanical properties measured in terms of Young's modulus and tensile strength (%) after treatment with keratin-based particles, compared with untreated virgin hair and untreated overbleached hair.

Formulation	Young's Modulus		Tensile Strength	
	Virgin Hair (%I)	Overbleach Hair (%R+I)	Virgin Hair (%I)	Overbleach Hair (%R+I)
K_{100}	14.24	23.26	6.92	14.91
$K_{100}P$	23.85	28.58	9.02	7.75
$K_{95}SF_5$	17.37	23.31	12.58	9.22
$K_{95}SF_5P$	16.45	23.68	11.13	14.14
$K_{90}SF_{10}$	28.94	30.74	11.98	22.68
$K_{90}SF_{10}P$	27.08	36.31	13.75	16.77
$K_{80}SF_{20}$	31.94	28.82	23.07	20.17
$K_{80}SF_{20}P$	41.84	36.14	27.74	19.34

The improvement and recovery of hair's mechanical properties could be directly related with the number and distribution of particles attached to the hair surface. Hair samples with more particles on their surface had the highest and more statistically significant values for Young's modulus. Despite this positive correlation between the amount of particles on the hair surface and the improvement its mechanical properties, the increase in Young's modulus could also be related with the ability of the keratin-based particles to penetrate into the hair cortex, contributing to the stabilization of keratin's α-helix, giving this structure a higher resistance to unfold during stretching.

Particle composition also influenced the improvement of hair's mechanical properties. Generally, there was an increase in Young's modulus values with increasing amounts of silk fibroin in the formulation. Such observation could be related with the secondary structure of silk fibroin. This protein is rich in β-

strands which are associated with higher resistance and stiffness profiles [173,182] while combining tensile strength and toughness [183].

The study of the effect of hair care products on hair's mechanical and thermal properties is essential when developing new formulations. Therefore, DSC measurements were performed to assess if keratin-based particles penetrate into the hair cortex and if they protect hair from thermal denaturation. One important parameter analyzed by this technique is the α-helix keratin denaturation, which is identified by a peak with a temperature between 210 and 250 °C [153]. The peak observed at 221.9 °C for the lyophilized keratin in **Figure V.8** and in **Figure V.9** is within this temperature range. Peaks around this temperature range were selected to evaluate the effect of the keratin-based particles treatment on the enthalpies values for virgin and overbleached Asian hair. If there is an increase in the enthalpy of α-helix keratin denaturation, the formulation is somehow interacting and stabilizing the keratin structure, protecting it from damage [184]. The thermograms of virgin Asian hair, overbleached Asian hair, lyophilized keratin and of hair samples treated with the keratin-based particles are shown in **Figure V.8** and in **Figure V.9**.

Table V.5 shows the enthalpy values for the α-helix keratin denaturation. The enthalpies values correspond to the difference between the virgin and overbleached hair treated with the keratin-based particles and the hair without treatment.

The treatment of hair with the particles resulted in an increase in the value of α-helix denaturation enthalpies. For the virgin hair, the best formulations were the $K_{80}SF_{20}$ and $K_{100}P$, while for the overbleach hair the best formulations were the $K_{80}SF_{20}$ and $K_{95}SF_5P$. It was already demonstrated by some studies that silk fibroin tends to establish intermolecular interactions with keratin, so it was expected that formulations with it would have higher denaturation enthalpies [167]. These results corroborate the positive effect of keratin-based particles on the mechanical and thermal properties of virgin and overbleached hair.

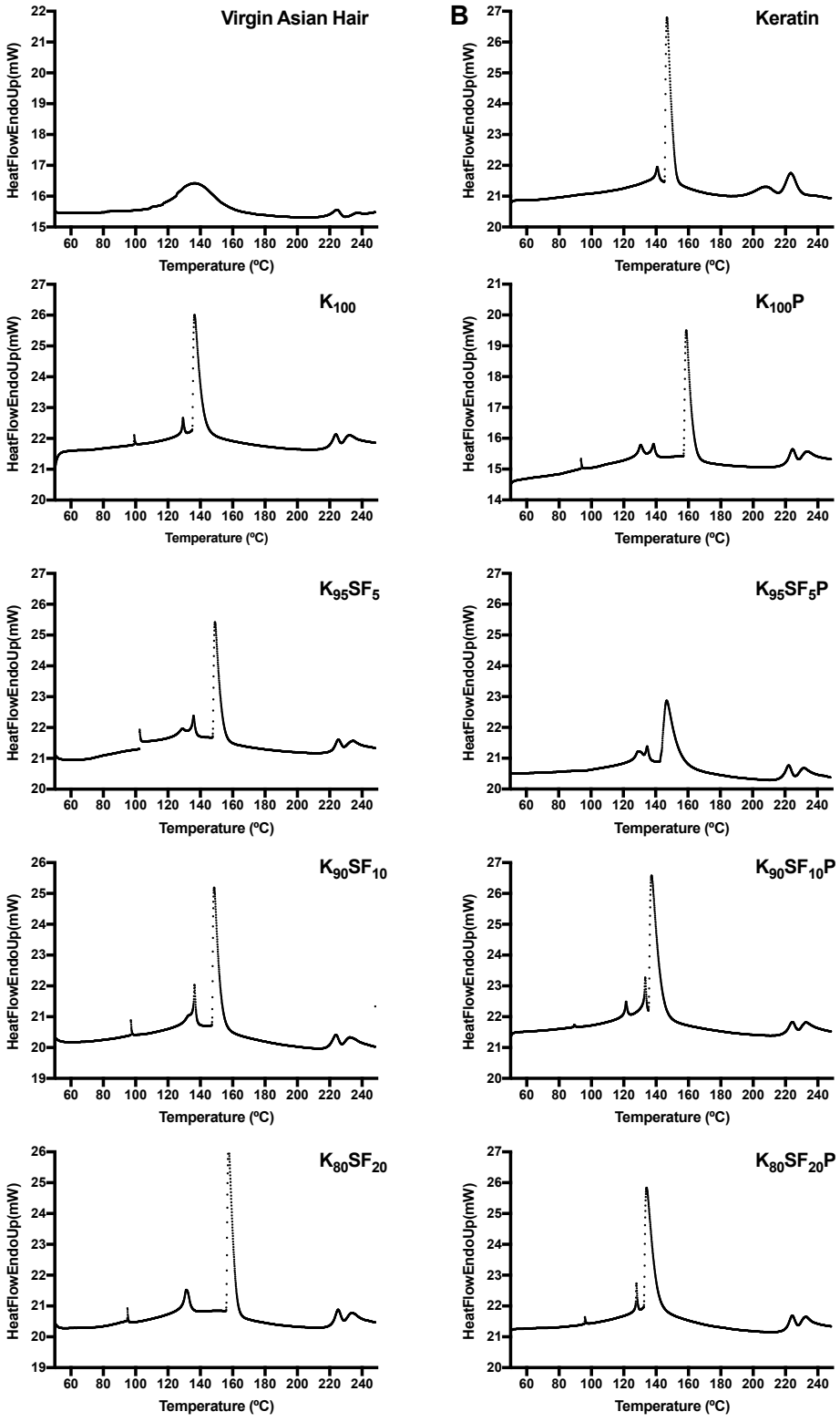


Figure V.8 - DSC thermograms of virgin Asian hair, lyophilized keratin and virgin Asian hair after treatment with the keratin-based particles.

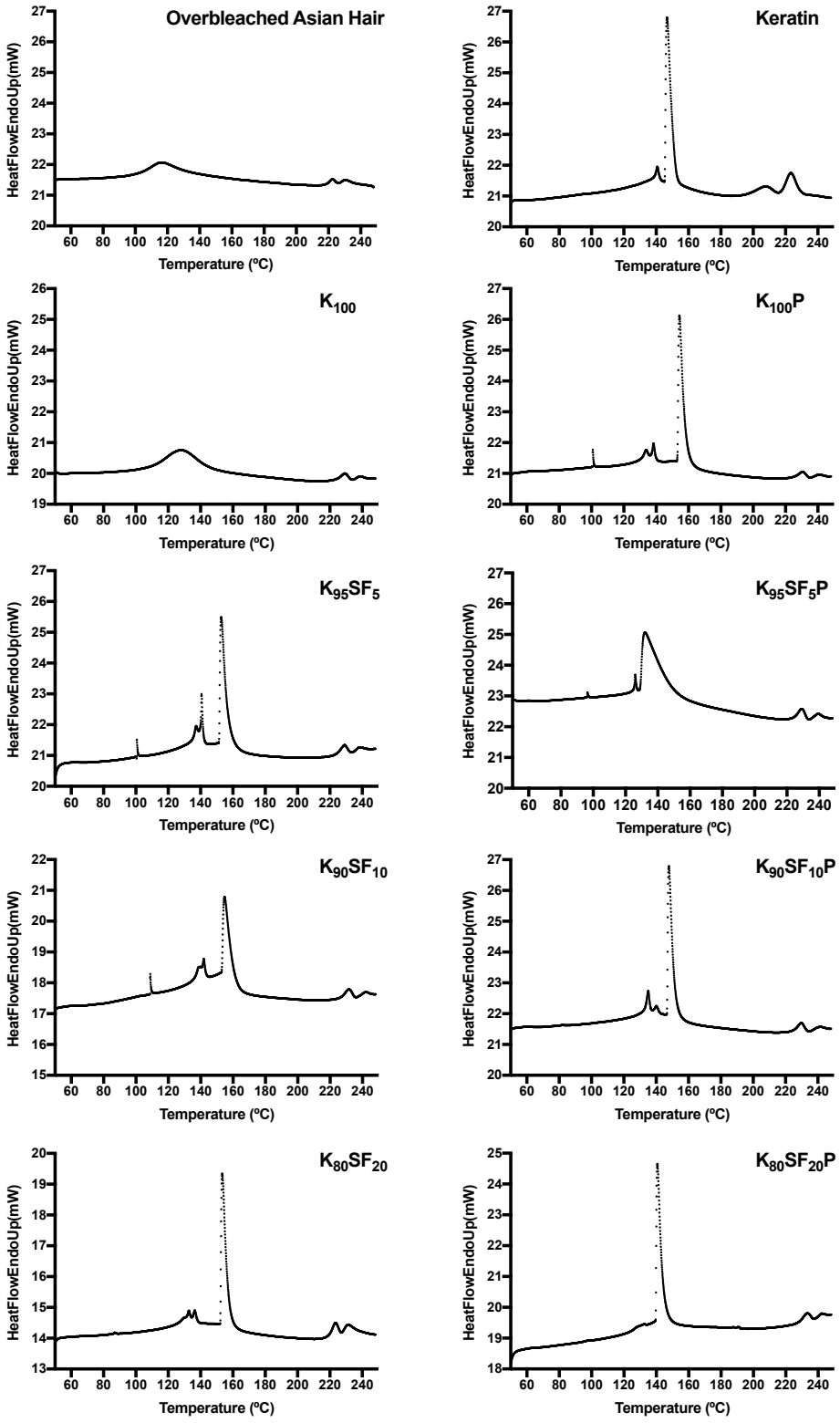


Figure V.9 - DSC thermograms of overbleached Asian hair, lyophilized keratin and overbleached Asian hair after treatment with the keratin-based particles.

Table V.5 - Keratin α-helix denaturation enthalpies of virgin and overbleached Asian hair after treatment with 0.5 mg/mL of keratin-based particles. The values were calculated and expressed as the energy increase between the samples treated with particles and the respective control: virgin or overbleached hair (n=3).

Keratin based particles	DeltaH (J/g) (Virgin Hair)	DeltaH (J/g) (Overbleached Hair)
K_{100}	2.18 ± 0.60	2.36 ± 0.97
$K_{100}P$	1.81 ± 0.19	1.85 ± 0.26
$K_{95}SF_5$	3.54 ± 1.32	1.51 ± 0.50
$K_{95}SF_5P$	4.23 ± 0.16	3.77 ± 1.75
$K_{90}SF_{10}$	4.61 ± 0.69	2.15 ± 0.95
$K_{90}SF_{10}P$	3.87 ± 1.38	2.58 ± 1.59
$K_{80}SF_{20}$	3.01 ± 1.28	2.29 ± 0.21
$K_{80}SF_{20}P$	3.43 ± 0.92	1.54 ± 0.28

5.3.7 Effect of keratin-based particles on hair smoothness

The effect of a formulation on hair smoothness is an essential parameter during the development of new hair cosmetic products. End users prefer products which turns hair softer to the touch over a product which does not increases hair smoothness. A subjective assessment with a cohort of 30 volunteers was performed to study if the treatment of virgin and overbleached with the keratin-based particles increases hair smoothness – **Figure V.10**. Analyzing **Figure V.10**, it was demonstrated the ability of all keratin-based formulations to improve hair's smoothness, with a direct relation with the increase of silk fibroin concentration in the formulation. This improvement in hair texture could be related with the moisturizer effect of silk fibroin, capacity already demonstrated and proved for other formulations applied on skin [160]. Besides, the accumulation of the keratin-based particles on the cuticles' edges **(Figure V.6 B)** or their ability to form film-like structures **(Figure V.6 D)**, attenuated the hair surface irregularities increasing hair smoothness. This behavior was similar to the described for hair conditioners [185].

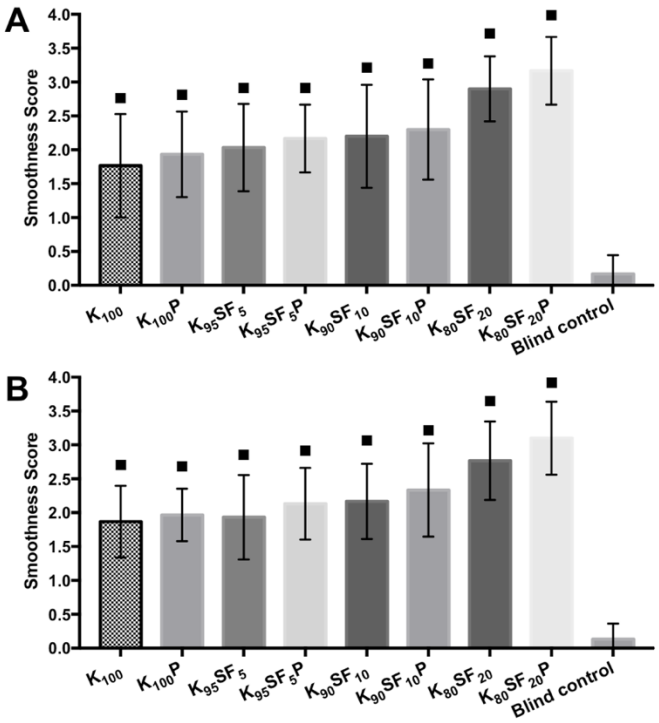


Figure V.10 - Increase of virgin hair (A) and overbleached hair (B) smoothness after treatment with the keratin-based particles. The qualitative score data were obtained from a cohort of 30 volunteers. Score: 0: no improvement of smoothness; 1: slight improvement of smoothness; 2: improvement of smoothness; 3: good improvement of smoothness; 4: great improvement of smoothness. The data represents the mean ± SD from the thirty volunteers. Data were analyzed by one way-ANOVA: ■ p-value ≤0.0001, when compared to the results of the Blind control (untreated hair).

5.4 Conclusions

The conventional chemical treatments used in hair cosmetic have detrimental effects on the mechanical and thermal properties of hair. The development of formulations capable of recovering and improving the properties of hair is mandatory. In the present study, keratin-based particles were successfully produced by HPH and applied to virgin and chemically overbleached hair. Generally, the particles were very stable over time and proved to be safe when tested in human keratinocytes. Depending on hair cuticle structure, hair surface chemistry and particle formulation, the keratin-based particles exhibited different deposition patterns on hair's surface. All the formulations were able to induce significant recovery of the stiffness and tensile strength of virgin and overbleached hair along with an increase of the α-helix denaturation enthalpy and of surface smoothness for both types of hair.

The keratin-based particles here presented are excellent candidates as new restorative hair care products, opening new perspectives on the use of functional particle-based formulations in hair cosmetic industry.

Additionally, these particles will be further explored to encapsulate fragrances and bioactive molecules, which will result in a set of multifunctional keratin-based formulations for hair restoration, hair targeting and hair beauty.

Chapter VI

Keratin:Zein Particles for Fragrance Controlled Release in Hair

Chapter VI – Keratin:Zein Particles for Fragrance Controlled Release in Hair

Abstract

Hair perfumes are becoming a trend all over the world and new cosmetic formulations have been developed to address this recent need. In the present study, we produced particles composed by keratin and zein for the encapsulation of two different fragrances, linalool or menthol. The Keratin:Zein particles presented a high stability along storage, related with the addition of the keratin to the particles formulation. When applied on virgin Asian hair, the particles formed a film-like structure over the hair fibers with some accumulation near the cuticles' edges. The Keratin:Zein particle formulations showed the capacity to improve hair's mechanical properties and hair's hydration degree. The fragrance diffusion from the hair treated with the particles encapsulating the linalool or the menthol was dependent on the releasing temperature, strategy used to produce the particles and the fragrances' physicochemical properties like melting point and vapour pressure. The formulation with linalool was selected has the most pleasant by a panel of volunteers when applied to the hair. The new Keratin:Zein particles reveal a high potential for the development of personalized hair cosmetic products as they can be produced according to personal sensory preferences.

This chapter is based on the following scientific publication:

Ana Tinoco, Filipa Gonçalves, André F. Costa, Artur Cavaco-Paulo and Artur Ribeiro, Keratin:Zein Particles for Fragrance Controlled Release in Hair, To be submitted.

6.1 Introduction

Hair cosmetic market has been looking for new ingredients and technologies to create formulations for the protection, treatment and adornment of hair. Its role in the protection and thermal regulation of human body, together with is social relevance, makes hair a target for the development of innovative and appealing products [186,187]. Fragrances have been extensively used in cosmetic products to an ample variety of consumer products including perfumes, soaps, and personal care products [188]. Unfortunately, most of the fragrances are very sensitive presenting a low stability, which is associated with the molecules' reactive groups and low solubility. Additionally, the fragrances' degradation do not only cause changes in their sensory properties but may also induce some allergenic reactions [189]. Their encapsulation is thus foreseen as an strategy to protect the fragrances against chemical, thermal and/or physical instability and expand their applicability in several cosmetic products [190–192]. Protein based-particles offer several advantages for the encapsulation and the delivery of fragrances. The encapsulation of fragrances in protein-based particles provides the stabilization and controlled release of the entrapped fragrance and improves the handling of the compounds and the fragrances' safety [189]. Systems composed by keratin and zein were developed in this study for fragrance encapsulation. Prior to the application of the encapsulated fragrances on hair, this new delivery system has to satisfy several conditions including: (i) high encapsulation efficiency; (ii) long-term stability; (iii) small particle size with narrow size distribution and (iv) binding capacity to the hair [192–195]. Zein comprises approximately 45-50% of the total proteins existent in maize, presenting high hydrophobicity [196]. Due to the special tertiary structure, composed 50-60% by α-helix, zein can self-assemble to form micro- and nanoparticles through liquid–liquid dispersion or solvent evaporation approaches [197,198]. The isolated zein is biodegradable, biocompatible and has high coating capacity which provides an advantage for its use as delivery system of drugs, enzymes, essential oils and other compounds [197,199–202]. Keratin proteins refers to a broad category of insoluble proteins organized as intermediate filaments that form the bulk of cytoplasmic epithelia and epidermal appendage structures like hair, horn, nails, wool and feathers [203]. Taking in consideration the protein structure and the function, keratin proteins can be classified as soft or hard keratins. Hard keratins are the main responsible for the tough structure of epidermal appendages like hair, nails and horns. These proteins contain a high cysteine content and are capable to form inter and intramolecular disulphide bonds, giving keratin its high strength and stiffness [204,205]. Soft keratins are found in the stratum corneum and in the callus and present a lower cysteine content than the hard keratins [203,206].

Due to its excellent mechanical and protective properties keratin is largely explored for the development of cosmetic formulations. Keratin and mainly keratin hydrolysates have been widely used in personal and hair care products [207]. Studies using functional keratin proteins demonstrated that these proteins were able to protect hair from the damaged caused by chemical treatments (relaxation and perming), while improving the mechanical strength of the hair fibers and decreasing the fading of color from dyed hair [208]. In the last years, our group has developed keratin-based particles for the improvement of the stiffness, tensile strength, thermal stability and smoothness of virgin and overbleached Asian hair [209]. Here, we developed a system for the release of fragrances from hair, using zein and keratin proteins to encapsulate linalool or menthol. The Keratin:Zein particles were prepared by two different strategies: in strategy A, the zein nanoparticles were formed and then the keratin was associated by electrostatic deposition; in strategy B the keratin was co-precipitated with the zein during particle's formation (**Figure VI.1**).

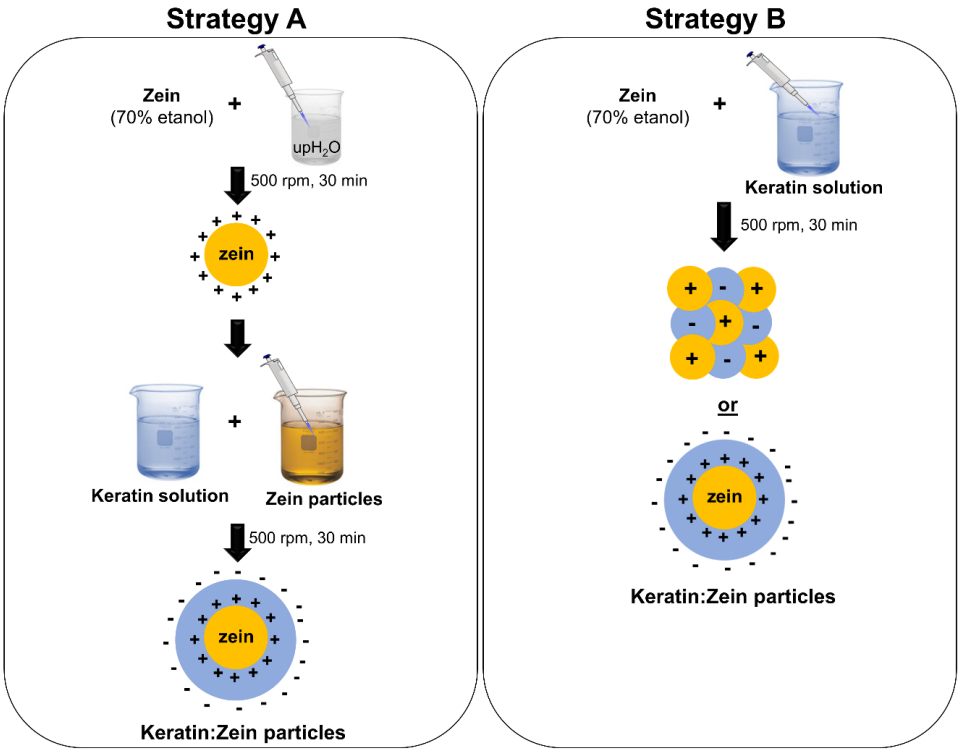


Figure VI.1 - Schematic representation of the procedures (strategy A and B) used to prepare the Keratin:Zein particles

The stability of the prepared Keratin:Zein particles encapsulating the fragrances was evaluated using dynamic light scattering (DLS) and the particles' application on hair was characterized by scanning electron microscopy (SEM). The effect of the treatments on hair properties was evaluated in terms of hair mechanical properties and hydration degree. Gas chromatography-mass spectrometry (GC-MS)

experiments and a human panel were selected to evaluate the fragrance release from the hair treated with the Keratin:Zein particles encapsulating the linalool or the menthol fragrances.

6.2 Materials and Methods

6.2.1 Reagents and materials

Zein from maize (powder) was purchased from MerckSigma, Spain. Linalool (96%) and (-)-menthol (99%) were acquired from TCI chemicals, Belgium. Ethanol HPLC grade was obtained from Fluka, Portugal. All other reagents were acquired from MerckSigma, Spain, and used as received. Natural Asian hair was provided by International Hair Importers & Products Inc. (Glendale, New York, USA). Gas chromatography vials (22.5×75.5 mm, septa of silicone blue transparent/PTFE white) were purchased from Enzymatic, Portugal.

6.2.2 Keratin extraction and purification

Hair donated from local hairdressing salons was used to extract the keratin, after washing the hair according to the IAEA/RL/50 1978 recommendations to remove contaminants and lipids [95]. An adapted protocol from Ayutthaya *et al.* (2015) was used for keratin extraction using a solution comprising 0.2 M sodium dodecyl sulphate, 8 M urea and 0.5 M of sodium metabisulphite in a ratio of 10 mL of solution to 1 g of dry hair [161]. The extraction solution was heated to 100 °C, the hair was added to this solution and the mixture was incubated for 30 min with agitation. After that time, the mixture was incubated over night at 37 °C, under constant agitation. The extraction solution was then centrifuged at 4000 rpm for 10 min, and the supernatant was filtered using paper filter to remove remaining hair fragments. The keratin solution was dialyzed for 5 days against distilled water using a dialysis membrane with a 14 kDa of cut-off [209].

6.2.3 Preparation of the Keratin:Zein particles

To prepare the Keratin:Zein particles, two strategies represented on **Figure VI.1** were used (strategy A and B). In strategy A, 2 mL of a zein solution (50 mg/mL, pH 5, in ethanol 70%) was added to a beaker containing 8 mL of ultrapure water (upH_2O), to obtain the zein particles. The particles were then subjected to agitation at 500 rpm for 30 min, at room temperature (RT). The zein particles were then coated with keratin, considering different Keratin:Zein ratios of 2:1, 1:1, 1:2; with the final zein concentration being maintained constant at 5 mg/mL (**Table VI.1**). The particles were then stirred at 500 rpm for 30 minutes, at RT. In strategy B, 1 mL of the zein solution (50 mg/ml, pH 5, in ethanol 70%) was added to a beaker containing 9 mL of a keratin solution and the particles solution was stirred at 500 rpm for 30 minutes, at RT. The final keratin concentration was determined considering the zein concentration of 5 mg/mL

and the Keratin:Zein ratios of 2:1, 1:1, 1:2. The concentration of zein and keratin, here used, were optimized to improve the particles' stability over time and particles' physical properties like size, surface charge and polydispersity index.

Table VI.1 - Final concentration of keratin and zein proteins in the Keratin:Zein particles formulation, according to different ratios used in particles preparation.

Keratin:Zein Ratio	[Keratin] mg/mL	[Zein] mg/mL
2:1	10	5
1:1	5	5
1:2	2.5	5

To quantify the particles formation efficiency, the free protein was separated using a centrifugal filter tubes with a 100 kDa of cut-off (Vivaspin 20, GE Healthcare, Spain). The free protein was quantified using DC™ Protein Assay (Bio-Rad, Portugal) according to manufacturer, and bovine serum albumin (BSA, MerckSigma) was used to perform the calibration curve. The efficiency of particles formation was determined by Equation VI.1:

$$Eff.particles\ (\%) = \frac{[IProtein]i - [Protein]f}{[Protein]i} \times 100 \qquad \text{(Equation VI.1)}$$

Being Eff.particles the efficiency of particles formation; $[Protein]_i$ is the protein concentration used to prepare the particles; $[Protein]_f$ is the free protein concentration quantified by DC™ Protein Assay.

6.2.3.1 Characterization of the Keratin:Zein particles

The mean size diameter, polydispersity index (PDI) and surface charge of the Keratin:Zein particles were measured using a Zetasizer Nano ZS (Malvern Instruments) at 25 °C. The samples were measured without any dilution, and read in triplicate, being the results described as mean ± standard deviation.

6.2.4 Preparation of the Keratin:Zein particles encapsulating the linalool or the menthol fragrances

Considering the Keratin:Zein particles physical characteristics and stability, the ratio 2:1 was selected for the fragrances encapsulation. Briefly, in strategy A, 2 ml of the zein solution (50 mg/mL, pH 5, in ethanol 70%) were added to 8 ml of the fragrance solution (previously prepared in ethanol 70% HPLC-grade) dissolved in upH_2O. The zein particles encapsulating the fragrances were then incubated for 30 min, with constant agitation at 500 rpm. The beaker was sealed to avoid the fragrance evaporation. The zein particles encapsulating the fragrances were then coated with the keratin as described in section 6.2.3. The Keratin:Zein particles encapsulating the fragrances were then incubated with constant agitation (500

rpm) for 30 minutes, at RT, and were stored completely sealed at 4 °C until further use. In strategy B, 2.5 mL of a zein solution (50 mg/mL, pH 5, in ethanol 70%) were added to 22.5 mL of keratin solution where the fragrances (prepared in ethanol 70% HPLC-grade) were previously dissolved. The particles formulation with the fragrances were then stirred at 500 rpm for 30 minutes, at RT, and were sealed and stored at 4 °C to avoid the fragrance evaporation until further use. The particles, prepared by the two strategies, presented the final concentrations of the components: $[keratin]_{final}$ = 10 mg/mL, $[zein]_{final}$ = 5 mg/mL, and $[fragrance]_{final}$ = 0.5 mg/mL.

6.2.4.1 Fragrance encapsulation efficiency

To determine the fragrance encapsulation efficiency into the Keratin:Zein particles, the free fragrance was separated from the particles formulations, by size-exclusion chromatography (SEC) using a 5 kDa of cut-off PD-10 Desalting Column (GE-Healthcare). The amount of fragrance encapsulated into the particles was indirectly quantified by GC-MS by the difference between the initial concentration of fragrance added to the particles (0.5 mg/mL) and the non-encapsulated fragrance separated through the PD-10 Desalting Column. Calibration curves using known concentrations of linalool and menthol were previously performed by GC-MS for encapsulation efficiency determination.

6.2.4.2 Characterization of the Keratin:Zein particles encapsulating linalool or menthol

The physicochemical characteristics of the Keratin:Zein particles encapsulating the linalool or the menthol fragrances were measured using a Zetasizer Nano ZS (Malvern Instruments), as described in 6.2.3.1. Changes in the chemical structure of the Keratin:Zein particles were studied by Fourier Transform Infrared Spectroscopy (FTIR). The spectra were obtained using a Bruker Alpha II (Massachusetts, USA) and acquired by the software Opus 8.22.28. Samples were mounted directly over the crystal and the spectra were acquired between 400-4000 cm^{-1} wavenumbers with a 2 cm^{-1} resolution.

6.2.5 Hair treatment and characterization

6.2.5.1 Hair treatment with the Keratin:Zein particles

Virgin Asian hair samples were washed using a commercial shampoo (Pantene® Basic, Portugal) and dried at room temperature, prior treatment. Hair tresses with about 90 mg of weight were treated with 1.5 mL of Keratin:Zein particles (~15 mg/mL for the 2:1 Keratin:Zein ratio), a volume sufficient to cover the hair fibers, at room temperature, for 1 h. After this period, the hair samples were removed from the particles' solution and let dry at room temperature.

6.2.5.2 Scanning Electron Microscopy analysis

The analyses of the hair samples treated and untreated with the Keratin:Zein particles were performed using a desktop Scanning Electron Microscope (Phenom ProX, Netherlands), with the ProSuite software. The hair samples were placed on aluminium pin stubs covered with electrically conductive carbon adhesive tape (PELCO Tabs™) and observed at 5 kV, with a spot size of 3.3. Before analysis, the samples were covered with 20Å of gold to improve samples electrical conductivity.

6.2.5.3 Hair fiber tensile test

Hair fibers' mechanical properties (Young's modulus and tensile strength) were assessed using a dynamometer Hounsfield machine, in order to study the effect of the Keratin:Zein particles on hair. The differences in hairs' mechanical properties were determined following the guidelines outlined in ASTM D1145-95 for fiber tensile testing. For these tests, 20 single hair fibers with low variability in diameter were selected and were individually attached to a tensile jig (paper template with a fixed gauge length of 20 mm). Initially, the hair fibers in the tensile jigs were placed in an excicator for 48 h to avoid excessive humidity. Before the tensile test, the paper template was cut across so the measurements could be performed in the middle section of the hair fiber. The stretching measurements were performed at a rate of 1.5 mm/min, with a 0.01 N preloaded force, until the fiber broke [209]. The data are presented as a mean ± SD of twenty samples.

6.2.5.4 Thermal gravimetric analysis

The water content loss from the hair fibers was determined using a TGA 4000 (Perkin Elmer, Waltham, MA, US) and acquired using the Pyris software (version 13). Hair samples (8-10 mg) were analysed between 25 to 800 °C at 20 °C/min under a nitrogen atmosphere (flow rate: 20 mL/min) and the weight loss, in percentage, and its derivative were represented as function of temperature [210]. The temperature calibration was done by Curie temperatures of reference materials: alumel, nickel and perkalloy at the same conditions of the tested samples.

6.2.5.5 Fragrance release from the hair treated with the Keratin:Zein particles encapsulating the menthol or the linalool fragrances

The release of the fragrances from the hair treated with the Keratin:Zein particles encapsulating the menthol or the linalool fragrances, was evaluated by headspace GC-MS using manual injection of the SPME fiber (100 µm polydimethylsiloxane), as performed in Gonçalves *et al.* (2019)[211]. Briefly, 500 µL of the Keratin:Zein particles (~15 mg/mL for the 2:1 Keratin:Zein ratio) encapsulating the fragrances were added to 30 mg of virgin Asian hair with 1.5 cm of length and were incubated at 25 °C for 1 h to promote the adsorption of the particles onto the hair. The hair samples were then transferred to a GC

vial, and a SPME fiber was inserted in the middle (~40 mm from top) of the vial. The samples were incubated for 16 h at 25 or 37 °C and the release of the fragrances was evaluated by GC-MS. The GC analyses were carried out using a GC Varian 4000 system with a split/splitless injector coupled to a mass spectrometer (MS). The GC-MS conditions for this analysis were based on Gonçalves *et al.* (2019) [211]. Injections were operated at 250 °C in the split mode, with ratio 1:10 and using a Rxi-5Sil MS (Restek) column (30 m Å~ 0.25 mm, and 0.25 μm film thickness), with a column-head pressure of 7.3 psi using helium as carrier gas. The oven temperature started at 45 °C for 5 min, and the temperature increased until 250 °C at a rate of 7 °C/min. A full scan mode (50-750 m/z) was applied for the identification of the target compound. The mass spectrometer (MS) was operated in the electron ionization mode at 70 eV with total ion chromatogram detection mode for quantitative determination and S/N ratio of 5. Calibration curves for linalool and menthol fragrances were performed using the same conditions of the samples (volume, temperature and time) and were measured in duplicate. The amount of fragrance released from the hair treated with the particles was determined by integration of the peaks from chromatograms and the area value was substituted on the calibration curves.

6.2.5.6 Sensorial assessment using a human panel

A sensorial olfactory test was performed with 31 volunteers to evaluate if the hair samples treated with the Keratin:Zein particles with linalool or menthol presented a pleasant smell. The cohort included 31 volunteers, 18 females and 13 males, with ages between 20 and 42 years. A blind control (untreated hair) was included in each set of hair samples to validate the results. The olfactory test was performed in the following order: the volunteer smelt first the untreated Asian hair and then the hair samples treated with the fragrance-encapsulated Keratin:Zein particles and the blind control in a random order. Afterwards, it was asked to the volunteer to select the sample (corresponding to a strategy and a fragrance) which conferred the best smell to the hair.

6.2.6 Statistical Analysis

Statistical comparisons were performed by one-way ANOVA with GraphPad Prism 5.0 software (La Jolla, CA, USA). Tukey's post hoc test was used to compare all the results between them, and a Dunnet's test was used to compare the results with a control. A p-value ≤ 0.05 was considered to be statistically significant.

6.3 Results and Discussion

6.3.1 Keratin:Zein particles characterization

Zein colloidal particles can be prepared by controlled precipitation in ethanol/water solutions of zein by changing the characteristics of the solvent using water as an antisolvent [212,213]. The stability of the zein particles is often limited because of its sensitivity to temperature, pH and ionic strength [214,215]. To circumvent these problems, copolymers like proteins are normally incorporated into the particles' formulation to improve the dispersion stability [214,216]. In this work, we selected keratin extracted from hair to stabilize the zein particles and to promote the interaction of the particles with hair based on the natural affinity of this protein towards the hair fibers [51]. Two strategies were explored to include the keratin into the particles' formulations. In strategy A, the keratin was associated to the zein nanoparticles by electrostatic deposition after zein particle formation [216] while, in strategy B, the keratin was co-precipitated with the zein during particles formation [212].

Particles formulation was optimized regarding the methodology used (strategy A and strategy B), zein concentration (1, 2.5 and 5 mg/mL) and keratin concentration (2.5, 5 and 10 mg/mL). When the zein particles (5 mg/mL) were prepared at pH 5, they presented positive charge (≈29-34 mV) and an average size of 148±14.11 nm, however at the end of 1 week of storage at 4 °C they started to precipitate and formed a clump at the bottom of the vial – **Figure VI.2.**

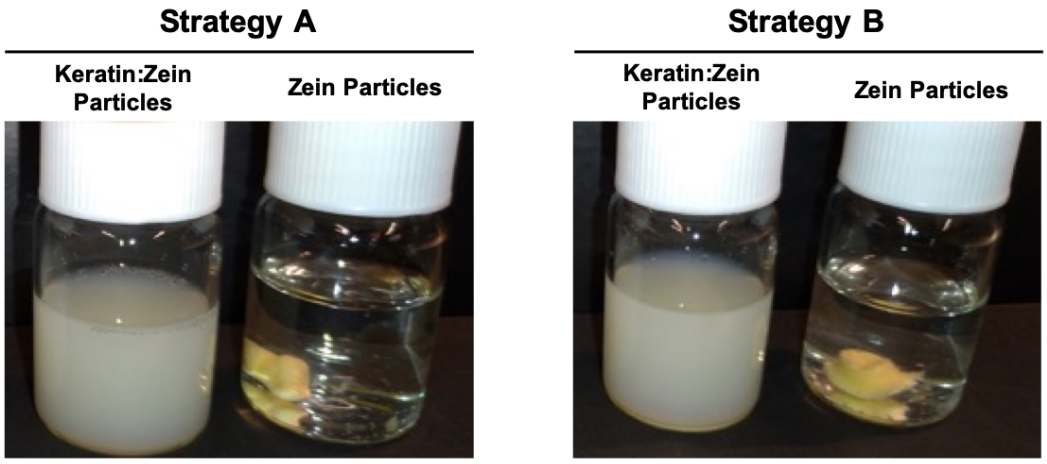


Figure VI.2 - Effect of keratin on the stability of the zein particles, prepared at pH 5 by the strategy A or B. The photographs were taken after 1 week of storage at 4 °C.

The same behaviour was observed in the work of Cheng and Jones (2017) where zein nanoparticles prepared at pH 5.25-6.75 presented significant precipitation [217]. However, when the keratin was included into the zein particles' formulation, the particles revealed a high stability for at least 90 days –

Figure VI.2 and **Figure VI.3**. The interaction between the two proteins was promoted by electrostatic forces since zein and keratin present, respectively, a positive and a negative charge at pH 5. It was also observed that, the addition of the keratin into the zein formulation caused the particles' surface charge to shift from positive (29.7±1.27 mV) to negative (≈ -24 mV), corroborating the hypothesis of the presence of keratin coating the zein particles. The mechanism involved in the stabilization of the zein particles by the keratin could be either by electrostatic repulsion, steric stabilization, or a combination of both effects that is known as electrosteric stabilization [212]. Keratin could have the tendency to localize and adsorb on the surface of zein particles due to their amphiphilic and charged nature, providing an electrostatic and steric repulsion against aggregation. This behaviour was also observed for sodium caseinate that stabilized zein colloidal particles [212].

The formation efficiency of the Keratin:Zein particles was determined by quantifying the free protein separated using centrifugal filter tubes (100 kDa cut-off) and the results are present in **Table VI.2**. Despite the particles' formation efficiencies higher than 80%, there was an influence of the strategy used for the particles' preparation. While for the particles prepared using strategy A, the formation efficiencies were higher than 96%, for the particles prepared by strategy B, the formation efficiencies were around 83.0%. The differences in the formation efficiency between both strategies could be related with the particles' structure: core-shell particles for strategy A or mixed particles for strategy B [214].

The formation of the core-shell particles is likely to be a more efficient process for the synthesis of the particles, which is corroborated by the higher particle formation efficiency obtained for strategy A.

Table VI.2 - Formation efficiency of Keratin:Zein particles prepared by strategy A and B and using different Keratin:Zein ratios. Physicochemical properties (size, PDI and surface charge) of the particles after 90 days of storage at 4 °C.

Strategy	Particles	Keratin:Zein ratio	Formation efficiency (%)	Size (nm)	PDI	Surface Charge (mV)
A	Zein (control)	-	97.41	-	-	-
	Keratin:Zein	1:2	96.88	243.7±4.8	0.166±0.066	-20.8±2.2
		1:1	97.51	231.8±2.8	0.132±0.066	-20.9±2.5
		2:1	96.89	233.6±8.1	0.108±0.018	-23.5±4.0
B	Zein (control)	-	97.66	-	-	-
	Keratin:Zein	1:2	83.58	251.6±4.6	0.096±0.005	-20.3±1.8
		1:1	82.34	239.9±3.2	0.150±0.029	-20.6±1.1
		2:1	84.64	259.7±6.6	0.127±0.035	-19.7±1.7

This phenomenon of particle formation could also affect the particles capacity to encapsulate different compounds/fragrances, as demonstrated later in this study.

The size, PDI, and surface charge of the Keratin:Zein particles were measured by dynamic light scattering (DLS), and the stability of the particles was evaluated until 90 days of storage at 4 °C (**Table VI.2** and **Figure VI.3**). affinity is associated with the high cysteine content of keratin protein and with specific binding amino acidic sequences present on the keratin [51].

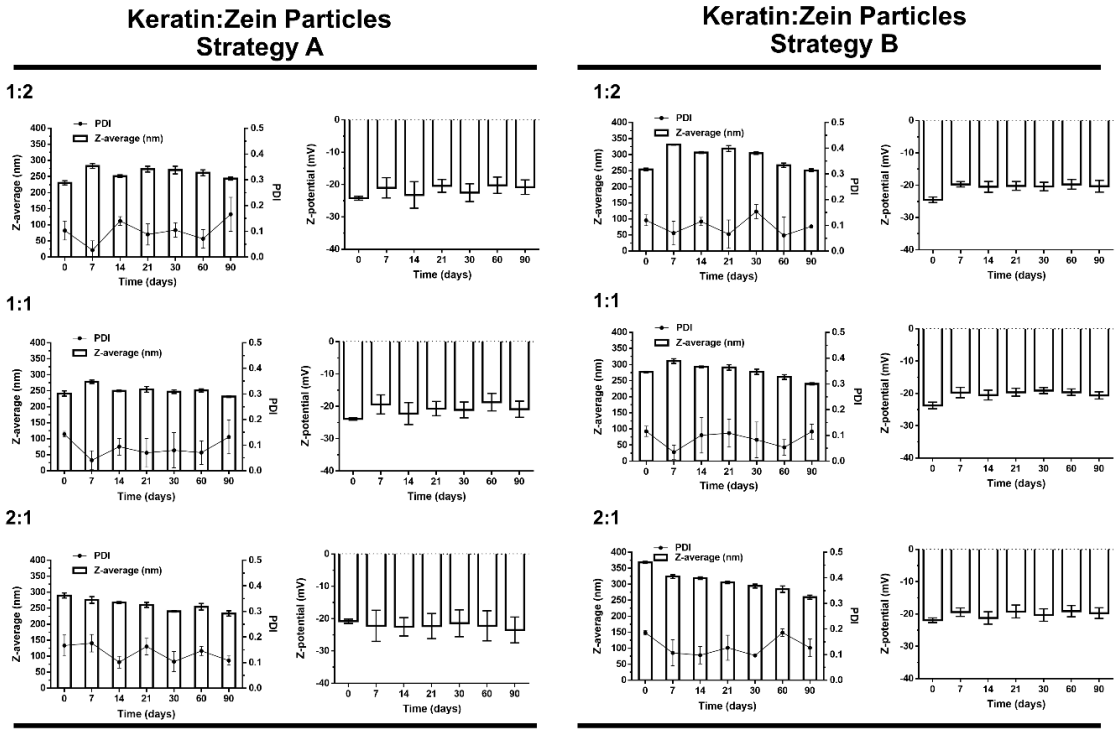


Figure VI.3 - Effect of storage at 4°C on the physicochemical characteristics of the Keratin:Zein particles. The particles were prepared by strategy A and B using different ratios (Keratin:Zein ratios – 1:2, 1:1 and 2:1) of keratin and zein proteins. Values represent the mean ± SD of 3 independent experiments.

Similar sizes, PDI and zeta-potential values were obtained for the Keratin:Zein particles prepared using different ratios of keratin and zein and the two strategies. After 90 days of storage at 4 °C, the size of the Keratin:Zein particles (ratio 2:1) obtained using strategy A varied between 231.8–243.7 nm with a PDI of 0.108–0.166, and the particles produced by strategy B varied between 239.9–259.7 nm with a PDI of 0.096–0.127. Due to the presence of the keratin on the particles' surface, all formulations presented a negative surface charge (~ from -19 to -25 mV). This combination was selected due to the high content of keratin, which might increase the particles' affinity to the hair. This greater affinity is associated with the high cysteine content of keratin protein and with specific binding amino acidic sequences present on the keratin [51].

Relatively to the particles structure, different organizations between the zein and the keratin could be obtained depending on the strategy used for the particles production. In strategy A, the particles' structure is most likely characterized by a positively charged core-shell of zein with the negatively charged keratin protein adsorbed on the surface. For strategy B, the final structure depends on the kinetics of two competing processes that occur during particle formation: precipitation of the hydrophobic protein (zein) and association of the keratin with the zein. Depending which process is predominant in the beginning of the particles' formation process, the Keratin:Zein particles could have a zein core-shell surrounded by the keratin or be mixed particles composed by keratin and zein. If the zein particle formation occurs more rapidly than the association between the keratin and the zein, it would be expected a zein core-shell surrounded by keratin. In another way, if the association between zein and keratin occurs more rapidly than the zein particle formation, it would be expected a mixed internal structure to be formed, with both proteins distributed through the particle matrix [212,214].

Although a detailed understanding of the particles' formation mechanism is outside of the scope of this paper, the absorption of keratin on the surface of zein particle during zein precipitation seems to be the most likely mechanism to happen on strategy B since zein is a highly hydrophobic protein that will rapidly organize and form particles when in contact with water [212,214].

6.3.2 Keratin:Zein particles encapsulating linalool or menthol fragrances

After particles optimization, relatively to the concentration of keratin and zein, the ratio 2:1 was selected to produce the Keratin:Zein particles with the linalool or the menthol fragrances. The strategies A and B were used to prepare the particles encapsulating the fragrances in order to evaluate the potential effect of the particles structure on the fragrance release. To determine the fragrance encapsulation efficiency into the Keratin:Zein particles, the free fragrance was separated from the particle formulations by size-exclusion chromatography using a PD-10 Desalting Column (GE-Healthcare) with a 5 kDa of cut-off. The free fragrance was quantified by GC-MS and this value was subtracted to the initial concentration of fragrance (0.5 mg/mL) used to prepare the particles. Encapsulation efficiencies upper than 77.6% were obtained for all the tested particles, with a higher encapsulating efficiency being verified for the particles prepared by strategy A when compared with the particles obtained using strategy B **(Table VI.3).**

These differences could be explained by the higher efficiency of particle formation obtained for strategy A (around 97%) and the core-shell structure of the particles prepared by the same strategy, that could promote a higher fragrance encapsulation when compared to the mixed structure obtained for the particles prepared by strategy B. The same behaviour was observed by Li *et al.*, (2017), where

higherencapsulation efficiencies were obtained for the core-shell zein particles encapsulating coumarin 6, when compared with the mixed structure zein particles [218].

Table VI.3 - Fragrance encapsulation efficiency (%) of linalool and menthol in the Keratin:Zein particles (ratio 2:1) prepared using strategy A and B. The fragrance encapsulation efficiency was determined by quantification of free fragrance using GC-MS and linalool and menthol standard curves.

Sample	Strategy	Fragrance	Fragrance encapsulation efficiency (%)
Keratin:Zein particles	A	Linalool	80.2
	A	Menthol	88.8
	B	Linalool	77.6
	B	Menthol	87.3

6.3.3 Particles size, size distribution and surface charge characterization

The particles produced through the two strategies remained stable for at least 90 days of storage at 4 °C (**Figure VI.4**), exhibiting along of these 90 days sizes ranging between 280-310 nm.

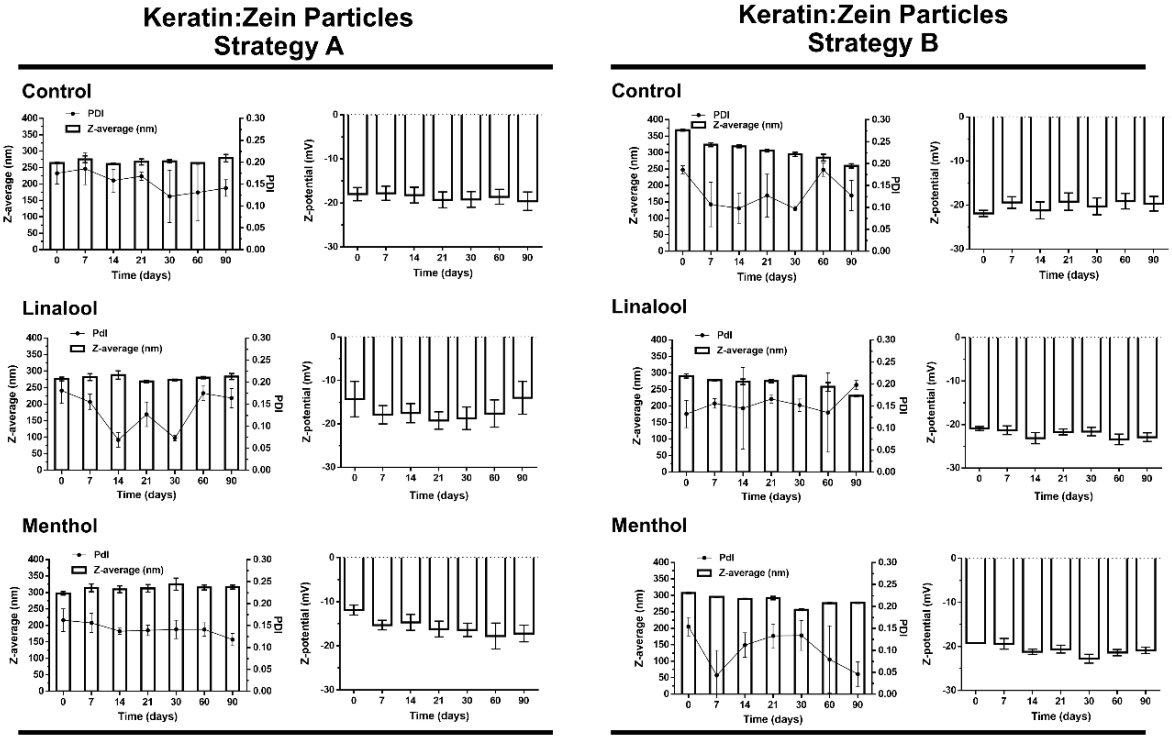


Figure VI.4 - Effect of storage at 4 °C on the physicochemical characteristics of the Keratin:Zein particles ('Control') and the Keratin:Zein particles encapsulating linalool ('Linalool') or menthol ('Menthol'), when prepared by strategy A or strategy B. Values represent the mean ± SD of 3 independent experiments.

Both strategies presented a monodisperse character and surface charge with approximately -20 mV, indicating that both strategies produced uniform and stable particles.

6.3.4 Particles analysis by FTIR

FTIR analysis was performed to assess the intermolecular interactions between zein and keratin proteins during the formation of the Keratin:Zein particles, and to evaluate the encapsulation of the linalool and menthol into the particles. The spectra of keratin and zein **(Figure VI.5)** showed the characteristic peaks of proteins, corresponding to the amide I and amide II (C=O bonds) peaks [214]. For the keratin, these peaks, amide I and amide II, appeared at 1653 and 1538 cm^{-1}, respectively; while for the zein a small shift in the peaks (1645 and 1585 cm^{-1}) was observed. Some other characteristic peaks were observed for these proteins. The peaks at 632, 1024 and 3282 cm^{-1}, which correspond to C-S bonds present in cystine, C-O stretching vibration and to N-H bonds, respectively, were detected for the keratin [219]. For the zein, it was detected a peak at 3304 cm^{-1}, which result from the stretching vibration of N-H and -OH from the amides in the amino acid residues [215]. Comparing the spectra of the keratin and zein proteins with the spectra of the Keratin:Zein particles, prepared by the strategy A or strategy B, it was observed the appearance of two new peaks at 878 and 2030-2043 cm^{-1} for the particles (* in **Figure VI.5**). These peaks could be due to the interaction between the proteins during the particle's preparation. The peak at 2030-2043 cm^{-1} could be attributed to an overtone, indicating the presence of aromatic compounds on the Keratin:Zein particles' surfaces, while the peak at 878 cm^{-1} could be due to the C=C bending from these aromatic compounds [220,221]. Moreover, the presence of the keratin and zein characteristic peaks, 632 cm^{-1} of the C-S bonds in the keratin and 3304 cm^{-1} of the stretching vibration of N-H from amides for the zein, in the Keratin:Zein particles spectra corroborate the presence of the two proteins in the final particles.

Regarding the fragrances, the menthol spectra presents peaks at 3226 cm^{-1} which corresponds to the OH group; at 2867 and 2929 cm^{-1} which are attributed to the C-H stretching vibrations; at 1344 cm^{-1} that corresponds to the isopropyl group and at 1024 and 1045 cm^{-1} which are attributed to C-O bonds [222]. For the linalool fragrance, the spectra show significant peaks at 3383 cm^{-1} that are attributed to the stretching of O-H bond of an alcohol; at 2924-2970 cm^{-1} corresponding to the C-H stretching vibrations and at 1109 cm^{-1} that is assigned to the C–O stretching vibration [223]. Most of the characteristic peaks of linalool or menthol are not detected or are masked when the fragrances were encapsulated into the Keratin:Zein particles. The absence of the fragrances' characteristic peaks, in conjugation with the encapsulation results determined by GC-MS **(Table VI.3)**, shows that the fragrances

were successfully encapsulated using both strategies. Also, the absence of new peaks in the particles encapsulating the fragrances suggested that the fragrances and the proteins might be physically attached without the formation of any new chemical bond [214].

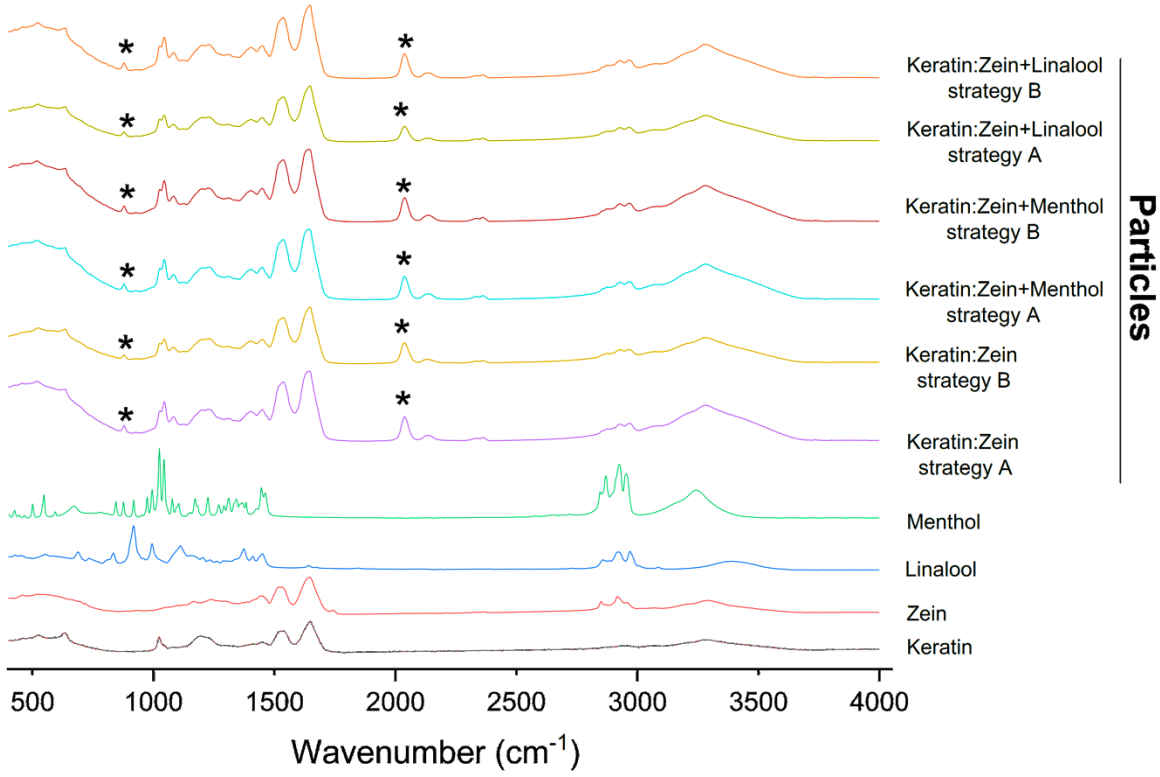


Figure VI.5 - FTIR spectra of Keratin:Zein particles and Keratin:Zein particles encapsulating linalool or menthol, when prepared by strategy A or strategy B. Keratin, zein, linalool and menthol were used as references. Bands marked with * represent changes observed during particles formation

6.3.5 Application of the Keratin:Zein particles in virgin Asian hair

After optimizing the conditions for the synthesis of the Keratin:Zein particles, the ability of the particles to adsorb to the hair fibers was tested. The hair was incubated with the particles' formulations and the ability of the Keratin:Zein particles to bind to the hair fibers was evaluated by SEM **(Figure VI.6)**. Comparing the fibers of the untreated virgin Asian hair (control) with the fibers treated with the Keratin:Zein particles prepared by both strategies, it is possible to verify the deposition of the particles onto the hair surface on the treated samples, while the control samples present a smooth surface with cuticles without any type of material deposited. No differences are observed in the particles' deposition pattern between the two strategies of particles preparation. In both samples the particles are tightly bound to the hair and present a random distribution along the hair fibers, with a higher accumulation in the interlayer zone of the cuticles. This deposition pattern is similar to the one observed for the hair conditioners which accumulate

near the cuticle edges, masking the damages caused by environmental factors, daily care routines or cosmetic procedures like bleaching, straightening, relaxing, coloration, among others [179,180]. A film-like structure was also observed, this behaviour was already expected due to the excellent film forming properties characteristic of the zein protein [224–226].

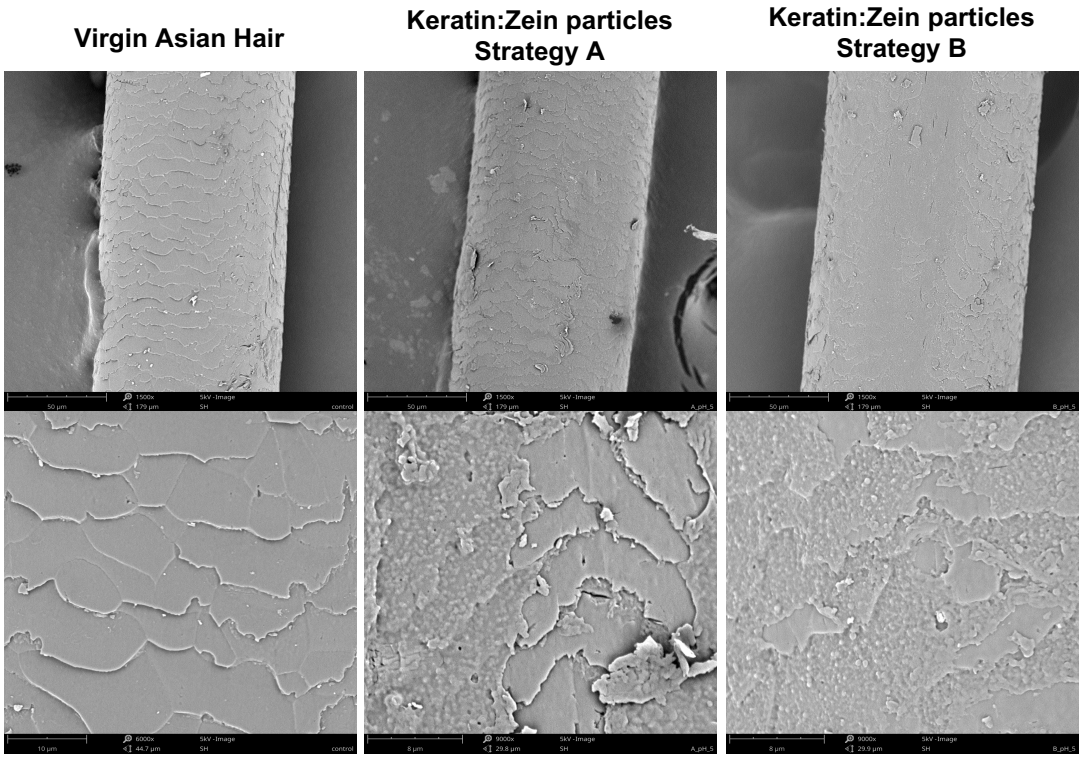
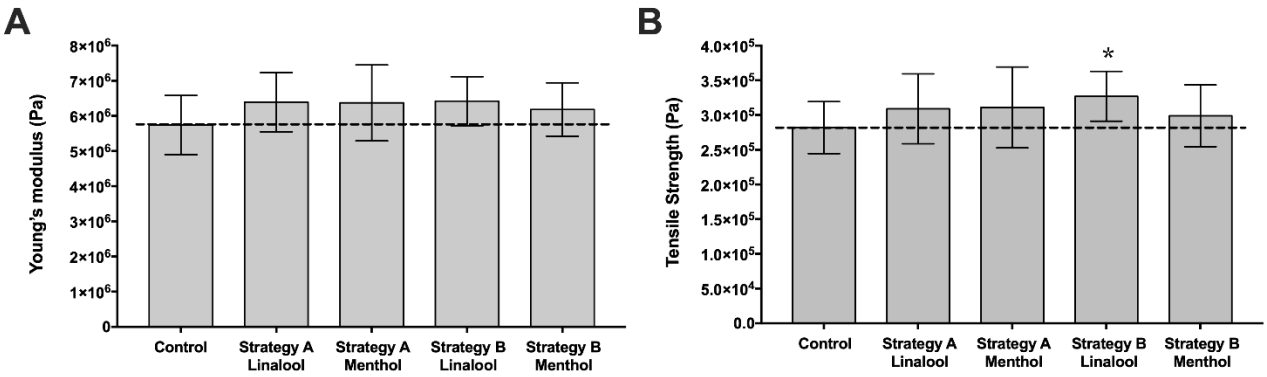


Figure VI.6 - SEM micrographs (1500x and 9000x) of untreated virgin Asian hair (left), Asian hair treated with the Keratin:Zein particles prepared by the strategy A (middle) and Asian hair treated with the Keratin:Zein particles produced by strategy B (right).

6.3.6 Effect of Keratin:Zein particles treatment on the mechanical properties of hair

Cosmetic formulations are described to affect the mechanical properties of hair. Depending on the composition, hair cosmetic formulations can protect hair from damage and recover its properties [95,227] or decrease the overall performance of the hair fibers [228,229]. To study the effect of the treatment with the Keratin:Zein particles on the hair mechanical properties, the hair mechanical resistance was determined taking in consideration the fibers' Young's modulus and tensile strength (**Figure VI.7 A-B**). Analysing **Figure VI.7,** all of the tested formulations increased the Young's modulus and the tensile strength values of the hair fibers when compared with the untreated Asian virgin hair (control). The improvement of the mechanical resistance caused by the Keratin:Zein particles application was determined comparatively to the values obtained for the control samples, and are presented in **Figure**

VI.7 C. Although an increase in both parameters was verified, no significant differences were obtained for the tested formulations, with exception for the hair treated with the particles encapsulating linalool and prepared using the strategy B (increase of 16% for the tensile strength parameter).



C

Sample	Strategy	Encapsulated fragrance	Young's modulus improvement (%)	Tensile strength improvement (%)
Keratin:Zein particles	A	Linalool	11.46	9.63
	A	Menthol	11.00	10.47
	B	Linalool	11.96	15.97
	B	Menthol	7.82	6.07

Figure VI.7 - Effect of the treatment with the Keratin:Zein particles encapsulating the linalool and menthol fragrances on the mechanical properties of Asian virgin hair: (A) Young's modulus, (B) tensile strength. Statistically significant differences from the control and the treated hair meshes are indicated as *p-value≤0.05. (C) Improvement of Asian hair mechanical properties measured in terms of Young's modulus and tensile strength after treatment with the Keratin:Zein particles, compared with the untreated Asian virgin hair.

6.3.7 Effect of the Keratin:Zein particles on hair hydration

Water content is an important factor in the maintenance of a healthy hair. However, daily routines and cosmetic procedures damage the keratin structure, inducing a decrease in fibers' water content [152]. The effect of the treatment with the Keratin:Zein particles encapsulating the linalool or the menthol, on the thermal stability and water content of hair was followed by thermal gravimetric analysis (TGA) (**Figure VI.8 and Table VI.4**). Analysing the TGA curves **(Figure VI.8)**, different decomposition profiles and temperatures were observed depending on the treatment. The first weight loss stage was observed between 25-165 °C due to the loss of loosely (25-65 °C) and tightly bound water (65-165 °C) [40,230]. The second loss weight loss is related to the organic degradation of the hair fiber and destruction of lateral

chains of keratin protein, happening in the range of 165-550 °C [231]. In the last step, from 550-800 °C, it was observed a complete degradation of the hair keratin structure [152,230]. The remaining weight percentage verified at 800 °C is associated with the carbon molecules existent in the protein and others structures that did not decompose at this temperature, and remain as ashes [232]. In the thermograms of the hair treated with the Keratin:Zein particles formulations, various loss steps were observed between 165-500 °C that were not observed in the control sample. The additional peaks could be related with the presence of the zein and the keratin proteins in the hair fibers, which can be localized in the hair surface or deeper in the hair cortex [233].

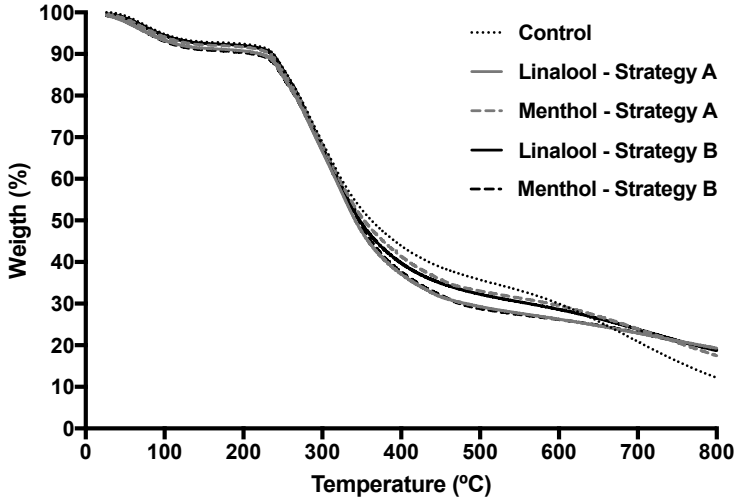


Figure VI.8 - Effect of temperature on the weight percentage of hair samples treated with the Keratin:Zein particles (2:1 ratio) encapsulating the linalool or the menthol fragrances, verified by TGA. Control: virgin Asian hair; Linalool – Strategy A: virgin Asian hair treated with Keratin:Zein particles prepared by strategy A and encapsulating linalool; Menthol – Strategy A: virgin Asian hair treated with Keratin:Zein particles prepared by strategy A and encapsulating menthol; Linalool – Strategy B: virgin Asian hair treated with Keratin:Zein particles prepared by strategy B and encapsulating linalool; Menthol – Strategy B: virgin Asian hair treated with Keratin:Zein particles prepared by strategy B and encapsulating menthol.

Considering only the temperature range between 25 and 165 °C from the thermograms, whose percentage corresponds to the loss of water molecules from the fibers, it is possible to evaluate the moisture content of the different hair samples [40,230]. Through the analysis of **Table VI.4**, we observe an increase of the total moisture content of the treated hair samples when compared with the untreated hair. The increase in the fibers' water content after application of the Keratin:Zein particles results from: (i) the ability of the proteins to bind water molecules, forming an appropriate environment for healthy hair

[48]; and (ii) the film-forming properties of zein that may confer a moisture barrier surrounding the hair, decreasing the water loss from the fibers [225,234].Considering the remaining weight percentage after hair samples heating at 800 °C, it was observed that the treated hair samples show a higher weight percentage comparatively to the control samples (increase between 5.4-7.3%). This increase could result from a stabilization of the keratin structure given by the mixture of zein and keratin proteins, making the hair fiber more resistant to higher temperatures.

Table VI.4 - Effect of Keratin:Zein particles application on the moisture content of hair characterized by TGA.

Sample	Strategy	Fragrances	Moisture content* (%)	Residue weight at 800°C (%)
Virgin Asian hair	-	-	7.18	11.85
Virgin Asian hair treated with Keratin:Zein particles	A	Linalool	8.31	19.14
	A	Menthol	7.72	17.22
	B	Linalool	7.36	18.50
	B	Menthol	8.46	18.84

*the moisture content is the fibers' weight loss calculated between 25-165 °C.

6.3.8 Fragrance release from hair treated with Keratin:Zein particles encapsulating linalool or menthol

The main goal of the present study was the development of an efficient system for the release of fragrances from hair. Two temperatures, 25 and 37 °C, were selected to perform the fragrance release experiments. These temperatures correspond to the room temperature and the body temperature or the temperature in a hot day in summer. The release of the fragrances was evaluated along 16 h, which corresponds to the normal period where an individual is awake. The calibration curves for linalool and menthol were prepared determining the area of GC-MS peaks of increasing concentrations of these fragrances at the same conditions of the samples **(Figure VI.9)**.

From the GC-MS data, a unique peak was obtained for linalool and for the menthol corresponding to a mass spectrum with retention time of 13.7 min and 15.8 min characteristic of linalool and menthol, respectively **(Figure VI.10)**. The chromatograms of the hair treated with the Keratin:Zein particles without the encapsulated fragrances did not reveal any peak (data not shown). This indicates that the matrix did not have any effects on the release of the fragrances, validating the GC-MS (SPME-HS-GC-MS) experiments.

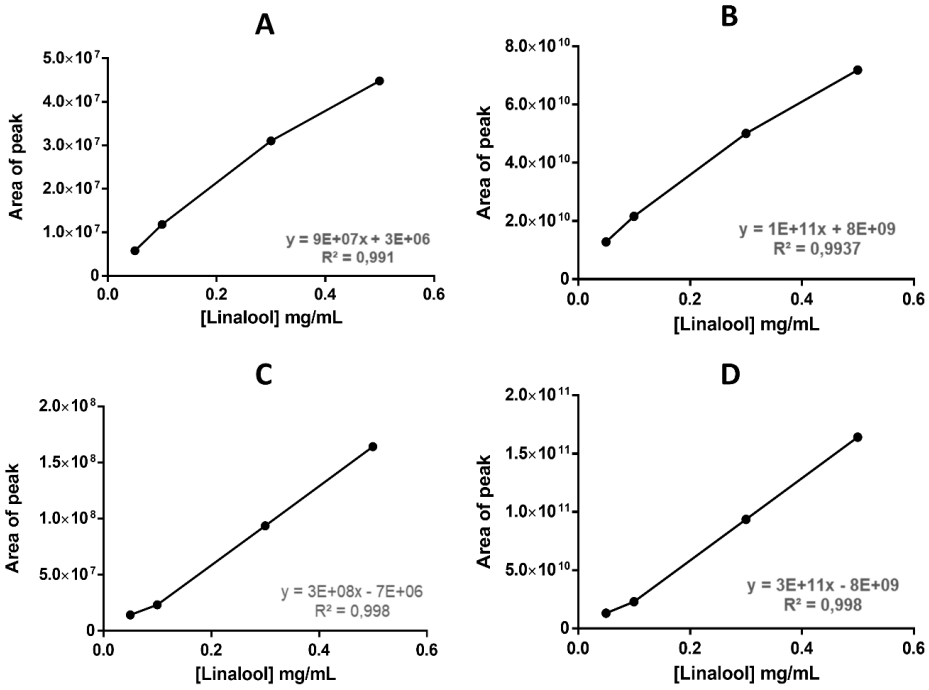


Figure VI.9 - Calibration curves of linalool at 25 °C (A) and at 37 °C (B), and of menthol at 25 °C (C) and at 37 °C (D) for 16 h of SPME exposition time (n=2), determined by GC-MS.

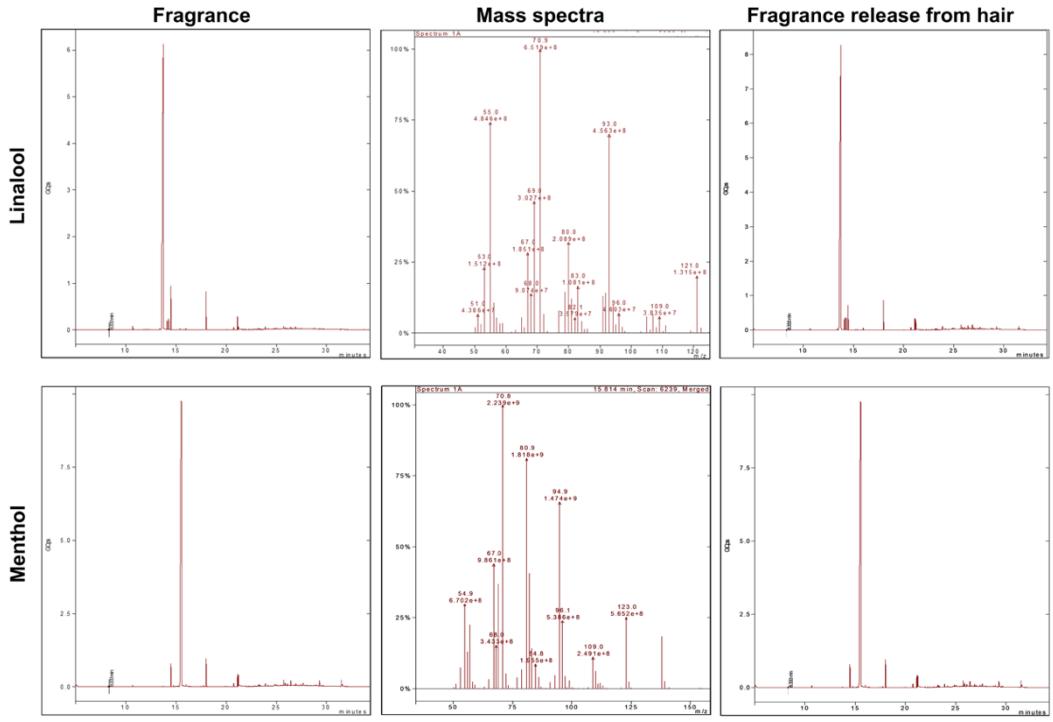


Figure VI.10 - Linalool and menthol chromatograms (RT=13.7 and 15.8 min, respectively) and mass spectra of linalool and menthol, observed by Headspace-SPME/GC-MS. The images on right showed the chromatograms for linalool and menthol released from hair samples treated with the linalool Keratin:Zein particles and with the menthol Keratin:Zein particles.

The GC-MS data showed an increased release of both fragrances when they were encapsulated into the Keratin:Zein particles following strategy B and at 37 °C **(Figure VI.11)**. Regarding the fragrance release at 25 °C, a release of 33% and 44% was measured for linalool encapsulated using strategy A and strategy B, respectively. While for this fragrance the method of particle preparation/fragrance encapsulation influenced the release profiles, no significant differences were detected for the menthol when comparing the two strategies of preparation. However, the increase of the temperature from 25 to 37 °C promoted a higher release of the fragrances from the hair. This higher release promoted by the temperature was particularly relevant for the hair samples treated with the Keratin:Zein particles encapsulating menthol, with an increase up to 68-70% when compared with the release measured at 25 °C.

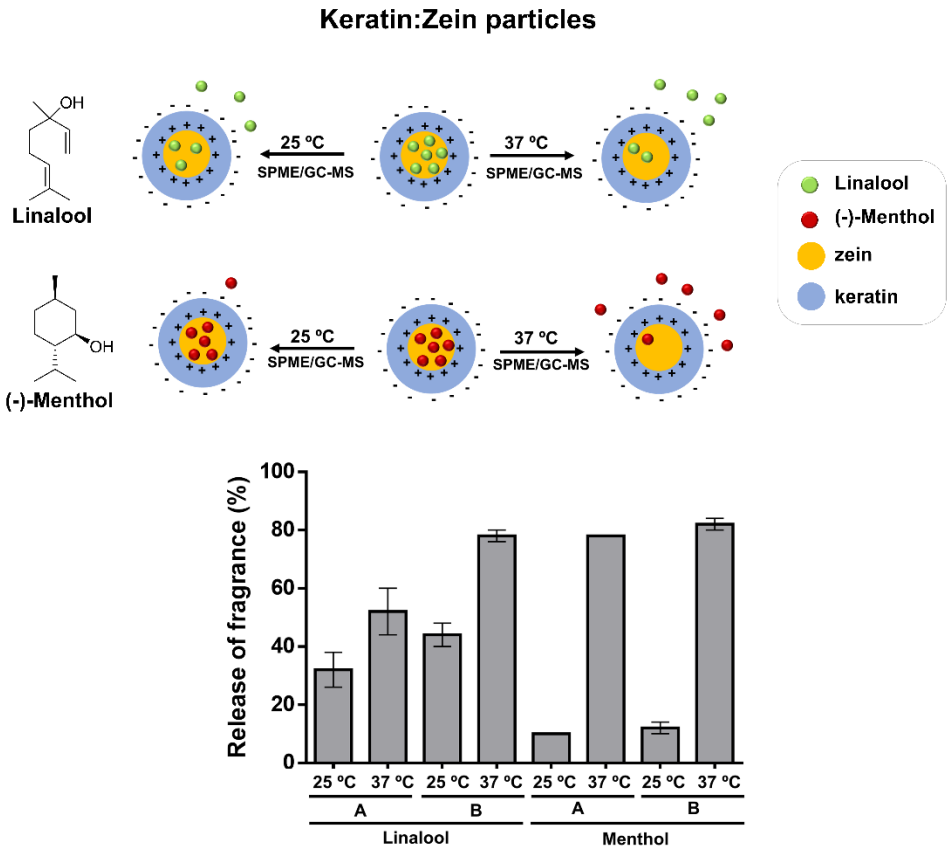


Figure VI.11 - Effect of temperature and method of encapsulation on the release of linalool and menthol from treated virgin Asian hair. The SPME fiber was exposed for 16 h at 25 or 37 °C and the fragrances were quantified by GC-MS.

The differences in the release profiles of linalool and menthol can also be associated with the structure and physicochemical properties of these fragrances. Despite the two fragrances belong to the class of monoterpenes, there are some structural differences between the linalool and the menthol. While the first

is linear, the menthol has a ring in its structure which confers the molecule with some molecular rigidity. This might influence the molecule mobility with the temperature – less mobility at room temperature and higher mobility near the menthol melting point (39 °C). During encapsulation, the menthol most likely formed crystalline structures in the Keratin:Zein particles core. However, when the temperature increased to values near the menthol melting point, the molecule mobility and volatility increased, boosting the release of this fragrance from the particles. Moreover, the vapour pressure at 25 °C for the linalool and menthol is 0.159 mm Hg and 0.00767 mm Hg, respectively (PubChem database). Therefore, the linalool can have a greater ability to evaporate/release from the particles at 25 °C comparatively to the menthol.

6.3.9 Sensorial assessment of the fragrance release from the hair using a human panel

The effect of cosmetic formulations incorporating fragrances on hair is an essential parameter during the development of new hair cosmetic products. Most of end-users prefer products that leave the hair with a pleasant smell instead of a neutral scent. A subjective assessment with a cohort of 31 volunteers (18 females and 13 males) was performed to study the effect of the Keratin:Zein particles encapsulating the linalool or the menthol on the hair odour **(Figure VI.12)**.

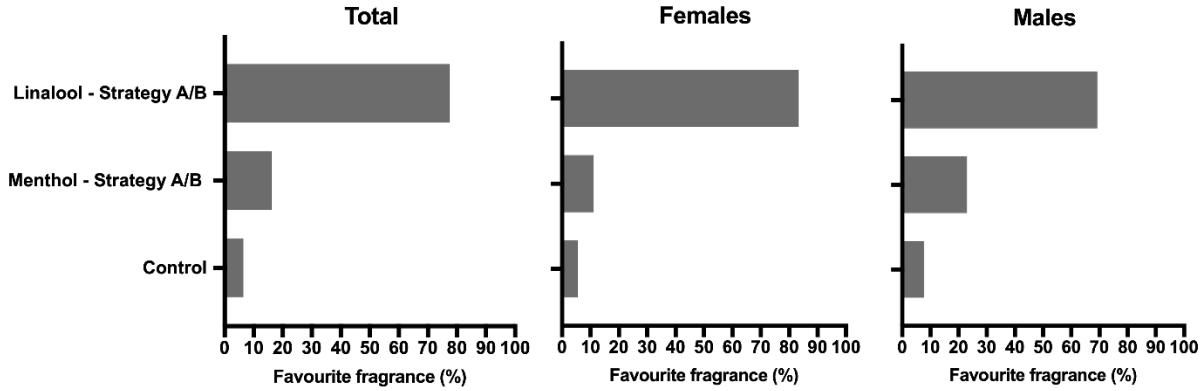


Figure VI.12 - Percentage of volunteers that classified as 'Favourite' the fragrance obtained from the hair treated with Keratin:Zein particles encapsulating linalool or menthol, when compared with the control sample (untreated virgin Asian hair). The olfactory test was performed by 31 volunteers (18 females and 13 males).

Analysing **Figure VI.12 'Total'**, 77% of the volunteers classified as 'Favourite' the formulations encapsulating the linalool (including both strategy A and B), while only 16% of the volunteers gave the same classification for the formulations encapsulating the menthol fragrance. Comparing the results regarding sex, 83 % of the females and 69 % of the males classified as favourite the hair treated with the formulations containing the linalool. Although the majority of the females and males' participants

classified as 'Favourite' the formulations containing linalool, only a few percentages of participants gave the same classification for the formulations containing menthol. The formulation containing menthol were classified as 'Favourite' by only 23% of males and by 11% of females. The differences between the results from the formulations containing linalool and menthol could be justified by the variances in the fragrance's perception according to the volunteer's gender. Donna (2009) described that fresh scents appear more in masculine fragrances and they are present in most personal care products for men [235]. This corroborate the results obtained in our study, where the hair treated with the Keratin:Zein particles with menthol presented a higher preference by the male gender. In the other hand, floral scents are clearly a feminine category and are present in 42% of woman's products and perfumes [235]. In this olfactory test, it was also observed that a small number of volunteers, 6.45%, classified the neutral scent (hair without fragrance application) as the 'Favourite' sample.

According to the volunteers, the low preference for the formulations encapsulating menthol was mainly due to the intensity of this fragrance. Therefore, a reduction of the menthol concentration could increase the pleasantness of the tested formulations with menthol.

6.4 Conclusions

This work showed a promising and low-cost delivery system for the release of fragrances from hair. The Keratin:Zein particles, prepared using two different approaches (strategies A and B), were stable when stored at 4 °C and were capable to encapsulate the linalool or the menthol fragrances. The inclusion of keratin on the particles' formulation increased the particles stability and the adhesion of the particles to the hair. Additionally, the formulations revealed high efficiency of fragrance release, and were able to protect the fragrances during storage. The treatment of Asian hair with the developed formulations demonstrated to be effective in the improvement of hair's properties, traduced on the increase of Young's modulus, tensile strength, and hydration degree. An olfactory test revealed, that the majority of the 31 volunteers preferred the formulations encapsulating linalool independently of the strategy used for the particles' preparation. The fragrances used in this study were selected as a model, proving the capacity of these particles to be personalized according to the personal perception of smell.

Chapter VII

Ohmic Heating as an Innovative Approach for the Production of Keratin Films

Chapter VII – Ohmic Heating as an Innovative Approach for the Production of Keratin Films

Abstract

Ohmic heating is a thermal processing method based on the application of electric fields directly into a semi-conductive medium. In this study, we explored for the first time the use of ohmic heating to obtain keratin films. The properties of the films prepared by ohmic heating and conventional heating were evaluated and compared under similar thermal profiles. A lower increase in free thiols'' concentration was obtained for the keratin solutions and keratin films submitted to ohmic heating (16% increase for the keratin solution extracted from virgin hair, pH 9, submitted to ohmic heating and 23% when submitted to conventional heating). Significant differences in the swelling results were observed for the films prepared with keratin extracted from virgin hair, with a swelling decrease in about 55% for the films prepared by ohmic heating. Generally, the keratin films obtained by ohmic heating showed distinct properties comparatively to the films produced by conventional methods. The application of a fusion protein on the keratin films demonstrated their capacity to be used as substitutes to hair fibers when evaluating the potential of new cosmetic products. This work suggests that ohmic heating show potential to tailor keratin films properties depending on an intended application or functionality.

This chapter is based on the following scientific publication:

Ana Tinoco, Rui M. Rodrigues, Raul Machado, Ricardo N. Pereira, Artur Cavaco-Paulo and Artur Ribeiro, Ohmic heating as an innovative approach for the production of keratin films. *International Journal of Biological Macromolecules,* 2020, 150:671–680.

7.1 Introduction

Protein unfolding and aggregation occur in different extents depending on the pH, ionic strength and temperature, affecting the final properties of the protein-based materials [236]. During heating of a protein solution, the three-dimensional structure of the protein is modified through the unfolding of the polypeptide chains, exposing the internal sulfhydryl groups and the hydrophobic side chains buried in the interior of the native molecule. These structural changes will influence the filming ability of the proteins and the properties of the protein-based films.

Ohmic heating was used in this study as a new and alternative procedure for the use of conventional heating methods – i.e. a water bath with controlled temperature - in the keratin denaturation process and keratin films' preparation. The final properties of the keratin films prepared by ohmic or conventional heating were compared in order to evaluate the effects of each heating method on the protein films. The ohmic heating is an emergent thermal process where electric currents pass through a semi-conductive solution allowing a direct or volumetric heating process. This electro-heating method is recognized by reducing excessive coagulation or the complete denaturation of the protein structure [237]. During ohmic heating, the non-thermal effects exerted by the presence of a moderate electric field has been described as potential vector of alteration of proteins interactions thus changing the final properties of the developed protein films [238].

Keratin proteins are found in the cytoplasm of almost all differentiated eukaryote cells, being mainly known as the principal structural proteins of hair, wool and skin [28]. The term 'keratin' includes a complex mixture of proteins, such as keratins and keratin-associated proteins [233]. Considering the disulphide bonds content, keratins can be classified as soft or hard keratins. The soft keratins, with a low content of disulphide bonds, are found in the stratum corneum and in the callus, whereas the hard keratins, with a high disulphide content, are found in epidermal appendages such as feathers, hair, nails, horns, and hoofs [28]. At a molecular level, the most distinctive feature of keratins is the high concentration of cysteine (Cys) residues (7-20% of the total amino acid residues) [28]. Cys is a non-polar amino acid that contains sulfur groups which can form sulfur–sulfur (S–S) cystine bonds with other intra- or intermolecular Cys molecules. The cross-links between intermolecular cystine bonds together with other protein structural features, like crystallinity and hydrogen-bonding, gives keratin its high strength and stiffness [205]. Due to its structure, keratin proteins are only soluble in solutions containing denaturing agents like urea, and reducing agents such as mercaptoethanol, which are capable to cleave the keratin disulphide bonds [28]. The keratin hydrolysates can be used in different applications: as odor-removing or deodorizing composition [239], included in skin-hair care cosmetic [209,240], in the

enrichment of wheat straw for ruminant diets [241] or applied in the packaging of goods [242]. Also, the keratin proteins are widely used in the biomedical field, for wound healing [243], ocular [244] and nerve regeneration [177] and bone tissue engineering [245]. Keratins were also included in the development of drug delivery systems with controlled release due to keratin stability, low reactivity, and good mechanical and structural properties [246]. In cosmetic applications, keratin-based particles were developed to improve the mechanical and thermal properties of virgin and overbleached hair [209].

The aim of the present study was to evaluate the effect of the heating method (conventional vs ohmic heating), pH and keratin source (virgin vs overbleached hair) on the properties of keratin films. We also assessed the potential of these keratin films to be used as models for the screening of the effect of protein-based cosmetic formulations on hair. To assure that the differences observed for the keratin film-forming solutions and the keratin films were only related with the type of heating, pH and keratin source, the concentration of the keratin solutions and the time and heating profile, were maintained identical along the study.

7.2 Experimental

7.2.1 Materials

Caucasian black human hair samples were provided by a local hairdresser. The detergent compatible (DC) protein assay kit and the Ellman's reagent were obtained from Bio-Rad, Portugal. Glycerol and formaldehyde were supplied by Fisher Scientific, Portugal, and all other chemicals were supplied by Merck, Spain.

7.2.2 Extraction and purification of keratin

The keratin used to produce the films was extracted from human hair donated from a local hairdresser salon. The hair samples were first washed according to the IAEA/RL/50 1978 recommendations to remove all contaminants and lipids and the protocol for keratin extraction was adapted from Ayutthaya *et al.* ,2015 [161]. The final keratin solution was quantified by DC method according to manufacture protocol and was concentrated to a final concentration around 30 mg/mL.

7.2.3 Hair overbleaching

Hair samples were subjected to five bleaching cycles using a commercial kit (Revlon Professional®) following the manufacture guidelines. After bleaching, the overbleached hair was washed with a commercial shampoo (Pantene® Basic) and dried overnight. The keratin was extracted from the overbleached hair using the protocol previously described.

7.2.4 Heating treatment of keratin solutions

7.2.4.1 Conventional heating

Conventional heating experiments were performed in a water bath (Grant Instruments, OLS200, Cambridge, United Kingdom) and the increase in the temperature during the heating process was measured using a digital thermometer placed at the center of the sample volume. The keratin solutions selected for the conventional heating had a concentration between 30-35 mg/mL and the treatments were performed for a total of 53 min: 23 min heating time plus 30 min at 85 °C, with constant agitation.

7.2.4.2 Ohmic heating

The ohmic heating of the keratin solutions was performed on a glass cylinder containing stainless steel electrodes at each edge, as described by Pereira *et al.*, 2010 [238]. The conductivity of the samples allowed studying the effect of the ohmic heating on the keratin films (conductivity between 20-120 μS/cm). The treatments were performed for a total of 53 min: 23 min of heating time plus 30 min at 85 °C. The heating kinetics and thermal profile were kept similar for ohmic heating and conventional heating treatments. The temperature was controlled by regulating the voltage output of a function generator (1 Hz–25 MHz and 1 to 10 V; Agilent 33220A, Penang, Malaysia) and then amplified on an amplifier system (Peavey CS3000, Meridian, MS, USA). The electric fields used ranged from 7 to 24 V/cm and applied as sine waves at a frequency of 25.000 Hz. Temperature was measured with a type K thermocouple (Omega Engineering, Inc., Stamford, CT, USA), connected to a data logger (USB-9161, National Instruments Corporation, Austin, TX, USA). During the treatments the samples were gently stirred (with a magnetically stirrer) to ensure homogeneity.

7.2.5 Preparation of keratin films

The keratin solutions treated by ohmic and conventional heating were used to produce the keratin films. A plasticizer agent (glycerol 1% (w/w)) was added to the keratin solutions and the samples were incubated in an ultrasound bath for 15 min followed by 10 min on ice. A volume corresponding to 29 mg of keratin was added to Teflon molding rings with 1 cm inner diameter [247]. The molding rings containing the protein solutions were placed on a vacuum chamber, at room temperature overnight, to minimize the appearance of bubbles on the films' surface. The keratin solutions were then dried at 40 °C for 24 h and then the temperature was increased to 110 °C for 4 h for the curing of the films. This curing was essential to remove the films from the mold and oxidize the disulphide bridges between keratin molecules, producing more stable films. The films were stored in an excicator until use. To study the effect of the type of keratin and the pH on the films' properties, we used keratin extracted from virgin and from overbleached hair with the pH of the keratin solutions being adjusted to 7, 8, 9 and 10 prior heating.

7.2.6 Keratin films' cross-linking induced by formaldehyde

To induce the cross-linking, the keratin films were treated with formaldehyde 37 wt.% in water. Initially, the keratin films were incubated overnight with formaldehyde vapor, at room temperature. Then, each film was incubated with 3 mL of formaldehyde 37 wt.% in water for 24 h. The films were then removed from the formaldehyde, dried with a filter paper and incubated at 50 °C until completely dried [248].

7.2.7 Characterization of keratin film-forming solutions

7.2.7.1 Formation of keratin aggregates

The average size of keratin aggregates formed during the heating treatments at 85 °C was measured by dynamic light scattering using a Zetasizer from Malvern Instruments, Nano-ZS. For this analysis, 50 μL of keratin solution was dispersed in 950 uL of ultrapure water and analyzed at 25 °C. The values for viscosity and refractive index were 0.8872 cP and 1.330, respectively. Each sample was measured in triplicate, and the results are presented as mean value ± standard deviation [95].

7.2.7.2 Determination of free thiol content

The amount of free thiol groups in the keratin samples was determined spectrophotometrically using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent). Briefly, 100 μL of keratin solution was added to 500 μL of 0.5 M phosphate buffer, pH 8.0, containing 10 μL of Ellman's reagent solution (4 mg/mL) in 0.5 M phosphate buffer, pH 8.0. The samples were mixed and incubated for 1 h at room temperature, in dark. Absorbance was then measured at a wavelength of 412 nm, and the amount of free thiol groups were determined taking in consideration a standard curve obtained using L-Cys [44].

7.2.8 Characterization of keratin films

7.2.8.1 Determination of free thiol content

The amount of free thiol groups in the keratin films was determined using the protocol previously described in section 7.2.7.2, with the keratin solutions being replaced by the keratin films and the solution volumes increased 10x.

7.2.8.2 Swelling ratio and water solubility

To measure the swelling ratio of the keratin films, three films of each condition were weighted and immersed in 10 mL of distilled water in petri dishes sealed with parafilm and placed in a thermostatic bath at 25 °C. After 1h of incubation time, the films were taken from the water, the excess of liquid on the surface was removed with a tissue paper and the films were weighted again. The swelling ratio (%) was calculated using the Equation VII.1:

Eq.1: $\text{Swelling ratio } (\%) = \frac{Weight_f - Weight_0}{Weight_0} \times 100$ (Equation VII.1)

, where $Weight_f$ corresponds to the film weight after 1h of incubation and $Weight_0$ correspond to the film's dry weight before immersion on distillated water.

The solubility of the films in water was determined after incubation of the keratin films in distillated water for 24 h. The films were removed from the water, dried at 100 °C for 24 h in a dry oven and then the solids content was measured. The water solubility (%) was determined using Equation VII.2:

Eq.2: $\text{Water solubility } (\%) = \frac{Weight_0 - Weight_f}{Weight_0} \times 100$ (Equation VII.2)

, where $Weight_f$ corresponds to the film weight after incubation and $Weight_0$ to the film's dry weight before immersion on distillated water. Both swelling rate and water solubility data corresponds to the average of three measurements [249].

7.2.8.3 Secondary structure analysis

Fourier Transform Infrared Spectroscopy (FTIR) measurements were recorded on a Perkin Elmer Spectrum-Two™ IR spectrometer with a deuterated triglycine sulfate detector and KBr beam splitter, coupled with an UATR (single reflection diamond, Perkin Elmer) accessory. Atmospheric CO_2/H_2O was automatically subtracted during spectra collection. Spectra were acquired at room temperature from 4000 cm^{-1} to 400 cm^{-1} and collected after 64 scans with a resolution of 4 cm^{-1}. Baseline subtraction, normalization, second derivative and band fitting were performed with OriginPro 9.0 (OriginLab, Northampton, MA).

7.2.8.4 Morphological observation

The samples were characterized using a desktop Scanning Electron Microscope (SEM) (Phenom ProX, Netherlands). All results were acquired using the ProSuite software. The keratin films samples were added to aluminium pin stubs with electrically conductive carbon adhesive tape (PELCO Tabs™). Before analysis, the samples were covered with 20Å of gold to improve samples electrical conductivity.

7.2.8.5 Thermal Gravimetric Analysis (TGA)

TGA of keratin films was performed with a TGA 4000 (Perkin Elmer, Waltham, Massachusetts, USA) using an alumina crucible with sample weights of 8-10 mg. The temperature calibration was done by Curie temperatures of reference materials: alumel, nickel and perkalloy at the same sample scanning rate. The measurements were performed from 25 to 800 °C at 20 °C/min under a nitrogen atmosphere (flow rate:

20 mL/min). The weight loss, in percentage, and its derivative were represented as function of temperature. The data were acquired using Pyris software (version 13).

7.2.9 Increase of films opacity using KP-Crystallin fusion proteins

For this assay, keratin films prepared at pH 9, and submitted to conventional and ohmic heating, were incubated with two crystallin-based fusion proteins (KP-Cryst Wt and KP-Cryst Mut) [227]. The increase of keratin films opacity was achieved by adding 100 uL of 1 mg/mL of KP-Cryst Wt/Mut solutions to the surface of the films. After application of the proteins, the films were incubated at room temperature until completely dried. This procedure was repeated 6 times. Films incubated with ultrapure water added to the surface were used as controls. Opacity was calculated through the ratio between reflectance and reflectivity (Chroma Meter CR-400, Konica Minolta, Japan).

7.2.10 Statistical analysis

Data are presented as average standard deviation (SD) of three assays. Statistical comparisons were performed by one-way ANOVA with GraphPad Prism 5.0 software (La Jolla, CA, USA). Tukey's post-hoc test was used to compare all the results between them, and Dunnett's test was used to compare the results with a specific control. A p-value ≤ 0.05 was considered to be statistically significant.

7.3 Results and Discussion

7.3.1 Characterization of keratin film-forming solutions

7.3.1.1 Proteins' aggregation profiles

The denaturation of proteins involves the breaking of individual covalent bonds between the atoms of the polypeptide backbone. This process is usually accompanied by the aggregation, coagulation or gelation of the proteins' molecules. The conversion between the folded and unfolded state of proteins can be induced by changes in temperature, pressure, pH, chemical denaturants or solvent nature [250].

When the keratin solutions were heated at 85 °C for 30 min, a change was observed on the average hydrodynamic diameter of the protein particles' size, independently on the type of heating and type of keratin – **Table VII.1.** For the keratin obtained from overbleached (OB) hair there was an increase on particles size regardless of the pH (data not shown). Nevertheless, for the keratin obtained from the virgin hair, the pH of the solutions governed the proteins aggregation profiles, with a decrease of particles size being observed for pH values between 7 and 9, while an increase was observed for pH 10. Alkaline pH values are described to contribute to the disruption of Cys in the keratin molecules, and favor the occurrence of some side reactions as the hydrolysis of peptide and amide bonds, and the formation of

new cross-links as lanthionine or lysinoalanine [251]. These factors could benefit the interaction between different molecules of keratin proteins at higher pH values, increasing the final aggregates size.

Regarding the variation of the keratin solution polydispersity index (PDI), there was a decrease in the PDI after heating for both types of keratin and for all the pH values. Before heating, the keratin solutions presented a polymodal distribution, however, after 30 min at 85 °C, the particles population becomes less polydisperse, and in some conditions the size distribution became monomodal (represented by 100% of percentage population in **Table VII.1**).

Table VII.1 - Characterization of keratin film-forming solutions in terms of average particle size and polydispersity index (PDI), before and after conventional and ohmic heating. The keratin solutions were heated for 30 minutes at 85 °C.

Keratin Hair Source	pH		Conventional heating		Ohmic Heating	
		Incubation time (min)	Size (nm)	PDI[b]	Size (nm)	PDI[b]
Virgin	7	0	565.5 (58.2%)[a]	0.598	523.0 (50.5%)[a]	0.506
Virgin	7	30	331.9 (78.6%)[a]	0.535	213.3 (75.4%)[a]	0.432
Virgin	8	0	1348 (69.2%)[a]	1.000	1987 (44.1%)[a]	1.000
Virgin	8	30	486.7 (100%)[a]	0.263	861.5 (71.7%)[a]	0.879
Virgin	9	0	735.5 (85.8%)[a]	0.510	1138 (47.3%)[a]	0.551
Virgin	9	30	547.7 (92.6%)[a]	0.402	957.5 (75.5%)[a]	0.424
Virgin	10	0	613.3 (71%)[a]	0.867	714.4 (73.3%)[a]	0.737
Virgin	10	30	780.2 (92%)[a]	0.462	776.9 (90.8%)[a]	0.606
OB[c]	9	0	623.7 (88.1%)[a]	0.600	623.7 (88.1%)[a]	0.600
OB[c]	9	30	979.2 (100%)[a]	0.500	700.6 (100%)[a]	0.481

[a] percentage of population corresponding to a specific size; [b] polydispersity index; [c] only reported the results for pH9, data is not shown for the other pH values.

Analyzing the effect of pH on the protein particles' size after heating, an increase in the aggregates size was observed with the increase in the pH of the keratin solutions. This behavior could be due to the fact that higher pH leads to an increase in protein charge, which could stabilize the aggregates by the formation of specific charge interactions like salt bridges or ion pairing [252]. Moreover, the differences in keratin samples' population observed on **Table VII.1** could also be related with keratin solution's conductivity [238]. An increase in solution's conductivity was observed with increasing pH of the keratin solutions. Also, the keratin solution extracted from overbleached hair, presented a very low conductivity when compared to the keratin extracted from virgin hair. The differences in solution conductivity could be

due to the different nature of the electrolyte (keratin protein vs degraded keratin), the protein hydration and the aggregates shapes [253], as well as the protein ions present in the solution [254]. These differences in the conductivity may also explain the observed variation of the particles size, since different electric fields were applied to the keratin solutions to achieve the same temperature profiles.

7.3.1.2 Free thiol determination

Keratin is a unique protein because it contains a large amount of the amino acid Cys when compared to other proteins. As referred previously, cysteine is a sulfur-containing amino acid and can form sulfur–sulfur (S–S) cystine bonds with other intra- or intermolecular cysteine molecules. Free thiol groups before and after heating were quantified using Ellman's reagent and are represented on **Table VII.2**.

Taking all data in consideration, it was observed, for both types of heating, an increase in the keratin free thiol content after heating. This was expected since thermal processing at denaturation temperatures involves changes in protein conformation that can convert masked SH groups into free SH groups, thus becoming available for the formation of covalent disulphide bonds [236] or reaction with the Ellman's reagent.

After incubation at 85 °C, significant differences in thiols' concentration were verified between the conventional and the ohmic heating for the keratin solutions prepared from virgin hair (p-value ≤ 0.01). For these solutions, a higher increase in thiols concentration was verified for the conventional heating when compared with the ohmic heating for all the tested pH values. However, when the keratin solutions were prepared from OB hair, no significant differences (p-value > 0.05) between the two types of heating were verified in the thiols' concentration after heating. The higher reactivity of SH groups for the solutions submitted to conventional heating, was previously described by Pereira *et al.,* 2010, using whey solutions to obtain protein films [238]. The results here obtained can be explained by the differences between the conventional and the ohmic heating, suggesting that the structural transitions and the keratin unfolding were more favored during the conventional heating, possibly due to more aggressive thermal effects [236,238]. Denaturation kinetics are primarily driven by covalent bonding of unfolded proteins, which in turn is closely related to the availability and reactivity of the free SH groups of unfolded proteins [236], being these more pronounced for the conventional heating.

Analyzing the effect of pH on thiols concentration, the same trend was observed for the two types of heating, with a higher sensitivity to variations on pH being obtained for the conventional heating. The higher sensitivity for the conventional heating could be related with the effect of the total net charges or local amino acids charge on the final protein structure, like the localization of free SH groups on the protein surface. For example, the effect of pH on Cys make it changes its net charge from neutral to

negative between pH 7 and pH 8, so its responsiveness and reactivity with other groups is affected by the variations on pH [255].

Table VII.2 - Effect of conventional and ohmic heating on the thiols' concentration of keratin film-forming solutions, after treatments at 85°C for 30 minutes

Keratin Hair Source	pH	Incubation time (min)	Conventional heating		Ohmic Heating	
			Thiols (mM)	Thiols increase (%)	Thiols (mM)	Thiols increase (%)
Virgin	7	0	0.431±0.002	-	0.351±0.015	-
Virgin	7	30	0.705±0.013	64.69	0.549±0.018	56.45
Virgin	8	0	0.454±0.01	-	0.437±0.015	-
Virgin	8	30	0.730±0.012	60.62	0.586±0.015	34.02
Virgin	9	0	0.666±0.019	-	0.516±0.009	-
Virgin	9	30	0.820±0.001	23.12	0.598±0.007	15.98
Virgin	10	0	0.334±0.0028		0.492±0.017	
Virgin	10	30	0.811±0.001	59.12	0.869±0.029	43.44
OB	9	0	0.0859±0.001		0.086±0.001	
OB	9	30	0.145±0.001	69.26	0.150±0.008	74.39

7.3.2 Characterization of Keratin Films

7.3.2.1 Free thiol determination

After keratin films preparation and insolubilization using the formaldehyde, the concentration of free thiols on the films was determined, with the results for the films prepared at pH 9 being presented in **Table VII.3**. Comparing with the keratin solutions before casting (**Table VII.2**), it was observed that the free thiol concentration on the keratin films is much lower. These lower values can be attributed to the films curing at 110 °C for 4 h, a process essential to oxidize the disulphide bridges between keratin molecules, producing more stable films with improved properties [247]. Also, the free thiols concentration is affected by films insolubilization with the formaldehyde that promote Cys cross-linking, also increasing the films stability [248]. The same behavior was observed for the keratin films prepared at pH 7,8 and 10, with a decrease in the thiols' concentration of films when compared to the keratin film-forming solution (data not shown).

Table VII.3 - Effect of conventional and ohmic heating on the thiols' concentration of keratin films*, after curing at 110 °C for 4 h and insolubilization with formaldehyde.

Keratin Hair Source	Thiols (mM)	
	Conventional Heating	Ohmic Heating
Virgin	0.0253±0.000557^{ac}	0.0187±0.00156^{ad}
OB	0.0108±0.000457^{bc}	0.00624±0.000446^{bd}

* the films were prepared with keratin extracted from virgin or overbleached hair and the pH adjusted to 9; The data with the same superscripts (a, b, c and d) are significantly different between them ($p < 0.05$).

7.3.2.2 Swelling ratio and water solubility

To understand the effect of heating on the ability of the keratin films to swell and/or degrade when in contact with water, we evaluated the swelling ratio and water solubility of the keratin films obtained from the keratin solutions heated by conventional or ohmic heating **(Figure VII.1** and **Figure VII.2).**

During the keratin films preparation, it was necessary to add glycerol to the keratin film-forming solutions to avoid films brittleness after drying. Glycerol was able to improve films flexibility by decreasing the inter and intramolecular attractive forces and increase chain mobility [249].

Ideally, the regeneration of disulphide bonds in the keratin films during the curing at 110 °C should be sufficient to impart water insolubility and good mechanical properties to the resultant films. However, as represented on **Figure VII.1** and **Figure VII.2**, it was observed that the insolubilization by temperature was not enough with regard to keratin films' water solubility and swelling ratio. The films prepared with keratin from virgin hair adjusted to pH 9 and submitted to conventional heating, presented water solubility and swelling ration values of 26.21±0.078% and 597.12±68.52%, respectively. To promote the insolubilization of the keratin films they were incubated with formaldehyde (vapor and liquid). Formaldehyde is described by many authors as the most effective cross-linker in reducing water solubility [256]. Formaldehyde have access to some reactive groups in amino acids like Cys, Hys and Lys, resulting in the formation of covalent bonds, what leads to an increase in the degree of reticulation and greater polymeric matrix structuring. As a consequence, a reduction in chain mobility is verified, producing more cohesive protein chains and more resistant films, resulting in films with lower water permeability [256].

The effect of insolubilization on the water solubility and swelling ratio of the keratin films, promoted by the formaldehyde, was evident for all the pH values, type of keratin and heating method. Regarding the water solubility, and comparing with the films before insolubilization, there was a decrease between 9-30% for the keratin films obtained by conventional heating and between 25-40% for the films obtained by

ohmic heating. The significant decrease on the water solubility was dependent on the pH (higher decrease for lower pH values) and on the heating method (higher decrease for the ohmic heating). The lower improvement observed for the higher pHs could be related with differences in the reactivity of the Cys groups available to interact with formaldehyde and to form stable linkages [255]. This amino acid shows a high responsiveness to changes in physiological states and environmental conditions which affect its ability to interact with other proteins or charged molecules [255].

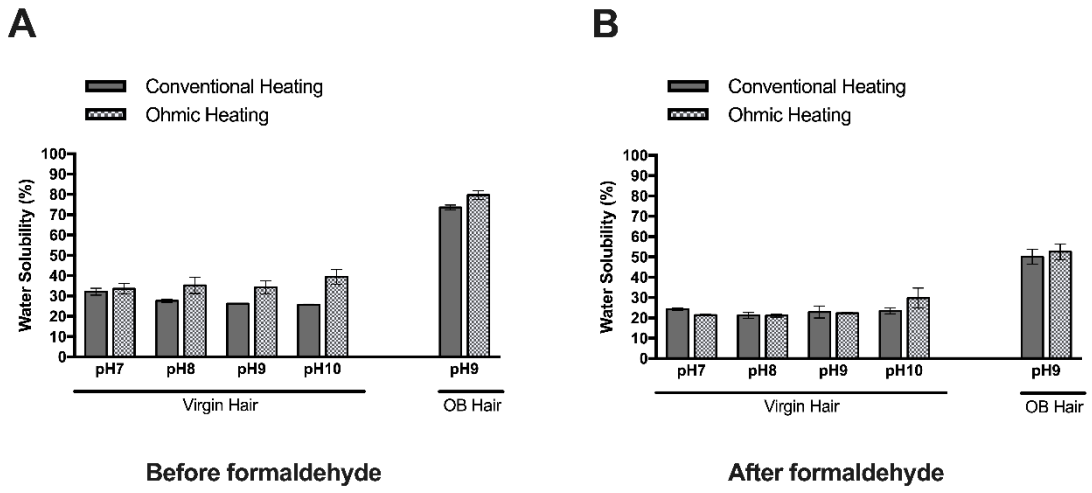


Figure VII.1 - Effect of pH and heating treatment (conventional or ohmic heating) on the water solubility of keratin films before (A) and after (B) formaldehyde treatment. The films were incubated in distilled water at 25 °C for 24 h and then dried at 100 °C. The data represent the mean ± SD from three independent experiments. Data were analyzed by one-way ANOVA.

The higher decrease on water solubility observed for the ohmic heating, could be related with keratin structural transitions and unfolding caused by the ohmic heating which can affect in a different way the availability and reactivity of the free SH groups from the keratin protein [236]. The differences in the availability and reactivity of the Cys groups can increase the cross-linking degree obtained from the reaction of formaldehyde with the free Cys residues on the keratin, what may also affect the alignment of the polymeric keratin structure resulting in a decrease in the films' water solubility [257]. The aggregates sizes of keratin film-forming solution could also affect the water solubility results. Ohmic heating was described to interfere with the protein aggregate pathways like reducing the aggregates size or change the aggregates shape [258], which could facilitate the keratin interaction with formaldehyde and increase the films' reticulation.

Analyzing the effect of formaldehyde in the swelling ratio of the keratin films, significant differences were observed for all the tested conditions (pH, type of keratin and heating method). Consistent with the water solubility, the swelling ratio is an indication of the level of protein cross-linking in protein-based films. Relatively to the films prepared with keratin from virgin hair and submitted to conventional or ohmic heating, it was verified a decrease between 84-95% in the swelling ratio while for the films prepared with keratin from OB hair, the effect of formaldehyde was more notorious, with a decrease around 97%. The decrease in films swelling was already expected since formaldehyde reacts with the protein amino groups, so they are not available to bind to water by hydrogen bonding. Also, due to the formation of cross-links in the protein structure, hydrophilic sites along protein chains become less exposed and, therefore, less accessible to water molecules [259].

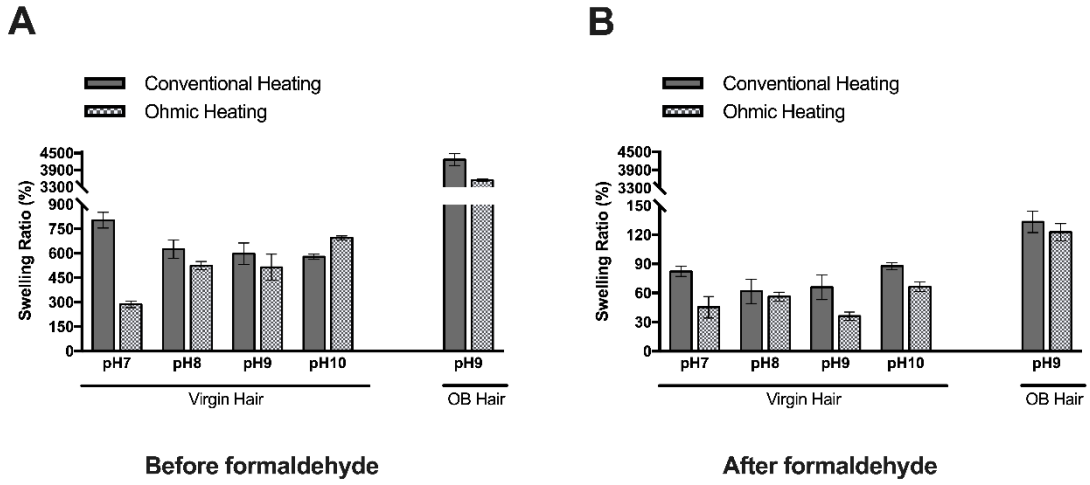


Figure VII.2 - Effect of pH and heating treatment (conventional or ohmic heating) on the swelling ratio of keratin films before (A) and after (B) formaldehyde treatment; the films were incubated in distilled water at 25 °C for 1h and then weighed. The data represent the mean ± SD from three independent experiments. Data were analyzed by one-way ANOVA.

Despite no differences were observed for the water solubility, the films prepared by conventional and ohmic heating after insolubilization presented significative differences (p-value≤0.001) in the swelling ratio when prepared from keratin extracted from virgin hair and adjusted to pH 7 and pH 9. These results could be an indication that the main differences between the films submitted to conventional or ohmic heating are related to the orientation of keratin molecules and the different proportion of reactive groups in the surface of the keratin films, available to cross-link. Due to the presence of moderate electric fields, ohmic heating can influence the denaturation and aggregation mechanisms of keratin proteins, affecting keratin films properties [238]. It was also demonstrated by Pereira *et al.,* (2010), for whey protein

solutions, that ohmic heating induces less protein denaturation and less activation of free SH groups, affecting the protein secondary structure in the films network and, consequently, the films properties. The reorganization of hydrogen bonds, hydrophobic interactions and ionic bonds are also responsible for some conformational disturbances on the tertiary structure of proteins during ohmic heating [236,238]. All these variations between ohmic and conventional heating could also justify the differences in the water solubility and swelling ratio results obtained for the keratin films.

Relatively to the effect of pH of keratin film-forming solutions on the properties of keratin films prepared by conventional heating and ohmic heating, different profiles were observed before the insolubilization with formaldehyde. For the films prepared by conventional heating there was a decrease on the water solubility and swelling ratio, increasing the pH of keratin solutions. For the films prepared by ohmic heating an increase of water solubility and swelling ratio was observed increasing the pH of keratin solutions. However, after insolubilization the differences observed for both heating methods became less evident.

From the results we can infer that the changes in keratin films properties after pH adjustment are related to physicochemical characteristics of keratin protein like surface hydrophobicity, conformation and net charge [252]. Moreover, the opposite tendencies between the films prepared by conventional heating and ohmic heating, could be related with different patterns of denaturation, interactions and aggregation of the keratin protein, which affect in a different way the inter- and intramolecular bounds and the water molecules interaction with the keratin films. It seems that the presence of moderate electric fields might have promoted an increase in film's surface hydrophilicity with the increase of pH [238], justifying the swelling results for the ohmic heating. In the same way, for the conventional heating, it seems that the increasing the pH promoted an increase of the amino reactive groups present at the surface of the keratin molecules, resulting in a higher percentage of cross-linking and a consequent decrease in water solubility and swelling ratio of the keratin films [257].

7.3.2.3 Secondary structure analysis

FTIR was used to evaluate structural changes in the keratin films prepared at different pH (7, 8, 9 or 10) and by different heating methods (conventional or ohmic heating) - **Figure VII.3**. As depicted in **Figure VII.3**, the FTIR spectra of the keratin films shows the main characteristic bands associated to the vibrational modes of peptide bonds in proteins namely, amide I (1700-1600 cm^{-1}, C=O stretching vibration), amide II (1600-1500 cm^{-1}, N-H bending and C-N stretching vibrations) and amide III (1200-1300 cm^{-1}, in phase combination of C–N stretching and N-H bending vibrations) [260,261]. For all

samples, the spectra present a peak with maxima at 1647 cm^{-1} for amide I, 1538 cm^{-1} for amide II, and 1215 cm^{-1} for amide III. Additionally, the broad band at 3600-3000 cm^{-1} corresponds to amide A and was assigned to O-H and N-H stretching vibrations [260]. The peaks with maxima at 2919 cm^{-1} and 2851 cm^{-1} were assigned to C-H stretching vibrations [262] and the two absorption bands with maxima at 1455 cm^{-1} and 1411 cm^{-1} were assigned to C-H bending vibrations [263]. Finally, the absorption band with maximum at 1024 cm^{-1} was attributed to C-O stretching vibration (related to alcohol functional group) and results from the presence of glycerol in the film samples [264]. Noteworthy, the absence of an absorption band at 1745 cm^{-1}, which is due to lipid ester carbonyl vibrations [265], indicates that lipids were effectively removed during the preparation of hair samples prior keratin extraction. The effect of ohmic heating was also evaluated by FTIR in order to determine if the heating source would induce any structural changes in the keratin films (**Figure VII.3 A**). In this case, all films presented a similar spectral as those obtained for the films submitted to the conventional heating, independently of the pH used for film production.

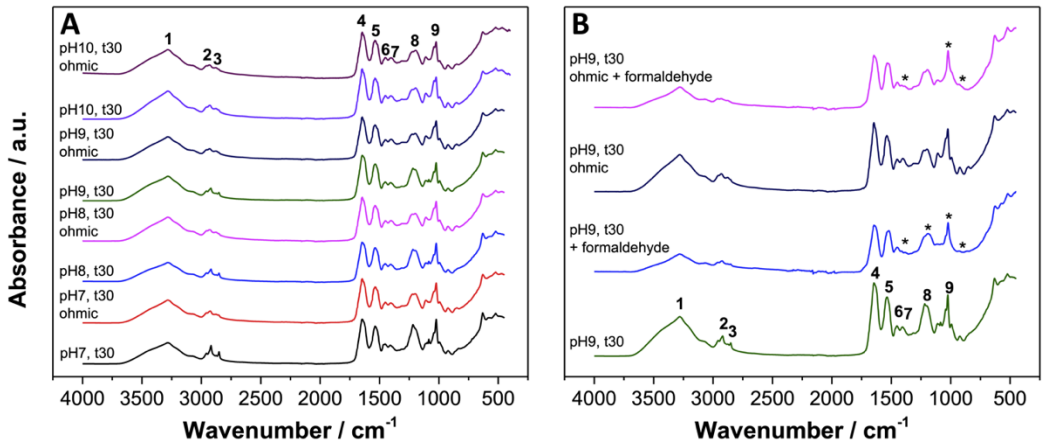


Figure VII.3 - A) FTIR spectra of keratin films produced by keratin solutions at different pHs and prepared by conventional heating ('pH7, t30'; 'pH8, t30'; 'pH9, t30'; 'pH10, t30') versus ohmic heating ('pH7, t30 ohmic'; 'pH8, t30 ohmic'; 'pH9, t30 ohmic'; 'pH10, t30 ohmic'); B) Effect of formaldehyde cross-linking on keratin films prepared at pH9 and submitted to conventional and ohmic heating. (1) amide A at 3600-3000 cm^{-1}; (2, 3) C-H stretching vibrations at 2919 cm^{-1} and 2851 cm^{-1}; (4) amide I at 1647 cm^{-1}; (5) amide II at 1538 cm^{-1}; (6, 7) C-H bending vibrations at 1455 cm^{-1} and 1411 cm^{-1}; (8) amide III at 1215 cm^{-1} and (9) C-O stretching vibration at 1024 cm^{-1}; Bands marked with * represent changes observed during solvent casting with addition of formaldehyde.

In order to improve films cohesion and resistance, keratin films were treated with formaldehyde, an effective cross-linker to protein films [256,259,266]. Addition of formaldehyde during solvent casting demonstrated to have minor effects at the structural level with changes being observed at 1411 cm^{-1} (C-H bending), 1215 cm^{-1} (amide III), 1024 cm^{-1} (C-O stretching) and 921 cm^{-1} (C-H out-of-plane deformation of alkene group $RCH=CH_2$) **(Figure VII.3 B).**

The position of the amide bands I and II are indicative of the secondary structure conformations and are commonly used to identify the protein secondary structure [267]. Accordingly, the peaks at 1647 cm^{-1} (amide I) and 1538 cm^{-1} (amide II) are indicative of the α-helix conformation [268]. However, the presence of shoulder bands at 1626 cm^{-1} and 1517 cm^{-1} also indicates some degree of β-sheet secondary structures after incubation with formaldehyde supporting the partial insolubilization of the keratin films [267]. This suggests that the keratin films are mainly characterized by α-helical conformation, independently of the pH and source of heating treatment, and that there is some degree of conversion of the α-helical into β-sheet after insolubilization with formaldehyde.

7.3.2.4 Thermal stability of keratin films

In order to study the thermal stability of the keratin films prepared by different heating methods, the thermogravimetric analysis of the films was performed between 25 and 800 °C – **Figure VII.4**. Comparing the films prepared by conventional heating with the films prepared by ohmic heating, the major differences were mainly noticed for temperatures higher than 500 °C. Initially it was observed, a weight loss between 5.1-8.1% for the conventional heating and 4.6-5.4% for the ohmic heating, related with the loss of bound water associated with the keratin films, for temperatures below 150 °C [232]. For temperatures between 150-500 °C there was a weight loss around 60-70% for the films prepared by the two heating methods. This weight loss was most likely associated with the destruction of lateral chains of the keratin protein molecules in the protein-based films [232]. At the end of the temperature scan, the films retained about 4.6-16.9% of their initial weight, being this percentage associated to the carbon molecules in the protein and structures that did not decomposed even at 800 °C, which remained as ash [232]. Also, all the keratin films, prepared by conventional or ohmic heating, presented various decomposition peaks in the analyzed temperature range due to the presence of proteins with different sizes, what is a characteristic of the keratin proteins [233].

In the thermograms for the keratin films prepared by conventional heating, it was determined a temperature between 207-210 °C corresponding to initial degradation of the films. This value decreased to 187-206 °C for the keratin films prepared by ohmic heating, what could be an indication of the lower

stability for these films as well as a lower amount of crystalline-sheet structures [230]. For these films, less energy is needed to start to disintegrate the protein matrix. Also, the multi-stage degradation profiles presented a main peak around 300-310 °C, corresponding to the maximum degradation rate, and a pronounced shoulder around 230-235 °C. Both peaks correspond to the disorganization and destruction of the crystalline regions of the keratin protein [232]. The intensity of this shoulder around 230-235 °C was less intense or inexistent for the keratin films prepared at pH 7, confirming the effect of pH on the formation of structures in the films mainly due to inter- and intramolecular bounds [232].

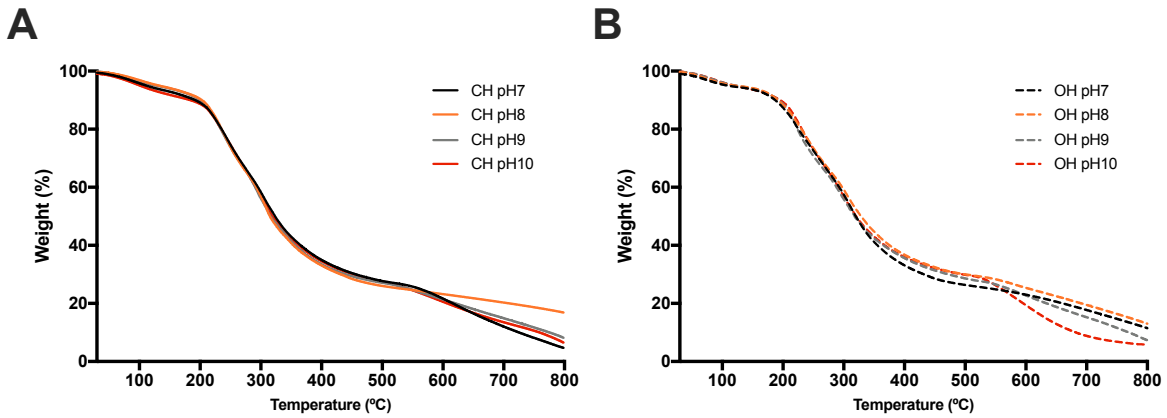


Figure VII.4 - Effect of pH of the keratin film-forming solutions on the thermal stability of keratin films prepared by (A) conventional heating (CH) or (B) ohmic heating (OH).

7.3.2.5 Morphological observation

The morphology of the keratin films prepared using keratin extracted from virgin or overbleached hair, adjusted to pH 9, and heated using the conventional and the ohmic heating were examined using SEM and the micrographs are represented on **Figure VII.5**.

Through the analysis of the SEM micrographs it was possible to verify that all the keratin films (Virgin and OB Hair) submitted to the different heating conditions form a compact and continuous structure and no phase separation between the keratin proteins and the glycerol was observed. The keratin films prepared by conventional heating shows more homogeneous surfaces and are more compact when comparing with the same films prepared by ohmic heating (Ohmic Heating/Virgin Hair: 75.14±2.65 μm versus Conventional Heating/Virgin Hair: 51.59±2.36 μm and Ohmic Heating/OB Hair: 74.43±1.52 μm versus Conventional Heating/OB Hair: 38.28±2.81 μm).

The micrographs of the keratin films after cross-linking with formaldehyde revealed the appearance of small aggregates at the surface of the films which are most likely related with the evaporation of the cross-linker. This evaporation effect was more marked for the keratin films prepared using keratin extracted

from virgin hair and submitted to ohmic heating, as they presented a surface with several wrinkle-like structures. These differences in films surfaces caused by cross-linking with formaldehyde were already reported by Carvalho and Grosso, 2006, and are caused by a cohesive arrangement of the polymeric matrix [266].

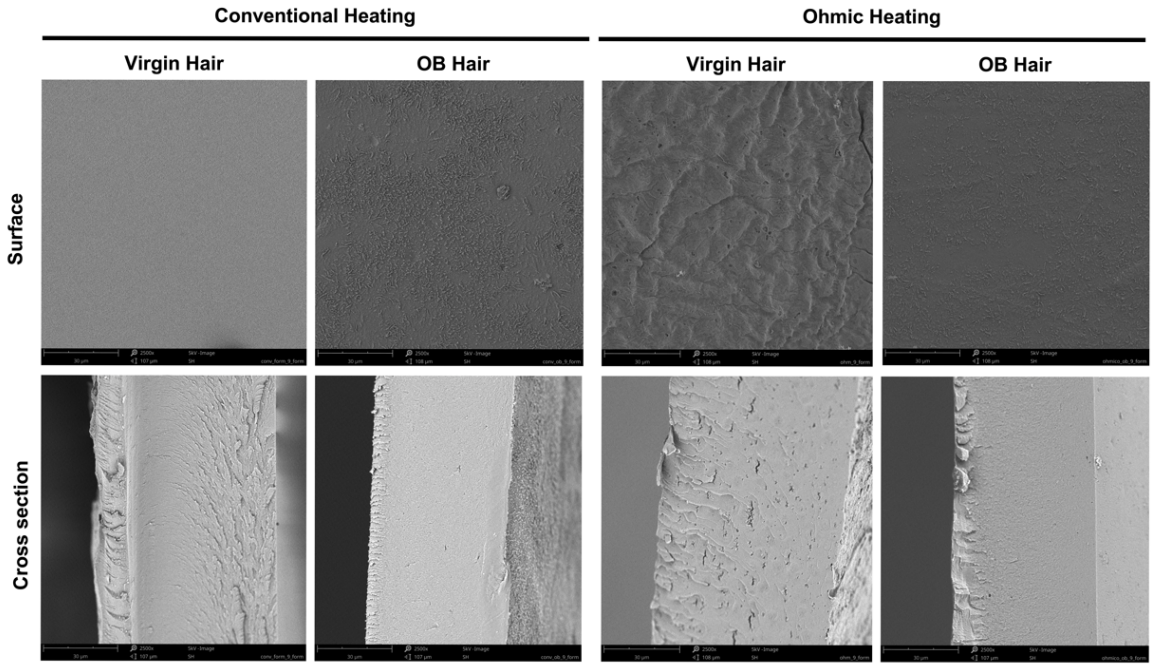


Figure VII.5 - SEM micrographs of keratin films, pH 9, treated with formaldehyde, when submitted to conventional and ohmic heating. Top section represents the keratin film surface and down section the keratin film cross-section that was mechanically fractured to obtain a view of the inner structure. Virgin Hair: keratin extracted from virgin hair; OB Hair: keratin extracted from overbleached hair.

7.3.2.6 Opacity using a fusion protein

Evaluating cosmetic treatments which modify some physical features of hair fibers can be challenging due to the intrinsic properties of human hair like size and shape, and also due to hairs' great heterogeneity. A fusion protein composed by human eye γD-crystallin and a keratin-based peptide (KP) was used to modify the opacity of hair fibers as a principle to develop new hair coloration strategies– **Figure VII.6**. Two fusion proteins were used: a wild type form (KP-Cryst Wt) based on the sequence human eye γD-crystallin and a mutant form (KP-Cryst Mut). The mutant form was constructed by the substitution of three arginine residues by three Cys, on the wild-type crystallin sequence, what would increase the formation of protein inter- and intramolecular bonds and its interaction with the hair keratin [227]. Proteins with Greek-key motifs like crystalline were already described to have the ability to form a coating around different structures, and to form opaque structures denominated cataracts when the proteins start

to unfold, promoting protein aggregation [227]. The combination of both factors, make the crystallin proteins perfect models to study fibers opacity caused by the application of these proteins.

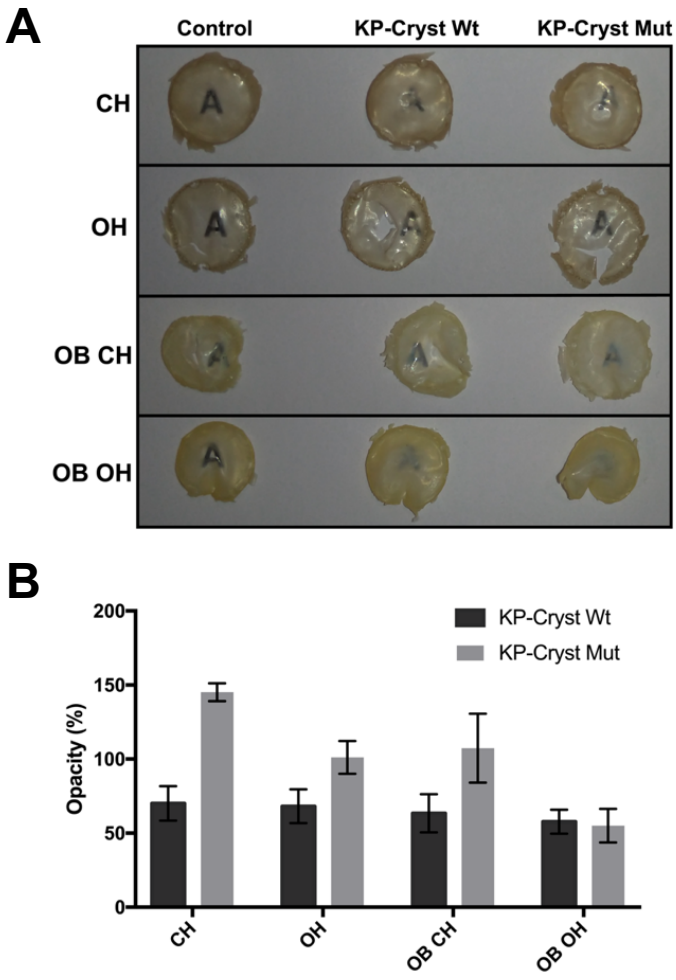


Figure VII.6 - A) Effect of KP-Cryst Wt and KP-Cryst Mut proteins application on keratin films opacity. A total of 0.6 mg of protein were applied to the keratin films. Ultra-pure water was applied in the same volume as the protein solution, for the control samples; B) Mean values of keratin films opacity after incubation with KP-Cryst Wt and KP-Cryst Mut proteins. The increasing percentages were calculated in comparison with the control samples. CH – conventional heating; OH – ohmic heating; OB – keratin extracted from Overbleached Hair.

A qualitative and quantitative analysis of keratin films opacity is shown in **Figure VII.6**, where it is possible to observe some changes in films opacity after application of KP-Cryst Wt and KP-Cryst Mut fusion proteins. Relatively to the KP-Cryst Wt, no significative differences were verified for the films prepared with keratin extracted from virgin or OB hair, neither the ones prepared by conventional or ohmic heating. However, a different behavior was observed for the films where KP-Cryst Mut was applied. Higher opacity

values were observed for films treated with KP-Cryst Mut protein, with a higher opacity degree obtained for the keratin films produced with keratin extracted from virgin hair and submitted to conventional heating. The higher opacity degree obtained for KP-Cryst Mut films could be due to structural characteristics of these protein, where the mutations were performed to induce the protein aggregation, mimicking the formation of cataracts in eye. Also, KP-Cryst Mut has three additional Cys which favors the binding of these molecules to keratin proteins, increasing the protein adhesion the films' surface [227]. Based on these results, we can assume that the keratin films can be used as models to determine the effect of cosmetic formulations specially developed to change the opacity of the hair fibers.

7.4 Conclusions

The heating of protein solutions influenced the unfolding and aggregation patterns of the proteins, affecting the film's features. Considering the ohmic heating, it was demonstrated that this heating method affected differentially the denaturation of keratin proteins, resulting in films with different properties comparatively to the ones prepared by conventional heating.

The application of KP-Cryst proteins over the keratin films, demonstrated the films' ability to act as a model to substitute the use of hair on the testing of new hair cosmetic formulations. With this work, it was demonstrated that both conventional and ohmic heating techniques are able to be used in the production of keratin films with adjusted properties according to the heating method. The keratin films show potential to be applied in the validation of hair cosmetic formulations as substitutes of hair fibers.

Chapter VIII

The Impact of Keratin-based Systems and Keratin-fusion Proteins in Hair Cosmetic Science Conclusions and Future Perspectives

Chapter VIII – The Impact of Keratin-based Systems and Keratin-fusion Proteins in Hair Cosmetic Science Conclusions and Future Perspectives

Abstract

Proteins and proteins hydrolysates have been widely used as ingredients in hair cosmetic formulations. Great focus has been given to the keratins due to their high cystine content and ability to interact with the hair fibers. This review is focused on the impact of formulations with keratin and keratin-based peptides for the development of new hair products. The potential of fusion proteins with a keratin-based peptide, designed to impart selected properties to the hair like color and thermal stability, is also explored. The outstanding results support the potential of the new keratin-based formulations to be incorporated in advanced multifunctional hair cosmetic products.

This chapter is based on the following scientific publication:

Ana Tinoco, Artur Cavaco-Paulo and Artur Ribeiro, The Impact of Keratin-based Systems and Keratin-fusion Proteins in Hair Cosmetic Science. To be submitted.

8.1 Hair cosmetics

Hair is an important aspect of body image and plays a key role in men's and women's confidence and self-image. Taking advantage of the great number of cosmetic products and cosmetic procedures available, we can easily change the length, color, shape, volume, shine, touch and gloss of hair [1,2]. However, the efforts to improve hair appearance may expose the hair to damage inflicted by the use of chemical solutions and styling procedures, which can irreversibly change the fibers properties. Moreover, daily hair care routines and environmental insults are also responsible for the damage and weakening of the hair fibers [3,48,269]. To respond to these challenges, there is a demand on hair cosmetic industry to develop new hair cosmetic formulations. The new products are expected to impart the desired features to the hair without compromising its properties, and to repair and prevent further deterioration.

Nowadays, cosmetic products are an essential part of most peoples' life, however the use of cosmetic is not exclusive of modern days. There are several reports of the use of cosmetics to enhance beauty and in religious rituals in ancient civilizations like Egypt, Greece and Rome [270,271]. Considering the cosmetic products developed for hair, they can be classified as shampoos, conditioners, colorants and hair styling preparations for temporary or permanent waving/straightening [17]. Cosmetic industries are now taking into consideration the growing interests of the worlds' society in the use of responsible, sustainable and eco-friendly products. These three pillars are nowadays the main driving forces for the need of constant innovation in cosmetic science research. Protein based formulations are seen as an exceptional solution since proteins can impart several functions to the hair while protecting it, and they can be obtained from several sources using green approaches.

8.1.1 Keratin based-formulations for hair cosmetic products

Cosmetic formulations containing proteins, protein hydrolysates and peptides, are extensively used in today's society. Proteins are now a common ingredient in cosmetic products, primarily due to their ability to bind water and the potential to be used as conditioning agents [37]. Also, the proteins capacity to prevent hair damage may be related with their amphoteric and buffering properties [40,272]. Keratin, a group of cysteine-rich structural proteins, are the main constituent of the human hair fiber [7]. Due to the high cysteine content and the capacity to interact with the hair fiber, keratin proteins and their hydrolysates have been widely used by the cosmetic industry in cosmetic products for hair application [45]. Keratin proteins can be obtained from different sources including feathers, hair, wool, nails and animal horns [273].

Barba et al., 2010, tested the effect of an intact keratin and a keratin-peptide with low molecular weight, both extracted from wool, on human hair fibers. It was verified that both keratin and keratin-peptide were able to restore fiber surface hydrophobicity, improve hair moisture and fibers' mechanical properties [42,274]. Keratin extracted from wool was also tested by Roddick-Lanzilotta et al., 2007, to evaluate its effect on human hair. The selected keratins comprised an intact protein fraction with a molecular weight around 55kDa and with reversibly protective cystine groups, responsible to permanently attach the proteins to the hair fibers. The treatment with these proteins was able to protect and restore the hair from the damage caused by the environment and chemical treatments. Positive effects on hair color protection using this formulation were also reported [208]. Savaides *et al.*, in US 2014/0261518 A1, developed a method for hair straightening where a keratin mixture was incorporated into the formulation. The keratin mixture was constituted by hydrolyzed keratins and oxidizing agents. This formulation provided long lasting curl reduction and styling results, even after grooming and washing of the hair [54]. Yoshioka and Kamimura, in US 4,279,996, also studied the effect of keratin hydrolysates into the modulation of hair shape. Water-soluble keratin hydrolysate having at least two mercapto groups per molecule, when applied to the hair, demonstrated to have an excellent waving and setting effect without the common fiber damage [62]. Keratin hydrolysates obtained from feathers were also applied on hair fibers, in conjugation with heat, and demonstrated to be effective on hair hydration and hair brightness improvement [76].

The main problems associated with keratin and keratin hydrolysates extracted from wool and feathers, is the lack of complete sequence identity with human hair keratin proteins, which might affect the final efficiency of these compounds [45]. In this perspective, keratin and keratin associated proteins (KAPs) extracted from human hair, their hydrolysates or peptides based on the keratins and KAPs sequences, might be used to improve the cosmetic formulations performance.

Basit et al., 2018, tested the effect of K31 keratin on the properties of virgin and bleached hair. K31 protein is the major component of the hair cortex, and shows a maximum homology with the keratins from hair, increasing the binding efficiency. The application of K31 protein increased bleached hair smoothness, diameter and improved the fibers' mechanical properties. It was also verified positive effects on the capacity of these protein to straight curly hair [45].

Keratin peptides derived from the human genome were designed to interact with the hair keratin and were tested on human hair by Cruz et al., 2017 [51]. These cysteine-containing peptides were based on the sequence of human hair keratin and keratin-associated proteins and demonstrated to be effective as hair straightening modulators. An effect in the fibers' mechanical properties was also observed after fibers treatment with each peptide, with an improvement in fibers' tensile strength and elasticity [51].

A peptide based on keratin type II cuticular Hb5, designated as KP, was firstly reported by Fernandes et al., 2012. The KP peptide was able to improve the mechanical and thermal properties of weakened relaxed hair [275]. When hair samples were placed in high relative humidity conditions, the KP also demonstrated to be an excellent agent in decreasing the volume change caused by the high humidity [276].

Taking advantage of the KP properties and proven benefits when applied to hair, this peptide was conjugated with other proteins, to design fusion proteins, with more than one function. The new fusion proteins explored the high affinity of KP towards hair, increasing the binding efficiency of the construct; together with the color of a chromogenic protein (KP-UM) [139] or the thermal stability of human eye γD-crystallin (KP-Cryst) [227].

Hair coloration is one of the most common cosmetic procedures, and is used for styling and to mask aging signs like the appearance of white hairs. However, the use of dyes to color hair has many disadvantages like hair damage, allergic reactions, skin irritation and environmental pollution. Also, bleaching procedures, normally applied before hair dyeing, are very aggressive to the hair, resulting in the loss of hair's mechanical properties, loss of hair smoothness, shine, texture and increase of hair porosity. The increasing concern of society about the environment and the desire to have a more natural lifestyle, stimulated the research on natural compounds for hair dyeing and for the substitution of the synthetic organic compounds currently used. To address this problem, we designed a fusion chromogenic protein constituted by the KP peptide and the ultramarine protein (UM). The UM is a monomeric and non-fluorescent protein obtained via five genetic mutations performed on Rtms5 protein, which presents an intense dark blue color [87]. The new KP-UM fusion protein was incorporated into three ethanolic formulations and its ability to be used as a coloring agent was tested using overbleached Asian hair. The KP-UM fusion protein was able to color the hair, and the final color was dependent on the formulation polarity **(Figure VIII.1)**, changing from pink to purple decreasing the ethanol concentration in the formulation [139]. Moreover, an improvement in the mechanical properties of the hair fibers treated with the KP-UM protein was also observed comparatively to the overbleached hair. The role of the KP, increased affinity of the fusion protein towards hair, was demonstrated by molecular dynamics. These studies proved the binding of the KP-UM protein to keratin protofibrils mediated by the KP peptide [139]. Using the KP-UM fusion protein has a model, we demonstrated the ability of 'KP-chromogenic proteins'-based formulations to be used for coloring of overbleached hair. This work opens the possibility to use other chromogenic proteins, with different colors, to color the hair according to users' personal taste.

Moreover, the chromogenic proteins show high potential to be incorporated into new ecofriendly cosmetic formulations for hair dying, decreasing the impact of conventional hair dyes in the environment [139].

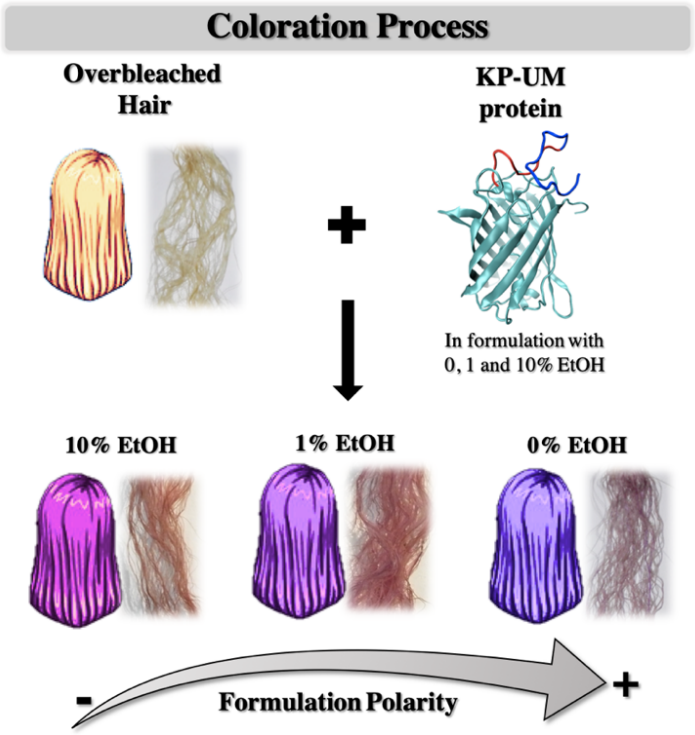


Figure VIII.1 - Coloration of overbleached hair using the KP-UM fusion protein. The hair color was influenced by the ethanol concentration present in the formulation.

Styling hair with straightening irons or curling tongs is a daily routine used by millions of people around the world. However, these procedures have a great negative impact over the properties of the hair fibers, with visible damage on its surface and internal structure. Despite there are some products available to minimize the effect of heat on hair fibers during heat styling procedures, most of them fail whenever necessary a full protection, from the surface to the cortex of the fibers. We have developed a new cosmetic formulation with a fusion protein to protect the hair during the application of high temperature procedures. The new fusion protein was based on the sequence of human eye γD-crystallin conjugated with the KP peptide [227]. Two proteins were designed, the first has the sequence of the wild-type γD-crystallin (KP-Cryst Wt) and the second is a mutant form of the first (KP-Cryst Mut). The mutant protein was obtained by the substitution of three arginine residues by three cysteine, in order to improve protein stability and protein interaction with the hair keratins. The capacity of the KP-Cryst fusion proteins to bind hair was demonstrated by confocal microscopy using FITC-labeled proteins. Both KP-Cryst fusion proteins, when

applied as a pretreatment before iron, were able to reduce the water loss from the fibers and prevent the conversion of keratin α-helix structure into β-sheets, preserving the hair's mechanical properties **(Figure VIII.2)**. Differentiated protective profiles were obtained for the KP-Cryst Wt and KP-Cryst Mut proteins, with the mutant form showing a higher structural and thermal resistance to higher temperatures which contributed to a higher degree of protection given by this protein when compared with the wild-type form. The protection of hair against high temperature imparted by the KP-Cryst proteins, support the use of these fusion proteins for the development of new hair protective formulations against high temperature insults [227].

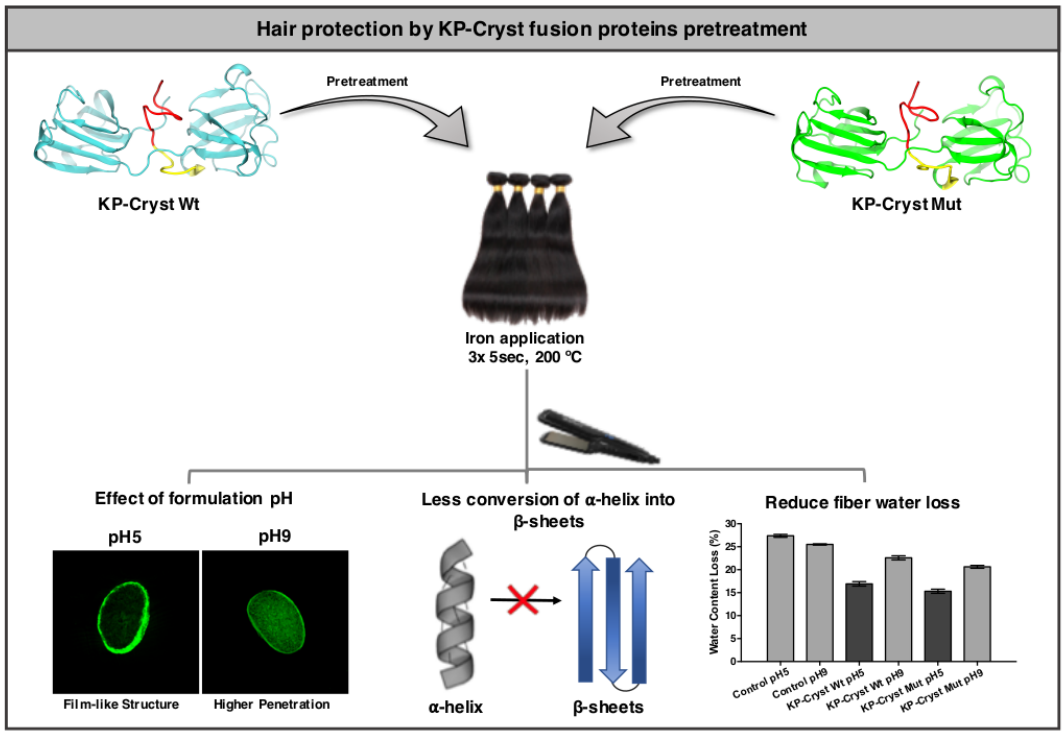


Figure VIII.2 - Protective effect of KP-Cryst Wt and KP-Cryst Mut fusion proteins against hair degradation induced by high temperature procedures.

8.1.2 Keratin nanosystems in hair cosmetic products

Nanoscale materials have been widely used in cosmetics due to the new properties given to the materials by the reduced size. These new properties are related with the color, transparency, solubility and chemical reactivity. Furthermore, the nanomaterials are used to improve the stability of some cosmetic compounds, generating a great interest of cosmetic industry partners in this area of research [277,278]. Despite of the great potential of keratin-based systems for the development of hair cosmetic products, no formulation containing keratin in this form can be found in the market or is described in literature.

We produced for the first time, particles composed by keratin, silk fibroin and poloxamer. The particles were obtained by high pressure homogenization and were applied over virgin and overbleached Asian hair [209]. The keratin and the silk proteins were selected as main ingredients of these innovative system due to their capacity to recover and protect hair from damage and to improve hair moisture, respectively. Considering the ratio between keratin and silk fibroin, we developed several formulations combining different amounts of the two proteins. Using different keratin/silk fibroin ratios we determined which combination conferred the best protection and properties to the hair. Although all the formulations recovered and/or improved the fiber's mechanical properties and the hair smoothness, the best results were obtained for the formulation with higher percentage of silk fibroin (20%). These improvements were most likely related with the secondary structure of silk fibroin, rich in β-strands, contribution for a higher resistance and stiffness, together with its capacity to interact with the keratin proteins from hair. An increase in the keratin α-helix denaturation enthalpy was also observed, proving the positive effect of these particles in hair's thermal stability. Using scanning electronic microscopy, we observed that the particles' deposition profile was dependent on the type of hair rather than on the type of particle (**Figure VIII.3)**.

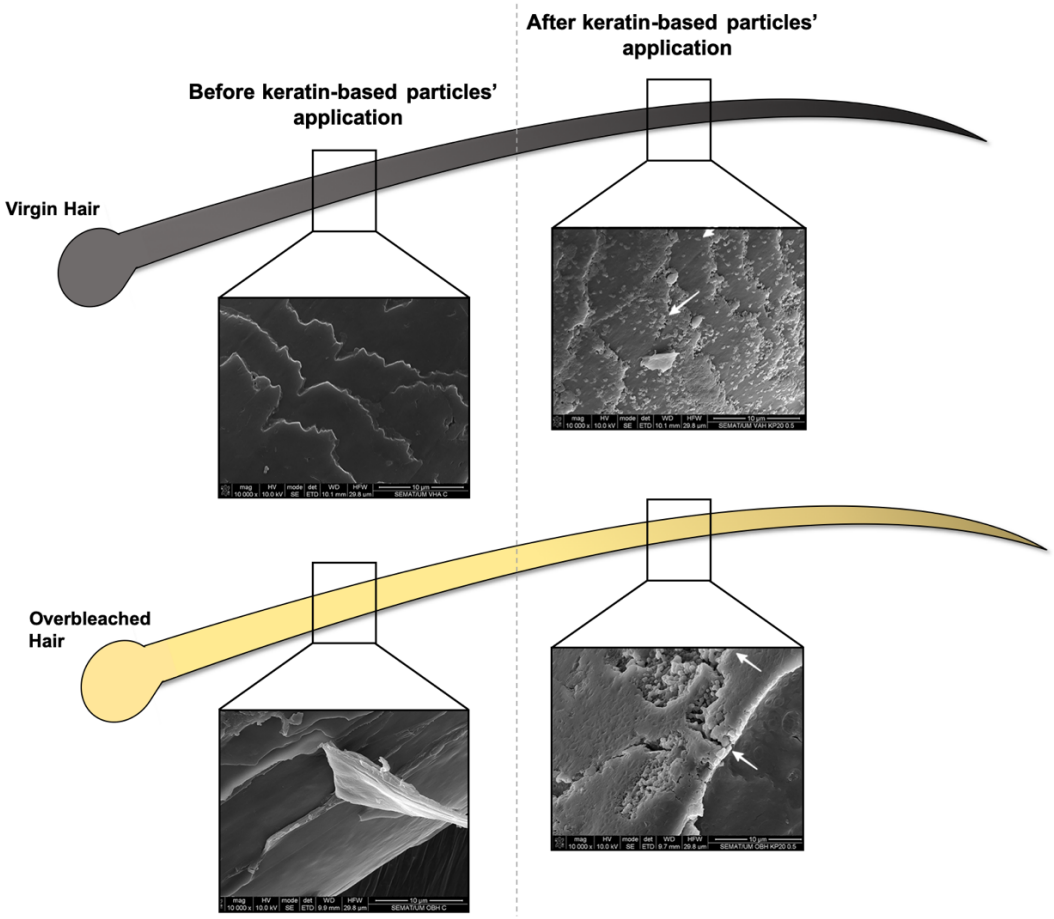


Figure VIII.3 - Pattern deposition of keratin-based particles on virgin and overbleached Asian hair.

For the virgin hair, the particles deposited preferentially in the interlayer zone of the cuticle, while for the overbleached hair the particles are evenly distributed along the hair fiber with a tendency to form film-like structures. Due to the new properties conferred to the hair by the particles, they show the capacity be incorporated into new cosmetic formulations for hair protection and restauration [209].

Taking advantage of the properties conferred to the hair by the keratin-based particles and responding to the recent need for new hair perfumes, we developed particles constituted by keratin and zein for the encapsulation of two fragrances, menthol or linalool. The Keratin:Zein particles were obtained using two different strategies: in strategy A, core(zein)-shell(keratin) particles were obtained while in strategy B, the particles could have a mixed structure and/or core(zein)-shell(keratin) structure. The inclusion of keratin in the formulation composition was fundamental since it stabilized the initial zein particles, avoiding its precipitation after 1 week of storage. The presence of the keratin at the surface of the particles was also proved by a shift on the particles' surface charge from positive to negative. When applied over Asian hair, the particles prepared by both strategies deposited over the hair surface and demonstrated to be effective in the improvement of hair's mechanical properties and hair's hydration degree. The Keratin:Zein particles were able to protect the fragrances during storage and revealed to be highly efficient in the release of the fragrance as response to a temperature stimulus **(Figure VIII.4).** The new Keratin:Zein particles can be used as new hair cosmetic perfumes, which can be adjusted according the personal taste of the end user, regarding the fragrance to be encapsulated and released.

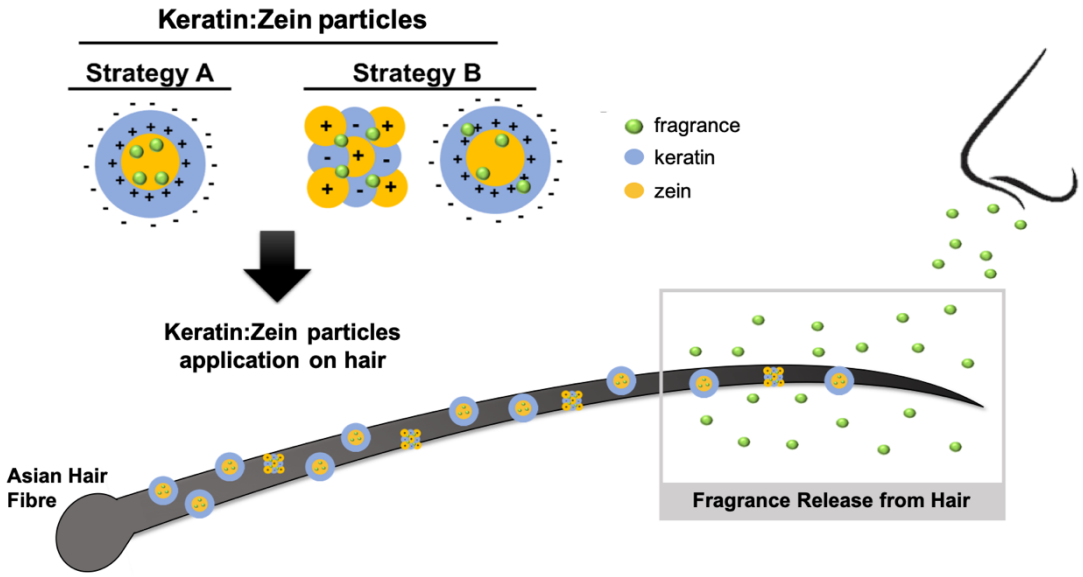


Figure VIII.4 - Schematic representation of Keratin:Zein particles' structure and fragrance release from hair.

8.1.3 Keratin films in hair cosmetic science

When developing hair care cosmetic products, is essential to accurately evaluate hair properties to determine the real benefits of cosmetic products application. However, a big number of hair samples are necessary in order to correctly evaluate the hair properties. This happens because hair is a very heterogeneous sample and differences are observed not only between individuals, but also in the same hair fiber, comparing for example the roots and the end tips of hair fibers. Because of the high hair fiber variability, it is important to develop new systems to accurately determine the effect of different cosmetics on hair properties. Ideally keratin films could be produced as a two dimensional and homogeneous sample. The keratin films respond to the heterogeneity of the hair fibers and might be used as hair substitutes for the validation of hair cosmetic formulations.

Keratin films were already developed by Fujii *et al.*, where they studied the effect of reducing and oxidizing agents, ultraviolet exposition and thermal treatments on the keratin films' properties. Comparing the results between the hair fibers and the keratin films, a higher sensitivity was obtained for the keratin films [279,280]. For example, the films presented 2000-3000 times more protein eluted than the hair fibers when exposed to reductive agents. So, an optimization in the keratin films properties should be performed in order to mimic more correctly the behavior of the hair fibers.

Considering the few numbers of protocols available, which use keratin obtained from virgin hair, we have developed a new protocol for the preparation of keratin films. We studied the effect of two heating methods on the properties of keratin films prepared in a pH range between 7-10 and with keratin extracted from virgin or overbleached hair [281]. The two heating methods included the conventional heating, using a water bath with controlled temperature, and the ohmic heating where a moderate electric field was used **(Figure VIII.5)**. The heating method influenced the film's free thiols' concentration, thermal stability and water swelling profiles. Furthermore, it was demonstrated that the properties of the keratin films could be regulated by changing the heating method, keratin solution pH and keratin source. The keratin films prepared by both methods also proved to be a good model to study the effect of cosmetics on hair using a cosmetic formulation containing the KP-Cryst Wt or the KP-Cryst Mut proteins.

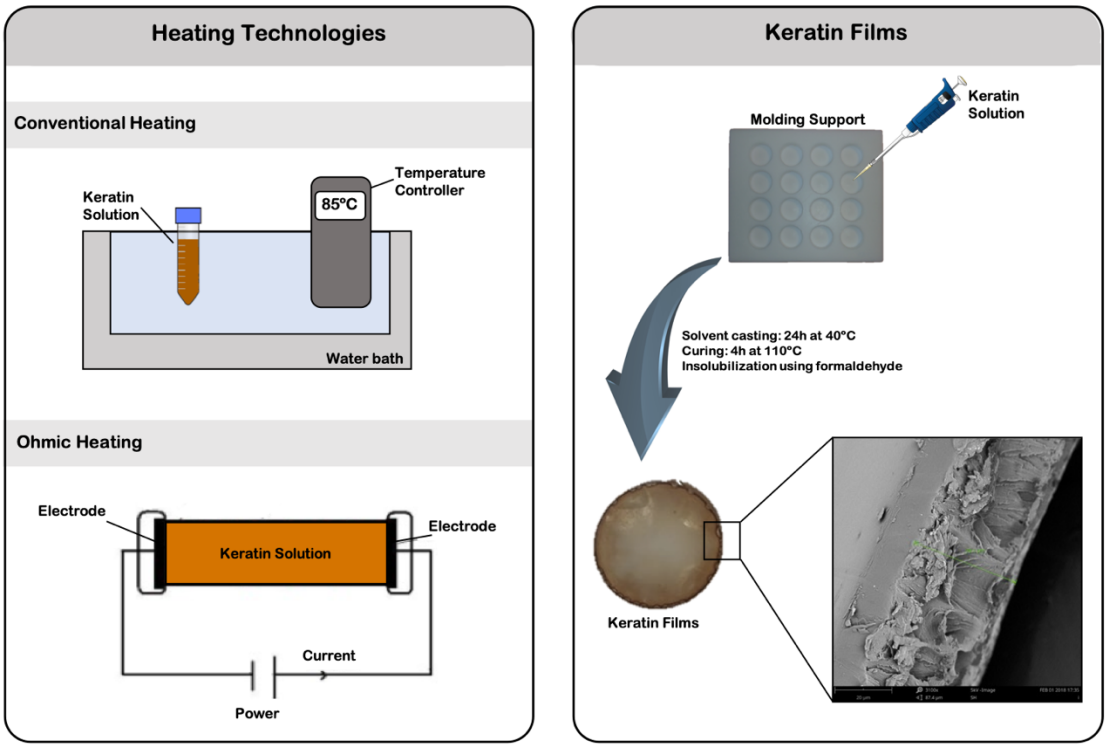


Figure VIII.5 - Schematic representation of the keratin films preparation using the conventional and ohmic heating methods.

8.2 Concluding remarks

Proteins, proteins hydrolysates and peptides have been widely used by cosmetic industries as ingredients for hair cosmetic products, since they are capable to protect hair and repair hair fibers. Recent studies using keratin protein, hydrolysates or fusion proteins containing keratin-based peptides demonstrated that they show potential to be incorporated into advanced cosmetic formulations capable to improve some hair properties like smoothness, strength, hydration and thermal stability. Fusion proteins with chromogenic binding modules, as is the case of KP-UM protein, could be used as coloring agents and be incorporated into hair cosmetic formulations for hair. This protein has the capacity to color hair while, simultaneously, improve the fibers' mechanical properties. When the chromogenic module was substituted by the γD-crystallin protein, a new fusion protein (KP-Cryst Wt/Mut) capable to protect the hair against high thermal procedures was obtained. The protective effect was more notorious in the prevention of water loss from the fibers and in the maintenance of hair mechanical properties (stiffness), after iron application. Besides the use of keratin-fusion proteins on hair, keratin-based particles were also developed for hair cosmetic applications. Particles composed by keratin, silk fibroin and poloxamer 407 were applied on virgin and overbleached asian hair and demonstrated to be able to improve the fibers'

mechanical properties, thermal stability and hair smoothness. Particles composed by keratin and zein were developed for fragrance encapsulation and to be used as hair perfumes. In this formulation, keratin demonstrated to have a surfactant behavior, avoiding zein particles precipitation and improving particles' stability over time. This formulation protected the fragrances during storage and revealed a high efficiency of fragrance release. A positive effect on hair's mechanical properties and hydration degree was also verified after the treatment of Asian hair with these particles. After developing a cosmetic formulation, is important to evaluated its effects on the hair fibers. To avoid the use of heterogeneous hair fibers, a two-dimensional keratin films' system was successfully developed. Both the keratin films prepared by conventional or ohmic heating could be used as models to study the effect of cosmetic formulations on hair.

In this review, it was demonstrated the potential of keratin-fusion proteins and keratin-based systems to be incorporated into several hair cosmetic products capable to protect hair and improve its color, mechanical strength, smoothness, thermal stability and hydration degree. The systems here developed present an infinite number of possibilities for the hair cosmetic field, depending on the properties of the proteins selected. Also, they could be able to substitute the synthetic organic compounds included nowadays into the cosmetic formulation, obtaining a more natural and eco-friendlier product.

8.3 Future Perspectives

In the last years, environmental protection and green marketing trends resulted in the change of consumer's demand and behavior. The interest of consumers about an environmentally friendly lifestyle has increased, where greener products should be developed by cosmetic industry, in order to respond to consumers' environmental and health awareness. The formulations developed in this thesis should be optimized in order to substitute the maximum components by green ingredients without compromising the formulation efficacy. The use of chromogenic proteins as a substitute of hair dyes is already an important step in the search of greener products for hair coloration, decreasing the dyes' negative impact in the water systems.

Chromogenic proteins with different colors should also be developed to increase the color palette available for hair coloring using recombinant proteins. Also, further studies about the potential capacity of KP-UM to be incorporated into a cosmetic system capable to change the hair color between yellow and purple, depending on the formulation pH, need to be performed.

Fusion proteins composed by a conjugation of different proteins could be an innovative system in cosmetics due to the simultaneously improvement of several hair properties. For example, a fusion protein

containing the KP-UM and the KP-Cryst proteins could simultaneously color the hair while protect it against high thermal procedures.

The keratin-based particles here described open new perspectives on the use of these formulations in hair cosmetic industry for hair restoration, hair targeting and hair beauty. These keratin-based particles could be used to encapsulate or bind several molecules like fragrances, bioactive molecules, vitamins, antioxidants, conditioners, among others. The inclusion of different proteins or even lipids could also be an interesting approach to increase the hair protection and fibers' mechanical strength.

Relatively to the keratin films prepared as a model to substitute the use of the hair on cosmetic testes, an optimization in the hair model should be performed. Ideally, a three-dimensional model using keratin solution should be prepared in order to mimic more correctly the hair fibers and obtain more reliable results.

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