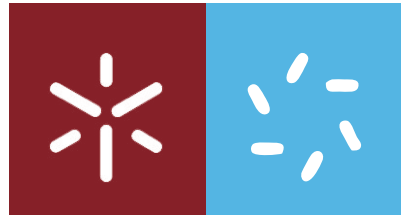




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**Development of protein-based formulations
for topical treatment of keratinaceous
appendices**

Universidade do Minho
Escola de Ciências





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**Development of protein-based formulations for
topical treatment of keratinaceous appendices**

Tese de Mestrado em Biofísica e Bionanossistemas

Trabalho realizado sob a orientação de
Doutor Artur Jorge Araújo Magalhães Ribeiro

e coorientação de
Professora Doutora Andreia Ferreira Castro Gomes

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Abstract

Keratinaceous appendices play an important role in the visual aspect of each individual. The chronic exposure of hair, nails and skin to external hazards have a negative impact on their structure. To prevent or revert the impact of such exposure, several cosmetic products have been developed and are now commercialized. Yet the use of cosmetic products is not restricted to this specific application as they are widely used in the adornment of the keratinaceous appendices, mainly for hair coloration.

In this thesis, two approaches for the development of new hair cosmetic formulations were tested. Keratin-based particles and two recombinant crystallins were designed and obtained. These were mainly focused on the restoration and improvement of hair mechanical properties.

Keratin-based particles composed by keratin (K₁), silk fibroin (SF₁) and Poloxamer407 (P₂) (K₁SF₁P₂) were obtained by high pressure homogenization. Particles size ranged between 150 and 250 nm with low polydispersity and a net negative surface, which was partially neutralized by the addition of poloxamer to particles' formulation. Homogenization process resulted in an increase on the percentage of β -strands. All formulations do not show cytotoxic effect on human keratinocytes (NCTC 2544 cell line). The effects of keratin-based particles over virgin (VH) and overbleached (8xOB) Asian hair, were monitored regarding particles ability to bind to hair fibres and their effect over hair mechanical properties. Keratin-based particles increased hair fiber physically by 62.7% for VH and 83.4% for 8x OB, which was directly related with the quantity of β -strands present in the particle system as well their binding capability.

Two crystallin forms, a wild type (CrysWt) and a mutant (CrysMut), based on the sequence of human eye γ D-Crystallin were expressed in *E. coli* using a commercial TB-AIM medium. The recombinant crystallins were obtained pure after purification with magnetic beads and the proteins had a molecular weight of 24.8 kDa and 24.6 kDa for *wild type* and *mutant* forms, confirmed by MALDI-TOF. Analysis of protein secondary structures revealed that the new crystallins retained the structure of human eye γ D-Crystallin, which is rich in β -sheets. This type of structure is typical of crystallin which belong to a family of proteins characterized by the presence of the Greek-key motif.

Outcome from protein formulation characterization supports the hypothesis that the keratin-based particles were able to recover and improve the mechanical properties of virgin and overbleached hair. Therefore, these particles can be considered as a very promising strengthening safe agent for the development of new restorative hair care products.

Resumo

Os apêndices queratinosos desempenham um papel importante no aspeto visual de cada indivíduo. A constante exposição a perigos externos para cabelo, unhas e pele tem um impacto negativo na sua estrutura. Para evitar ou reverter o impacto dessa exposição, vários produtos cosméticos foram desenvolvidos e agora são comercializados. No entanto, o uso de produtos cosméticos não é restrito a esta aplicação específica, uma vez que são amplamente utilizados no adorno dos apêndices queratinosos, principalmente para a coloração do cabelo.

Nesta tese, foram testadas duas abordagens para o desenvolvimento de uma nova formulação cosmética para o cabelo. Partículas à base de queratina e duas cristalinas recombinantes foram projetadas e obtidas. Estas estratégias distintas são principalmente focadas no restauro e melhoramento das propriedades mecânicas do cabelo.

Partículas à base de queratina compostas por queratina (K_α), fibroína de seda (SF_γ) e Poloxamer (P_z) ($K_\alpha SF_\gamma P_z$) foram obtidas através de homogeneização de alta pressão. As partículas possuíam tamanho entre 150 e 250 nm com baixa polidispersividade e carga superficial negativa, parcialmente neutralizada pela adição do poloxamer. Nenhuma formulação apresentou efeito citotóxico na linha celular NCTC 2544 de queratinócitos humanos. A capacidade das partículas se ligarem com as fibras e a sua deposição em cabelo virgem (VH) e cabelo descolorado (8xOB) foi analisada. A melhoria das propriedades mecânicas das fibras foi estudada através de testes de tensão. Em cabelo virgem houve um aumento de 62.7% e em cabelo descolorado de 83.4%. Estes aumentos estão diretamente ligados à quantidade de estruturas β - no sistema particulado assim como a capacidade de ligação com a superfície da fibra do cabelo.

Os dois tipos de cristalinas, *wild type* (CrysWT) e *mutante* (CrysMut), baseadas na sequência da γ D-Cristalina do olho humano foram expressas em *E. coli* em meio comercial TB-AIM. As cristalinas recombinantes purificadas, obtidas após a purificação com “beads” magnéticas, apresentam peso molecular de 24.8 kDa e 24.6 kDa, para CrysWT e CrysMut respetivamente, confirmados pela técnica de MALDI-TOF. Análise à estrutura secundária destas proteínas puras revelou que estas mantêm uma estrutura rica em folhas β , tal como é característico da estrutura em chave grega desta família de proteínas.

O resultado da caracterização da formulação proteica suporta a hipótese de que as partículas foram capazes de recuperar e melhorar as propriedades mecânicas dos cabelos virgens e excessivamente descolorados. Estas podem ser consideradas como um agente de fortalecimento muito promissor e seguro para o desenvolvimento de novos produtos reparadores para cabelo.

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The results of Amide I deconvolution, expressed in percentages, were analyzed using OriginPro 8.5 software.

List of Abbreviations

BSA	Bovine Serum Albumin
CrysWt	Wild-type Crystallin
CrysMut	Mutant Crystallin
DLS	Dynamic Light Scattering
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulphoxide
DOC	Deoxycholate
FWHM	Full Width Half Maximum
HPH	High Pressure Homogenization
K	Keratin
KAPs	Keratin Associated Proteins
KP	Keratin Peptide
LB	Lysogeny Broth medium
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-of-Flight mass spectroscopy.
MTS	3- (4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H-tetrazolium
PBS	Phosphate-buffered saline
P	Poloxamer 407
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Eletron Microscopy
STEM	Scanning Transmission Eletron Microscopy
SF	Silk Fibroin
TB	Terrific Broth medium
TB-AIM	Terrific Broth Auto Inducible medium
TEMED	Tetramethylethylenediamine
V	Vegetable oil

Chapter 1: Introduction

1.1. Main Goal and Motivation

Many cosmetic products for keratinaceous appendices like skin, hair and nails do not show viable results regarding their final application. This is mostly related to how products are applied and how they interact with the keratinaceous appendices. Barriers like hair cuticles and nails plate play an important role in preventing the entry of compounds in aqueous solutions and thus the use of solvents becomes necessary.

In this work two strategies for the treatment of damaged appendices were pursued. The new approaches are solvent free and constituted by biological materials. Keratin was extracted from hair waste of barbershops while fibroin was extracted from *Bombyx mori* cocoons and recombinant crystallins, based on the sequence of human γ D-crystallin, were expressed in *Escherichia coli*.

The work presented here was carried out at the BBRG laboratory at Department of Engineering Biological and was divided in two parts:

- 1- Production, characterization and application of keratin-based particles
- 2- Production and characterization of recombinant crystallins

The production of keratin-based nanoparticles for hair restoration and strengthening was pursued due to the affinity of keratin and keratin-based peptides to hair fibres. [1]

The effect of γ D-crystallin on the properties of virgin and damage asian hair [2], was the motivation for producing two new recombinant crystallins. To increase the anchorage and the film forming properties of the proteins on hair fibres a keratin-like peptide (KP) was included in the crystallins sequences (*wild type* and *mutant* forms). The KP peptide will improve the crystallins polymerization on hair surface creating a protein film. This film could be used for the anchorage of other functionalities like chromogenic proteins for hair colouring.

1.2. Keratinaceous Appendices

Keratinous appendices are structures characterized by the presence of high amounts of keratin on their constitution or have keratinocytes which are responsible for the production and accumulation of keratin.

These appendices, apart from conferring protection, regulate body temperature and act like sense organs, have a central role in the esthetic of the human being. Thus, the development of new formulations for the treatment and improvement of the properties of these appendices is of great importance with a significant economic and social impact. [3]

Hair, nails and skin are differentiated by the structural type and the type of keratins. [4] Skin on stratum corneum have a variety of “soft” epithelial keratins opposite to the keratins found in hair and nails. These are built up from a separate subfamily of hard keratins which are rich in sulfur content, in their non- α -helical head and tail domains, that are responsible for the high degree of filamentous cross-linking by keratin associated proteins (KAPs). [5–7]

1.2.1. Hair

Human hair has an important and undeniable relevance in society due to its important role in visual appearance and social communication. Hair is mainly composed of structural proteins mainly keratin, keratin associated proteins, water, lipids, trace elements and pigments (Table I).[3]

Table I - Hair shaft composition

❖ <u>Structural proteins</u>	90%
• Keratin	
❖ <u>Water</u>	10%
❖ <u>Trace elements</u>	
❖ <u>Pigments</u>	
• Melanine	
❖ <u>Lipids</u>	
• 18-MEA	

Hair is generally divided into two main parts: the hair follicle and the hair shaft. The study of the hair follicle is mainly addressed by biological sciences while the hair fibre is mainly studied from a physicochemical perspective by cosmetic sciences. The hair shaft is divided in cuticle and cortex with hard keratins, and the medulla made by soft keratins. These three regions act like a unit and are represented at Figure 1. [3,8]

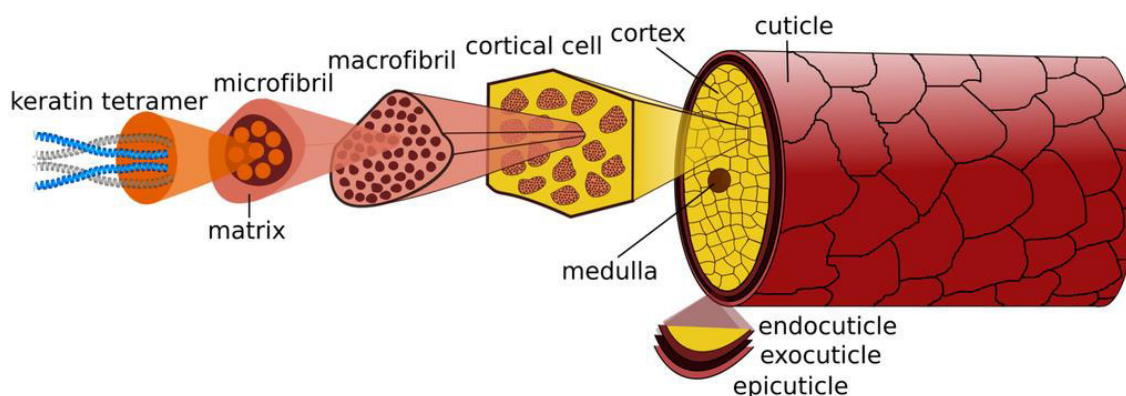


Figure 1- Scheme of hair structures.[8]

The cortex is surrounded by the cuticle, has a thickness of 45–90 μm and corresponds to 90% of the total hair mass. It is composed of elongated cortical cells, which enclose melanossomes, and the cell membrane complex (CMC). The cortical cells are tightly packed contain macrofibrils which are parallel and longitudinal oriented to the fiber axis. These macrofibrils are constituted by microfibrils (intermediate filaments (IF)) and a matrix with keratin associated proteins (KAPs). IFs are surrounded by KAPs and are constituted by high sulphur proteins, ultra-high sulphur proteins and high glycinytyrosine proteins. The KAPs, rich in cysteines, interact with IF through intermolecular disulphide bonds, providing high mechanical strength, inertness and rigidity to the keratin fibres.[3,8,9]

The medulla (5–10 μm) is located on the core of the hair fiber composed by spherical hollow vacuoles, surrounded by CMC, and do not play an important role in hair mechanical properties. They can be continuous or discontinuous and absent. In coarse hair, like beard, it is usually continuous, but in fine hairs it appears discontinuous while is absent in African hair. [8,10]

The hair cuticle is a protecting barrier of cortex from external environmental damage, containing 2-6 layers of overlapping scales. The cuticle's proximal end is firmly attached to the cortex (CMC cells) and the distal open end of the overlapping tiles points towards the tip of the fibre.

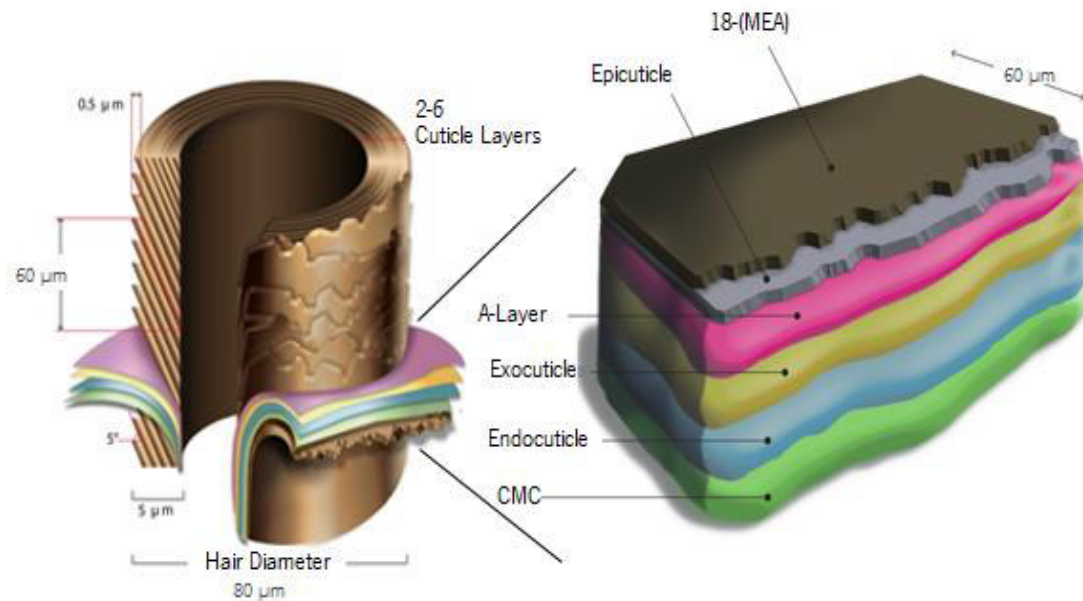


Figure 2- Illustrative image of the cuticle's layered structure with its 5 different sublayers.[11]

The cuticle is divided into 5 different strata (Figure 2), among which stand out A-Layer, Exocuticle and Endocuticle. Compositional differences between them render the cuticle a very reactional structure to external elements like water, heat, chemicals and others.

The A-layer is the region of the hair fiber most highly enriched in disulfide (cysteine) and isopeptide (amide) cross-links and has an average thickness of 110 nm. This layer is attached to the epicuticle which supports 18-MEA (18-methyl-eicosanoic acid). The presence of 18-MEA is associated with cuticles' hydrophobicity (figure 3) [11–13].

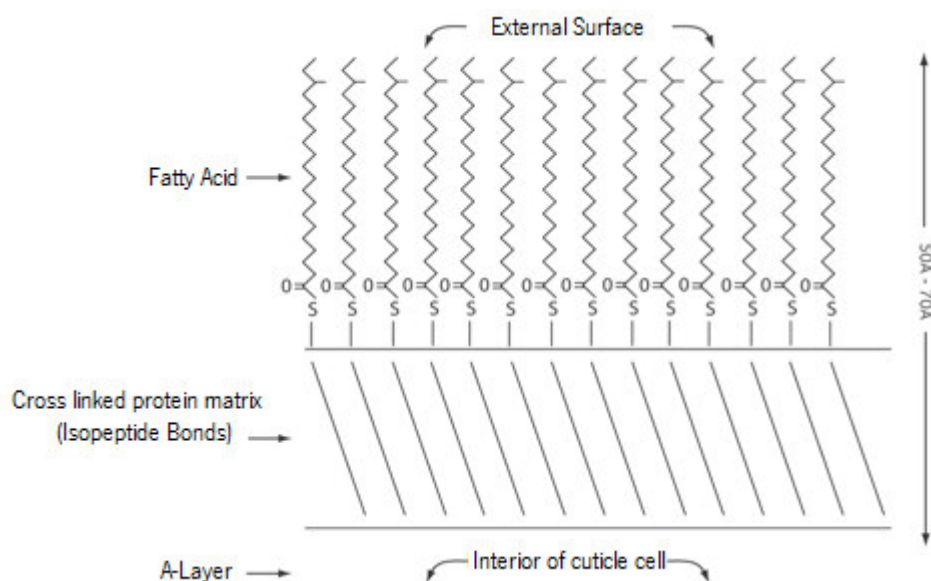


Figure 3 – Scheme of 18-MEA structure present on the epicuticle.[11]

The exocuticle usually has a thickness of 200 nm and is composed essentially by cysteines and non-polar amino acids and virtually does not show any isopeptide cross-links. However in interaction with A-Layer, the exocuticle will be fitting in the role of protecting the other surfaces of the hair from mechanical damage [14].

Endocuticle consists of the remaining cellular debris. This layer is very poor in cysteine residues, does not present cross-linked sections, but the presence of high levels of acid and basic amino acids makes this section the most prone to swelling.[12,14]

1.2.1.1. Types of Hair

Hair is a unique characteristic of each individual and differs in terms of growing rate, size and shape. It is possible to distinguish hair from different ethnic backgrounds such as Asian, Caucasian and African. Despite this phenotype variation, the amino acid composition appears to be uniform. In other cases, the percentage and location of lipids differs from each ethnical group [3,7,12]. In Figure 4 are presented the three types of ethnic hair and corresponding cross-sections.

Hair fibre shape is dependent on hair follicle structure. Large and straight follicles with a circular cross section produce thick straight hair, and flat follicles make curly elliptical/ ribbon shaped cross section hair.[15,16]



Figure 4 - Different hair fibres types with respective cross-sections. A - Asian; B - Caucasian; C - African.

Asian hair presents higher density and a rougher surface when compared with the other types of hair. It is usually straight with a round cross-section and is very similar to the caucasian hair regarding the lipidic constitution.

Caucasian hair can be quite variable in its shape going from straight to wavy or curly. The fibre is elliptical and is, on average, thinner than the Asian fibre. African hair is frequently tightly looped and twisted. In cross section, it is almost flat and ribbon-like in some cases. Presents higher concentration of lipids which is related with the higher surface hydrophobicity of this type of hair.[3,12,17]

The mechanical properties of hair are also influenced by the hair pattern. Curly hair, due to the smaller curve diameter, is more likely to break under external forces, than straight hair.[18]

1.2.1.2. Effect of bleaching in hair

Chemical bleaching is commonly used in cosmetics, which is apply to lighter the hair fibres and, more usually, to prepare the hair for dyeing. However, repeated hair bleaching results in hair damage.[10]

Many studies report the effect of chemical oxidation on hair structure and composition. After chemical treatment, there is a change in hairs' amino acid composition, hydrophobicity, melanin content, matrix and fibre diameter. Moreover the process due to the oxidation of cysteines results in an increase of the negative groups on hair surface. [12]

The overbleaching using H_2O_2 results in very fragile hair fibres with lower fibre resistance mainly related with the disruption of cross-linked cysteines in the cortex region along with the appearance of cracks and holes in the cuticle (Figure 5) [19].

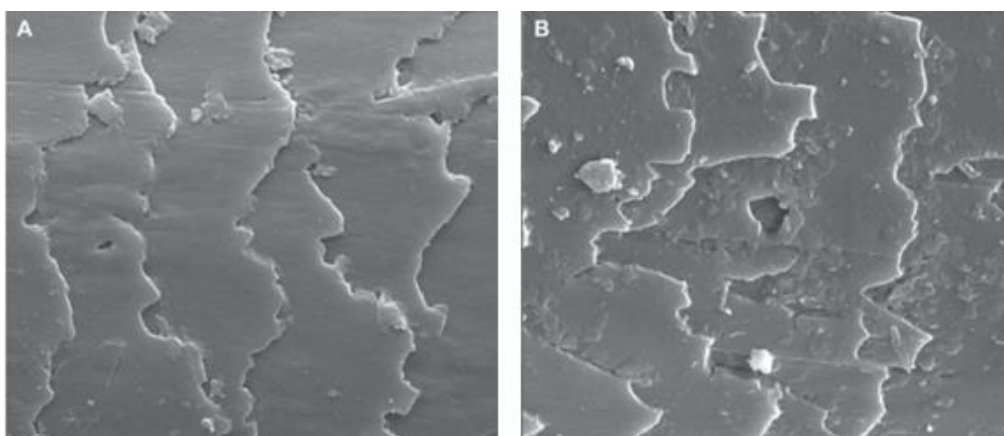


Figure 5- Surface electron microscopy (SEM) micrographs of normal Asian virgin hair (A) and overbleached hair (B). [19]

It is important to develop new strategies for hair colouring and products that reverse the undesired collateral effects of hair bleaching. Protein-based products are now seen as excellent candidates to restore hair fibre aspect and strength.

1.3. Nanotechnology for personal care

Engineered nanomaterials such as fullerenes, nanotubes, liposomes, quantum dots, solid lipid nanoparticles (SLN), starch lipid composites (SLC), nanoemulsions, among others are employed nowadays in cosmetic industry due to their nano-scale unique properties. Cosmetic industry is replacing the cosmeceuticals for nanocosmeceuticals by incorporating nanotechnology in the manufacturing processes resulting in nanotech based cosmetics and some of these new products are already available to the consumer.[20–22]

Nanotechnology can be defined as the study of matter on an atomic or molecular scale handling with exact size and shape structures which must be measured on a nanometric scale. Nanotechnology aims to create products with both special and improved chemical–physic features compared to conventional products. Consequently, the goal of nanobiotechnology is the development of nanoscale biomolecular components and analytical instruments for the investigation of cell. [20]

Nanomaterials may exhibit unique biochemical properties when compared with microparticles of the same chemical compound. Due to larger surface area to mass ratio they could have unique bioactivity profiles. Nanocosmetics are cosmetic products with nano-based structures used as delivery vehicles. There is an increasing interest in this type of cosmetic formulations, and major cosmetic companies such as L'Oréal, Christian Dior and Estee Lauder are focused on the development and commercialization of new functional nanocosmetics. [23,24]

1.3.1. Nanoparticles in cosmetic formulations

The use of nanoparticles brings many advantages for the cosmetic industry. Several advantages can be pointed out: large scale production, long-term stability, possibility to lyophilize and controlled drug release. Nowadays cosmetic industry applies engineered bio-nano-systems in cosmetic products, which are mostly created by nanoemulsion because of its transparency and pleasant to touch. However, there is a variety of nanostructures that are used for personal care purposes like liposomes, solid lipid nanoparticles (SLN), nanocapsules, dendrimers, cubosomes,

hydrogels, mineral nanoparticles and polymeric nanoparticles. Some examples of nanostructures are represented in Figure 6.[20,22]

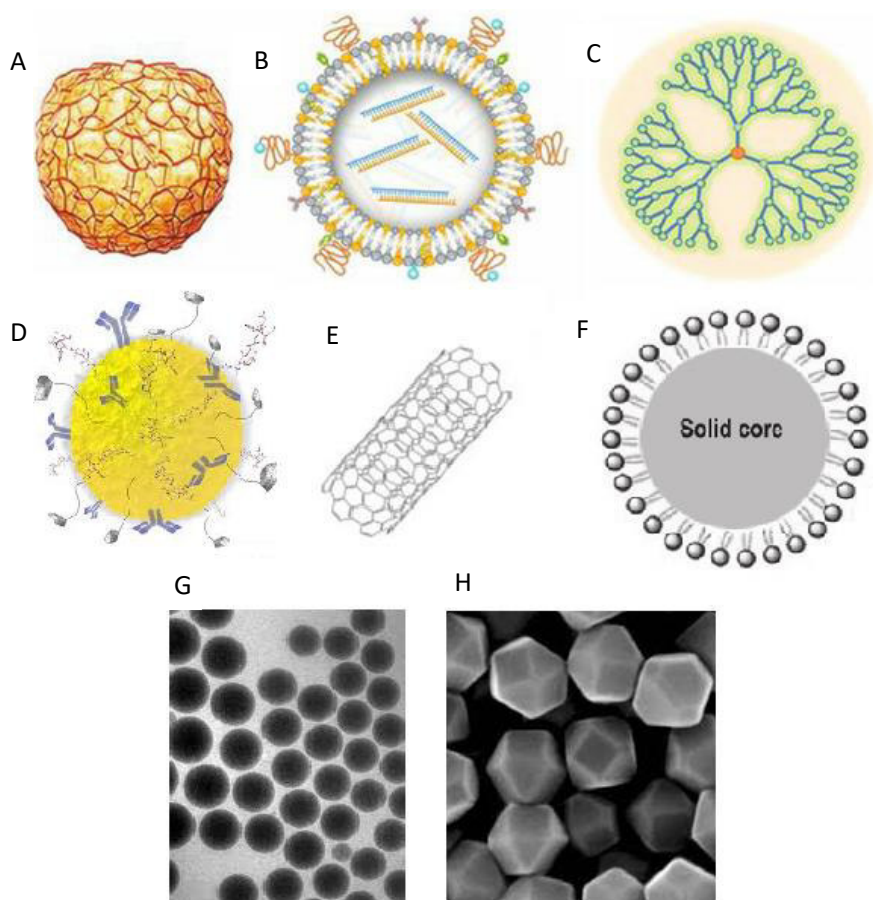


Figure 6 - Examples of nanoparticles used in the cosmetic industry. Polymeric nanoparticles (A); liposomes (B); dendrimers (C); mineral nanoparticles (D); nanotubes (E); SLNs (F); nanoemulsions (G) and nanocrystals (H).

Nanoemulsions are normally oi-in-water dispersions of nanoscale droplets and are used in odorants, shampoos and skin/hair care products. These are suitable for drug delivery since their small particle size provide higher stability, which also increases the shelf life of the cosmetic product. Some products with nanoemulsions are used for hair care improving cuticle structure and maintaining hair lubrication. Cationic nanoemulsions with silicone oil and seracin were tested and results showed a significant improvement of the cuticle's aspect even after several washes (Figure 7).

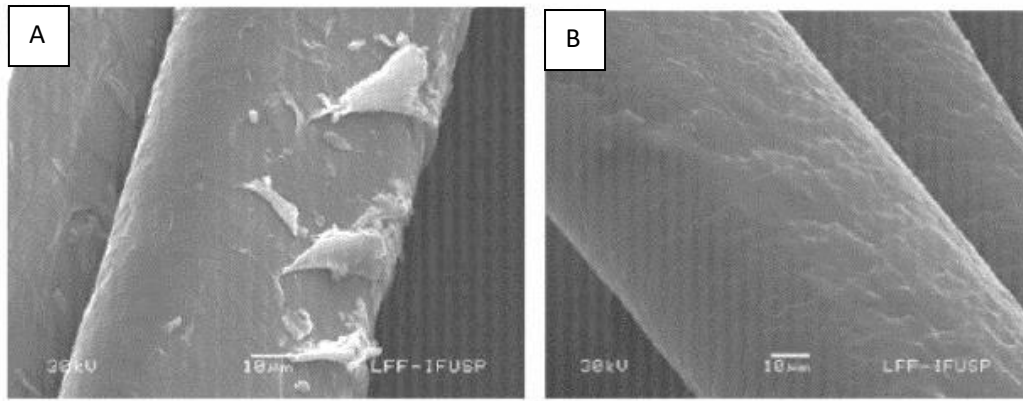


Figure 7- Micrographs of damage hair fibres before (A) and after (B) treatment with seracin nanoemulsion.[25]

Nanoemulsions structure can be manipulated based on the method of preparation (high pressure homogenization and ultrasound homogenization) and the nature of components.[25–27]

Gold nanoparticles (Figure 8) and hyaluronic nanoparticles with p-phenylenediamine dye had already been used as new hair colouring reducing the toxicity and enhancing cuticle penetration. Also, luminescent silica quantum dots were applied to hair resulting in the lightening of the hair shafts. [25]

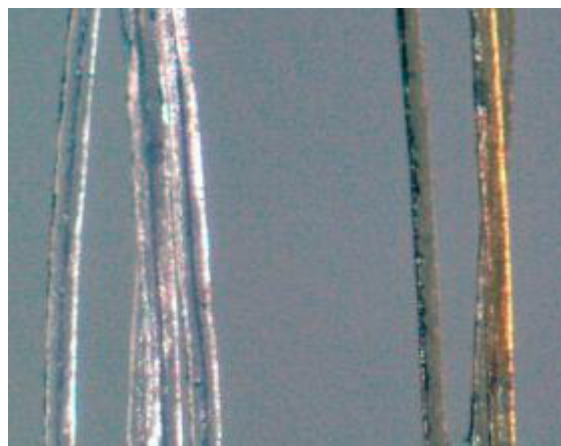


Figure 8 - Hair fibres before (left) and after (right) treatment with gold nanoparticles. [25]

1.3.2. Proteins in cosmetic formulations

The use of proteins (from the Greek word *proteios* = of primary importance) in cosmetic products is easy to understand as these play dominant roles in almost all biological processes. They act as enzyme catalysis; are involved in the transfer of small molecules and ions; coordinated movement, are involved in mechanical support and are associated with the control of growth and differentiation. The proteins can also be applied in the development of protein-based cosmetic products for the treatment of skin and/or hair. [28]

Since great ancient civilizations protein materials are used for cosmetics purposes. There are several examples in history like Cleopatra's legendary use of donkey milk and the use of soy flour to prepare facial masks by Hokkaido island fishers. Nevertheless, the first rational use of proteins on cosmetics dates back to the 1950's. Since then, many authors have recognized the effects of protein-rich substances for the topical treatment. Proteins were quickly considered useful ingredients to produce a suitable environment for healthy skin, hair and nail (i.e. conditioning agents).[28,29]

Protein derivatives are used for a variety of cosmetic formulations like emulsions, lotions, gels, creams, make-ups among others. Native proteins and hydrolysates with high molecular weight usually tend to form biofilms that result from protein polymerization providing continuous softness and smoothness. The possibility to anchor other types of proteins or peptides to the films, improving film properties like stability and visual appearance makes this type of approach very attractive in the development of new protein-based cosmetics. Some examples of proteins used in cosmetic products are: collagen, elastin, silk fibroin, keratin and casein.[30]

1.3.2.1. Human eye γ D-crystallins

Crystallins are a group of proteins located in the cytoplasm of fibrous cells of the ocular lens and are divided in two super families: gamma (γ -) and beta (β -). Both have an antiparallel β -sheet protein structure, also known as Greek key motif due to the similar pattern with pottery from ancient Greece. The crystalline structure and the Greek key motif are represented in Figure 9. The Greek key structure is represented in pairs and in two domains that interact between themselves. This interaction leads to a high thermodynamic stability which is not usual for proteins [2,31,32].

Gamma-Crystallin superfamily can be clearly differentiated in a chromatographic gel of the ocular lens compounds, appearing in the last peak, due to its monomeric structure and low molecular mass. This superfamily has an isoelectric point between pH 7.1 e 8.6 and are characterized by the existence of cysteine residues that are highly sensitive to the formation of disulfides in oxidative stress. Complementary studies distinguished this crystallins in two groups, γ A/B/C-Crys and γ D/E/F-Crys, which are differentiated by state of hydration. These states correspond to the critical temperature of separation which is superior in the γ D-Crys group and it is explained by the presence of three amino acid residues (Leu⁵¹, Ile¹⁰³ and His¹¹⁵) [33,34].

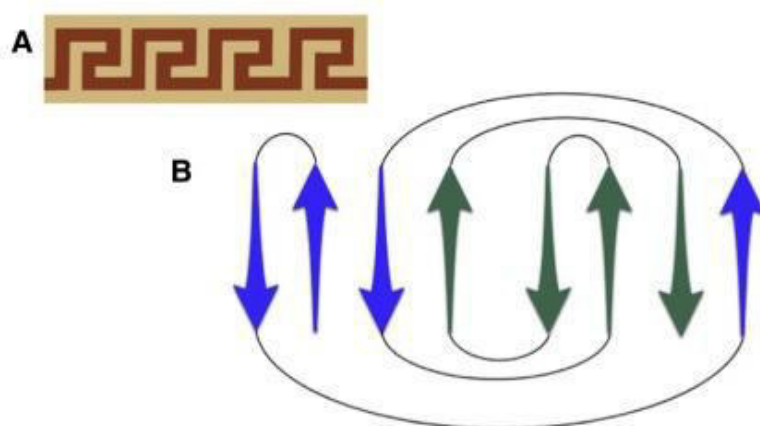


Figure 9 - Greek key motif structure of crystallins. Pattern from Greek pottery (A) and bi-dimensional representation of crystallin motif (B).[35]

The γ D-Crystallins contain 173 amino acids and two terminal domains (NH₂- e COOH-) unified by an amino acidic sequence of 6 amino acids and interact by contact between lateral chain and domain interface. Studies realized in physiological conditions that tested the capacity of this protein to fold and unfold showed that the termination COOH reacquire the natural form faster and consequently these domains do not present the same stability [36,37]. In Figure 10 is represented

the ribbon scheme of γ D-crystallin (A) and the amino acid sequence with color differentiation for the four structures of Greek key motif (B).

The γ D-crystalins are found in high concentration in the nuclear lens region [>400 mg/mL] and some studies indicate that its intermolecular interaction are directly related with lens transparency. In the occurrence of mutations, diseases like ocular cataract can be developed due to the opacity of the lens. Owing to the Greek key structure, these proteins are involved in the formation of coatings, like in the protein S present in the coating of spores of *Myxococcus xanthus*, which can be useful for the development of cosmetic formulations. This was already demonstrated in the production of crystallin-based films on hair[2,35,38,39].

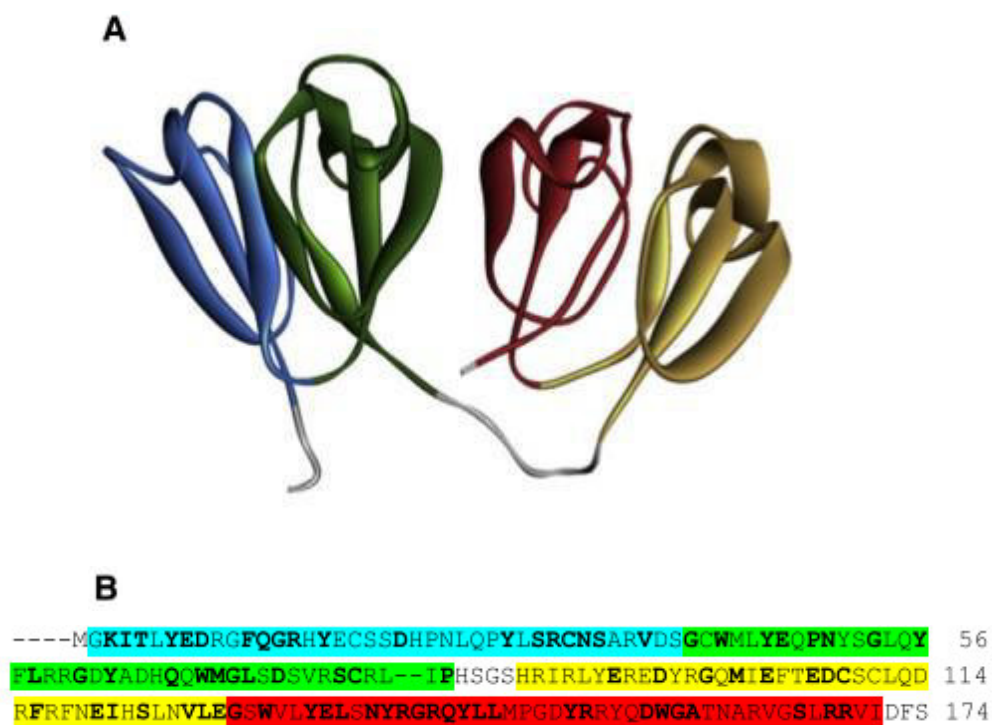


Figure 10 - Schematic ribbon diagram depicting the overall structure of human γ D-crystallin (-Crystallin. A) Tri-dimensional representation of Greek Key motif, each domain has a respective colour; B) Amino acid sequence highlighting each domain sequence.[35]

1.3.1.2. Human hair keratin

Keratin is an abundant biological material, represents a group of insoluble, usually with high-sulphur content and filament-forming proteins. They constitute the bulk of animal epidermal appendices such as hair, nails, claws, horns, beaks, and feathers. These “dead tissues” will end up in the trash or simply degrade so recovering keratin from “waste” could make it a bio-recoverable material with unique characteristics. It has been reported that approximately 300,000 tons of keratin-rich resources are discarded from salons, hospitals and slaughterhouses.[40,41]

Hair keratins, also known as hard keratins, are a family of structural proteins present in hair and fingernails. These are self-assembled into fibres in hair follicle and are controlled by many growth factors (≈ 30) in a highly proliferative process. They are sub-divided into two large groups: type I and type II, distinguished by their isoelectric point (Type I: acid, Type II: basic/neutral).[42,43]

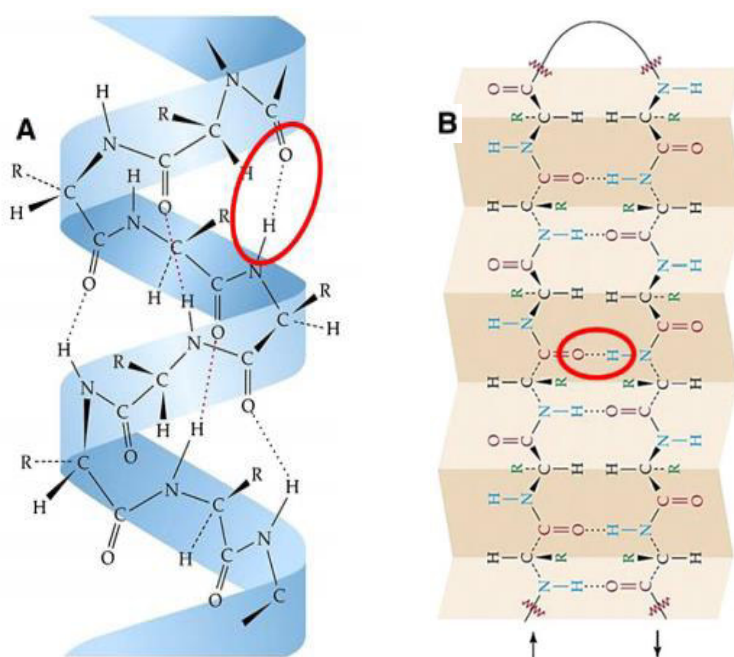


Figure 11 - Molecular structure of α -keratin (A) and β -keratin (B). Hydrogen bonds are highlighted (red circle) for each conformation.

Keratin in human hair is composed by 400 to 600 amino acids and contains a variety of cell-binding sites like LDV (Leu-Asp-Val) and RGD (Arg-Gly-Asp). It can be classified into three groups depending on the localization and structure. α -keratins are mainly structural and are the most abundant component of human hair. They present a α -helical structure, are low in sulfur content and have molecular weights between 60 and 80 kDa. The α -helical structure consists in two helical

polypeptide chains, usually found in right-handed direction, stabilized by the hydrogen bonds causing chain twisting (Figure 11 A). β -keratins form most of the cuticle and when extracted do not form any type of useful reconstituted structures. These involves laterally packed β -strands which can be parallel or antiparallel, held by hydrogen bonds stabilizing pleated sheet structures (Figure 11 B). [40,41,44]

The contribution of α - and β - strands to the mechanical properties of hair fibre was studied (Figure 12). Between 2 – 5 % strain, a near linear Hookean region, α -helices are stretched but no substantial structural changes happen. A transition from α -helix to β -sheet occurs in the yield region (5 – 50 %), at this point α -coiled coil conformation starts to disentanglement and β -sheets formed. In the post-yield region, there is a total refolding of α to β conformation. An increase in the area of stress-strain curve shows that these conformations enhance the energy-absorption capability of hair fibre. [40]

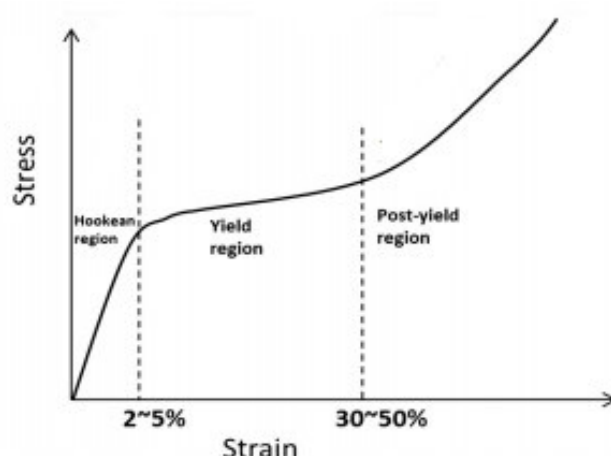


Figure 12 - Standard stress-strain curve profile for hair fibre tensile-test.[40]

Keratins extracted from human hair, wool or chicken feathers are reported as biocompatible, biostable and biodegradable materials. Keratins possess the physical and chemical characteristics for spontaneous self-assembly and polymerization, which allows the development of various types of biomaterials like scaffolds, films and hydrogels.[45]

Materials for cell culture coated with keratin improved cell adhesion and cell proliferation through cell-binding sequences such as LDV and RGD. [41] Keratin-based hydrogels were developed as hemostatic agents, wound healing dressings and as nerve conduit supports for

peripheral nerve regeneration. [46] Also, keratin thin films were developed to be used as *in vitro* nail plate models in permeability tests. [47]

1.3.1.3. Keratin-based peptide (KP)

Keratin-based peptide (KP) is based in an amino acidic sequence of KRT85 (Keratin, type II cuticular HB5) with peptide sequence $X_3CX_5CX_3$. It is composed by 13 amino acids with two residues of cysteine separated by 4 amino acids. In 2012, Fernandes & Cavaco-Paulo studied the ability of KP peptide to penetrate and restore some of the properties of damaged hair. [19]

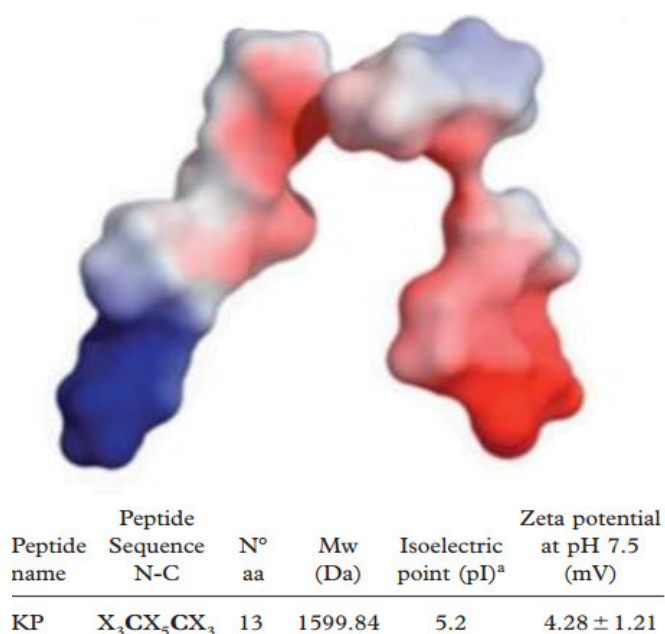


Figure 13 – Keratin-based peptide [$KP(X_3CX_5CX_3)$] surface charge analysis, from N- to C- terminal, as well as some KP characteristics at table. Red zones are identified for negative residual amino acids and at blue are the positive ones.[9]

In a posterior study, the effect of KP was tested in the mechanic and thermic properties of hair as well as the toxicity for skin in order to develop a new hair product. The results showed that KP penetrated in the hair fiber and improved its properties, even when the hair was not damaged. The easy penetration was related with the similar composition between KP and hair. Being like type II α -keratin, it can transfer its elasticity and malleability to the cortex of hair fibers. The toxicity results reveal that, when tested alone, irritation towards the skin was not present which could lead to the development of a cosmetic hair product. The structure of KP is depicted in Figure 13.[9]

1.3.1.4. Silk fibroin

Silks are fibrous proteins that are spun into fibres by some arthropods such as silkworms, spiders, scorpions, mites and fleas. Silk Fibroin (MW $\approx$ 391kDa) as a natural protein polymer presents advantages over synthetic polymers. It has unique qualities such as biocompatibility, biodegradability and bioresorbability which makes it environmentally friendly. Silk is involved in cocoon (i.e. *B. mori*) and web spider formations and in raw state it is composed by two main proteins: Sericin (glue-like) and Fibroin (filaments).[48–51]

Silk fibroin protein is mostly constituted by layers of antiparallel β -sheets with a regular amino acid sequence (Gly-Ser-Gly-Ala-Gly-Ala) $_n$. It has a high content of glycine and alanine and a tight packaging which improves silk's rigidity and tensile strength. Silk fibroin rigidity is also related with hydrophobic domain predominance as result of homogeneity in secondary structure. Combining stiffness and hardness make this a material that is used in several areas like biotechnology, biomedicine and cosmetics.[52,53]

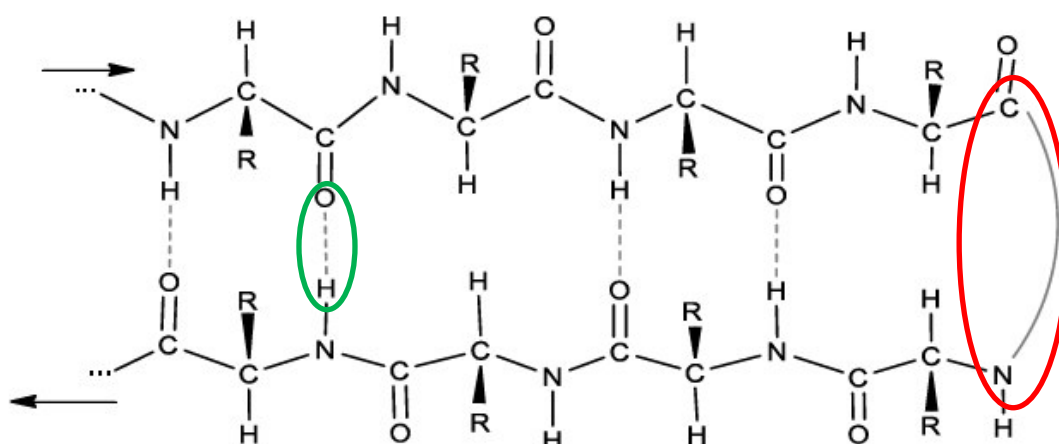


Figure 14 - Silk Fibroin β -sheet with hydrogen bonds (green) and van der Waals forces between C and N terminal (red) highlighted.

Three different crystalline structures have been described for silk fibroin: glandular state (silk I); crystalline silk structure and air/water interface (silk II and III respectively). Silk II presents a β -sheet conformation with the chains in an antiparallel orientation linked by hydrogen bonds between hydrogen side chains of glycine and methyl side chain of alanine, establishing strong intermolecular bonds and Van der Waals forces that will lead to a thermodynamically stable structure (Figure 14). [54]

Unique properties of fibroin make it a very versatile material for producing biofilms, modified textiles, scaffolds, contact lenses, hydrogels, micro and nanoparticles. [52]

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Chemical Reagents

All reagents used in this work are listed in Table II.

Table II – Reagents used in the work presented in this thesis.

Product	Brand
Acetic Acid glacial	Chem-Lab
Acetone	PanReac AppliChem
Acrylamide/Bisacrylamide (30 %/0.8 % (w/v))	Bio-Rad
Antibiotic Antimycotic Solution (100×)	Sigma
β -mercaptoetanol	Bio-Rad
Bovine Serum Albumin (BSA)	Sigma
Coomassie Blue	Merck
DC Protein Assay	Bio-Rad
Deoxycholate (DOC)	Sigma
Dimethyl Dulphoxide (DMSO)	Fisher Scientific
Dulbecco's modified Eagle's medium (DMEM)	Sigma
Ethylenediamine Tetraacetic Acid (EDTA)	Fluka
Ethanol Absolute	PanReac AppliChem
Fetal Bovine Serum (FBS)	Biochrom
Folin Reagent	Sigma

Glycerol 99 %	Sigma
Hydrochloric Acid (HCL)	Fisher Cientific
Hydrogen peroxide (H2O2)	Sigma
Imidazole	Sigma
IPTG	Fluka
L- Glutamine	Sigma
Lithium bromide (LiBr)	Sigma
Lowry Reagent	Sigma
Magnetic Beads	Biotoools
Methanol	Fisher Cientific
MTS	Promega
Protease cocktail inhibitor	Biotoool
Plus Protein All Blue Standard	Bio-Rad
Poloxamer 407	Sigma
Potassium Hydrogen Phosphate (K2HPO4)	Merck
Shampoo	Pantene
Sodium carbonate (Na ₂ CO ₃)	Sigma
Sodium dodecyl sulfate (SDS)	Sigma

Sodium Chloride (NaCl)	Panreac
Sodium metabissulfite	Sigma
Tetramethylethylenediamine (TEMED)	Bio-Rad
Trichloroacetic Acid (TCA)	PanReacQuimica
Tris(hydroxymethyl)aminomethane (Tris)	Fisher Cientific
Trypsin	Biochrom
Tryptone	PanReac
Urea	Sigma
Vegetable oil	Pingo Doce
Yeast extract	Sigma

2.1.2. Buffers and Solutions

Table III – Buffers and solutions used in experimental work presented in this thesis.

Running Buffer (1x)	25 mM Tris-HCl (pH 7.4), 192 mM glycine, 0.1% SDS
Laemmli Sample Buffer (5x)	250 mM Tris-HCl (pH 6.8), 50% Glycerol, 10% SDS, 0.1% Bromophenol Blue.
Binding buffer	20 mM Sodium Phosphate, 500 mM NaCl, 10 mM Imidazole, pH 7.4.
Washing buffer	0 mM Sodium Phosphate, 500 mM NaCl, 20-100 mM Imidazole, pH 7.4.
Elution buffer	20 mM Sodium Phosphate, 500 mM NaCl, 250-500 mM Imidazole, pH 7.4

Hair keratin extraction solution	8 M Urea, 0.2 M SDS, 0.5 M Na ₂ S ₂ O ₅
Phosphate Buffer Saline (PBS 1x, pH 7.4)	137 mM NaCl, 2.7 mM KCl, 8.5 mM Na ₂ HPO ₄ , 1.5mM KH ₂ PO

2.1.3. Culture media for bacteria

Several culture media were used throughout this work and are listed in Table IV.

Table IV – Growth media for *E. coli* cultures.

	Composition for 1liter of dH ₂ O, pH=7.0	Used for
LB	-10 g of Tryptone; -10 g of NaCl; -5 g of Yeast Extract	Overnight pre-inocules
LB-IPTG 0,1M	-LB composition; -100µm IPTG	Protein expression
TB	-12 g of Tryptone; -24 g of Yeast Extract; -2.5 mL of Glycerol;	Protein expression
TB AIM	-12 g of Tryptone; -24 g of Yeast Extract; -150 mg of MgSO ₄ ; -3.3 g of (NH ₄) ₂ SO ₄ ; -6.5 g of KH ₂ PO ₄ ; -7.1 g of Na ₂ HPO ₄ ; -0.5 g of Glucose; -2 g of alpha-lactose	Protein expression
TB-IPTG 0.1M	-12 g of Tryptone; -24 g of Yeast Extract; -2.5 mL of Glycerol; -100µm IPTG	Protein expression

2.1.4. Biological Materials

2.1.4.1. Bacterial strains

The *E. coli* strains used on this work have the following genotypes:

- XL1-Blue (Stratagene): endA1 supE44 hsdR17 thi1 recA1 gyrA96 relA1 lac [F' proAB lacI^q ZΔM15 Tn (Tet)]
- BL21 (DE3) (Novagen): F ompT hsdSB (r_B m_B) gal dcm (DE3)

2.1.4.2. Cell lines

In this project the human skin keratinocytes NCTC2544 cell line was used. The NCTC2544 cell line was supplied by Istituto Zooprofilattico Sperimentale Della Lombardia e Dell'Emilia-Romagna, Pavia (Italy).

2.2. Methods

2.2.1. Extraction of keratin and fibroin

2.2.1.1. Extraction and purification of keratin

Keratin was used as the core constituent of keratin-based particles. It was extracted from donated human hair obtained from a local barbershop. The hair samples were first washed according the IAEA/RL/50 1978 recommendations to remove contaminants and lipids. 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.

For the keratin extraction, a solution of 8 M urea; 0.2 M SDS; 0.5 M sodium metabisulfite in a ratio of 10:1 (solution to dry hair) was used. The mixture was heated at 100 °C for 45 minutes and then incubated overnight at 37 °C with constant agitation.

After extraction, the hair/keratin solution was centrifuged at 5000 rpm for 10 min and the supernatant was then filtered to remove hair fragments. The keratin solution was then dialysed for 5 days in distilled water using a dialysis membrane with a 14 kDa cut-off. The water was renovated twice per day. After dialysis, the keratin solution was quantified by Lowry's method and DC method

2.2.1.2. Extraction and purification of silk fibroin (SF)

The silk fibroin was extracted from *Bombyx mori* cocoons donated by Dr. Silvia Cappellozza from "Sezione Specializzata per la Bachicoltura" (Padova).

Obtaining a pure solution of fibroin is a time-consuming process in which the withdrawal of sericin by a degumming process, increases the biocompatible properties of the protein.

About 5 g of 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 silk fibroin fibres were then incubated in a solution of 9.3 M lithium bromide at 60 °C until total solubilisation. The soluble SF was then filtered to remove undissolved fibres and dialysed 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 keratin solution was quantified by Lowry's method and DC method. Processing of silk fibroin is summarized in Figure 15.

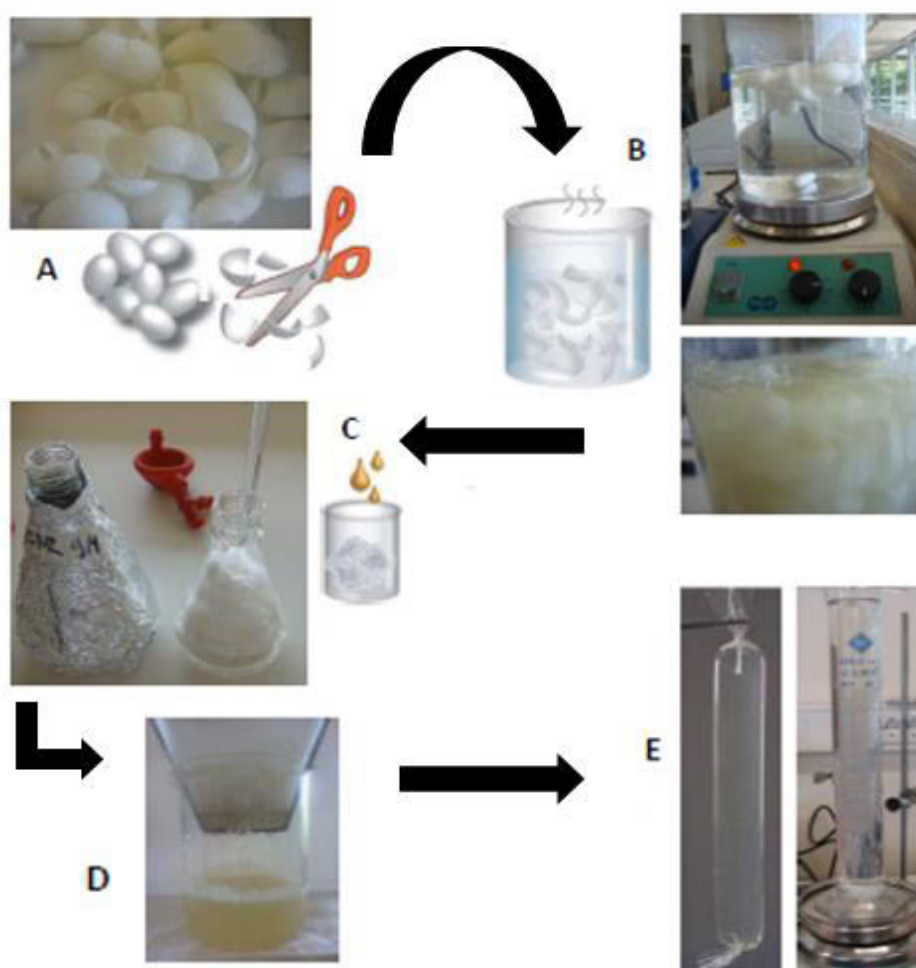


Figure 15 – Schematic representation of silk fibroin processing. *Bombyx mori* silkworm cocoons (A); Degumming process (B); Dissolution of degummed silk in LiBr 9 M (C); Filtration process (D); Dialysis process (E).

2.2.2. Protein quantification

Keratin and silk fibroin solutions were quantified by three methods: Lowry's method, DC method and Lyophilisation.

2.2.2.1. Lowry's method

The Lowry's method quantifies protein by its absorbance at 750nm against a standard curve. To quantify the extracted keratin and the SF, diluted solutions of both proteins were used as well as other solutions: DOC (1,5 mg/ml), TCA 72% (v/v), Vial of Lowry's reagent and Follin 20% (v/v).[55]

The steps of Lowry's protocol are resumed on Table V. The protein concentrations were expressed in mg/ml.

Table V – Steps of Lowry's protocol.

Step 1	Add 50 µl of DOC solution to 400 µl of diluted protein solutions
Step 2	Vortex and rest for 10 min
Step 3	Add 50 µL of TCA
Step 4	Centrifuge for 10 min at 5000 rpm and discard supernatant
Step 5	Add 400 µL of distilled water and 400 µL of Lowry's reagent
Step 6	Vortex and rest for 20 min
Step 7	Add 200 µL of Follin reagent
Step 8	Vortex and rest for 30 min
Step 9	Place 200 µL of Lowry treated solution in a 96 well microplate
Step 10	Read the absorbance at 750 nm

2.2.2.2. DC method

The DC method (Biorad) uses two different solutions: 200 µl of solution A and 25 µl of solution B and 20 µl sample of protein solution to be quantified. This process has a range from 0.2 to 1.5 mg/mL and sample dilution was required. Protein samples were prepared in a 96 well microplate and after 15 minutes of incubation in dark at room temperature, the absorbance was measured at 750nm. The protein concentrations were determined against a BSA standard curve.[56]

2.2.2.3. Lyophilisation

The lyophilisation or freeze-drying was invented in 1906 by Arsène s'Arsonval and consists in a dehydration process that works by freezing the material and reducing surrounding pressure to allow frozen water in the material to sublime directly from solid phase to gas phase.[57] 1 ml of protein solutions were stored in previous weighted centrifuge tubes and sent to lyophilisation process. After three days, the tubes containing the lyophilized proteins were weighted and the difference was expressed as the final protein concentration in mg/ml.

2.2.3. Production of Protein-based Particles

Eight different formulations of keratin-based particles were obtained by High Pressure Homogenization (HPH). The particles had the general formula:

$$\text{Keratin}_x\text{SilkFibroin}_y\text{Poloxamer407}_z \text{ (K}_x\text{SF}_y\text{P}_z\text{)}$$

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 0.05% of Poloxamer 407, when present. The homogenization process occurred at 240 – 580 bar pressure during 10 – 13 mins (3 – 5 mins of open cycles and 7 – 8 mins of close cycles) for each particle. The nanoemulsions obtained after homogenization cycles were centrifuged at 4000 rpm for 10 mins at 4 °C in Amicon tubes (Amicon Ultra-15 Millipore) to separate the free protein from particles. The free protein was quantified by Lowry's method and the nanoparticle formation efficiency was estimated for each formulation.

2.2.4. Particles Characterization

To study the physical properties and stability along time the keratin-based particles were characterized regarding their size, net charge and structure.

2.2.4.1. Dynamic light scattering (DLS)

Dynamic light scattering is a technique used to get size distribution, polydispersity index (PDI) and Zeta-Potential of particles.[58] Particles samples were analysed, by correlation spectroscopy using a Nanosizer Malvern ZS, at 25 °C with values of viscosity and refractive index of 0.8872cP and 1.330, respectively, for protein standards (pH = 7.4).

Each sample was measured in triplicate and presented as mean value with standard deviation. Keratin-based particles were kept at 4 °C and measured weekly until month one and then were measured monthly until month three.

2.2.4.2. Scanning Transmission Electron Microscopy (STEM)

To study size and surface distribution, keratin-based particles and treated hair fibres were coated with 80% Au and 20% Pd and observed at 5.0 kV with SEM (NOVA NanSEM 200 FEI).

2.2.4.3. 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 analysed 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 was allowed to vary, then, the base line and bandwidth were fixed and fitted again, and finally, the base line and centre 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.

FTIR characterization was performed for keratin particles formulations and for recombinant proteins with the same specifications.

2.2.5. Cellular viability assays

2.2.5.1. 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 25 and 75 cm² tissue culture flasks at 37 °C in a humidified atmosphere with 5 % CO₂.

The cell culture medium was renewed twice per week. For subcultures and plating, the adherent cells were detached with a trypsin solution 0.05%, and fresh medium was added in order to neutralize the trypsin. The cell suspension was centrifuged 5 min at 160 g. The supernatant was discarded and fresh medium was added to obtain a new cell suspension. The cell suspension was loaded in a Neubauer chamber and the concentration of cells present in the suspension was estimated.

2.2.5.2. Cell viability assessed by MTS assay

The MTS assay is a colorimetric method used to measure the toxicity of different substances in cells. The principle of this assay is that, in viable cells, mitochondrial activity is constant and therefore an increase or decrease in the number of viable cells is linearly related to mitochondrial activity. MTS is a tetrazolium reagent that can be reduced by viable cells resulting in formazan products soluble in cell culture medium. This conversion is thought to be carried out by NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells. [59][60]

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.

NCTC 2544 cells were incubated at 37 °C in a humidified atmosphere with 5 % CO₂. At the end of 24 and 48 h of contact, cell metabolic activity was assessed by MTS viability assay. After

incubation, the medium with the particles was removed and 100 μ l of fresh medium with 100 μ l of MTS were added to each well, and cells were incubated at 37 °C for 2 h. The medium with reduced MTS was transferred to a 96 microwell plate and colour intensity was measured with 96-well plate reader at 490 nm in a microplate reader SpectraMax Plus (Molecular Devices).

2.2.6. Hair Fibres studies

2.2.6.1. Hair fibre washing

Black Asian hair fibre samples (0.1 g) were washed before treatment with a classic commercial shampoo (Pantene). After washing, hair fibres were placed at 30 °C until completely dry.

2.2.6.2. Hair fibre bleaching

After washing, hair samples were subjected to eight cycles of bleaching (8xB). Bleaching consisted on the application of 12% H₂O₂ (v/v) in the presence of 0.1 M Na₂CO₃/NaHCO₃, pH 9.0 buffer at 50 °C for 1 h, in a bath ratio of 1:10 at 20 rpm, using a Rota-wash laboratory machine (MKII Series 7227, Shirley Developments). Treatments were applied to the same tresses of hair.

2.2.6.3. Hair fibre treatment with keratin-based particles

Hair samples (virgin and overbleached Asian hair) were 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. Treated hair samples were analysed by SEM as described previously.

2.2.6.4. Hair fibre 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 fibre tensile testing. For this test, a set of hair fibres 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 fibres 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; $70 \pm 5\%$ humidity) at a rate of 2.5 mm/min with 0.01 N preload force, and stretched until fibres broke. All measurements were made in the middle part of the hair fibre.

Results were presented in Young's module (mPa), and define the relationship between stress and strain, which is related to material strength and elasticity.[61]

2.2.3. Recombinant Crystallins Production and Purification

For the wild-type crystallin (Cwt) and mutant crystallin (Cmut) biosynthesis, the genes based on the sequence of human eye γ D-crystallin were synthesized by GenScript USA Inc. (Piscataway, NJ, U.S.A.) and cloned on the expression vector pET-28a (+). *E.coli* XL1-Blue and BL21 (DE3) strains were transformed with the recombinant vectors, pet28a (+)::KP-(GA)₅-CrysWt and pet28a (+)::KP-(GA)₅-CrysMut.

2.2.3.1. Transformation of Competent Cells- TSS method

The TSS “Transformation and Storage Solution” method is a combination of two steps from the transformation procedure, firstly competent cells are obtained and secondly the cells, using the same solution, are transformed resulting in transformation efficiency that goes up to 10⁷-10⁸ cfus (colony forming units) per microgram of DNA. This method was applied for the preparation of competent cells of the *E. coli* BL21(DE3) and for XL1-Blue MRF' cells.

A single colony, isolated and grown in a LB-agar plate, was used to inoculate 10 mL of LB medium and was grown at 37 °C with shaking (250 rpm), until a OD₆₀₀=0.3-0.4 was reached. At this point the metabolism and cell growth was stopped by incubation on ice for 5 minutes. The cell suspension was centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 1 mL of cold x1TSS solution. About 1-10 ng of plasmid in final volume of 1-10 μ L were added to the mix. The cellular suspension plus the plasmidic DNA were kept on ice 1 h and subsequently the cells were subject to a heat shock by exposition of the mix at 42 °C for 2 minutes. The heat shock was stopped by immersion on ice for 2 min. After it 1 mL of warm LB medium was added and the suspension was incubated 1 h at 37 °C with shaking (250rpm) in order to activate and express the gene that confers resistance to the selective marker (kanamycin).

For last, 50-200 μ L of the transformation mix were plated in LB-agar-kanamycin plates and were incubated for 16-20 h at 37 °C.

2.2.3.2. Glycerol Stock

To maintain and store the clones with interest, glycerol stocks were made. The selected colonies were grown at 37 °C with shaking (250rpm) on LB until an $OD_{600}=0.6-0.8$ was reached. Cells were then centrifuged for 5 minute at 500 rpm. The pellet was then resuspended in 30% sterile glycerol and the cells were kept on cryovials at -80 °C.

2.2.4. Plasmid Purification

For plasmid purification, the GeneJET Plasmid Miniprep from ThermoFisher Scientific kit was used following the manufacturer's instructions. The method was based on bacteria alkaline lyses followed by a partial purification and selective DNA adsorption to a silica gel.

2.2.4.1. Plasmid Quantification

The Thermo Scientific NanoDrop 1000 spectrophotometer is an instrument with high accuracy and precision. For DNA quantification 1 µl of sample was applied in receiving fibre. Source fibre was then brought into contact causing a liquid bridge connecting the fibres gap (1 mm). Focusing a pulsed xenon flash lamp and measuring absorbance at 280 nm, in triplicate, was possible to get the concentration and purity of our plasmids. [62]

2.2.4.2. DNA digestion with restriction enzymes

Reaction conditions (temperature, concentration, time of reaction, buffer) for the DNA digestion are supplied by the enzyme manufacturer. The rate of digestion was controlled by DNA agarose gel electrophoresis. Plasmids digestion were performed with *NdeI* and *NotI* restriction enzymes (ThermoFisher Scientific) to verify the size of Cwt and Cmut constructs in pet-28a (+) plasmid. Plasmids digestions were made with 6µL of DNA, 1 µL of FastDigest buffer, 0.5 µL of respective enzymes, 2 µL of UpWater and digested for 1 h at 37 °C. In this period a 1% agarose gel was prepared. 5 g of agarose were dissolved in 50 mL of TAE 1x buffer and boiled to complete dissolution. Boiled agarose solution was placed in the gel kit and 2µL of Gold View stain were added. The agarose solution was let to set in dark for 45 min. Electrophoresis was ran at 90W for 50 min at room temperature and the gel was analysed with Chemidoc machine software and molecular weight was determined using a DNA molecular weight marker.

2.2.5. Recombinant Crystallins Expression

The expression strains used are bacteriophage λ DE3 lysogenic. The λ DE3 is a recombinant phage carrying the cloned gene for T7 RNA polymerase under *lacUV5* control, and IPTG inducible. The differences between the strains lays in the absence of the *recA* recombinant complex on the BLR(DE3) strain. This complex is associated with *in vivo* recombination of highly repetitive DNA.

The strains were transformed with the recombinant vectors by the TSS method. And the selected colonies were plated on LB-agar medium supplemented with the kanamycin.

2.2.5.1. Expression screening

To assure a good protein expression, the best transformant needs to be chosen. Eight transformants of each construct were inoculated in a ratio 1:5 of culture media (LB) for air, and were grown overnight at 37 °C with constant agitation (200 rpm). The transformants were then inoculated in auto-induction media to a final $OD_{600}=0.1$ and were grown overnight at 37 °C with constant agitation (200 rpm). To analyse the total protein fraction composition 1 mL of culture media of each transformant was taken. The cells were spin down by 1 min cold centrifugation at 12000xg. The pellet was resuspended in 100 μ L of running buffer and 25 μ L of loading buffer. The samples were boiled for 5 minutes and after a spin-down, a volume corresponding to a $OD_{600}=0.1$ was loaded on a SDS-Page. To select the best expression media the best transformant was inoculated in LB, LB+IPTG, TB, TB+IPTG in the same condition as described previously.

2.2.5.2. Crystallins expression

Expression of recombinant crystallins was achieved from an overnight inoculum. For autoinduction media BL21 cells were grown at an initial $OD_{600}= 0.1$ in 1L of media for 24 h at 37 °C with constant agitation (200 rpm). For induction, 100uL of 1M IPTG were added when cultures reached $OD_{600}=0.8-1.0$, and cells were grown for 4h at 37 °C with constant agitation (200 rpm). Final cultures were transferred to centrifuge tubes and centrifuged at 10000 rpm for 30 min at 4 °C. The pellets were then resuspended in Phosphate solution (Phosphate Buffer pH 7.4, 10mM Imidazole) for further sonication.

2.2.5.3. Analysis of soluble and insoluble protein fractions

After 24 h production in TB-AIM at 37 °C the bacteria growth was stopped by cooling down the cells to 4°C. Final cultures were transferred to centrifuge tubes and centrifuged at 10000 rpm for 30 min at 4 °C. The pellets were then resuspended in Phosphate solution (Phosphate Buffer pH 7.4, 10mM Imidazole) for further sonication.

Bacteria were disrupted by sonication cycles (6 s on - 8 s off) at 40Hz in a Sonicator 3000 from Misonix. During sonication, samples were kept on ice to avoid protein denaturation. Last, the suspension was centrifuged at 4°C for 30 minutes at 10000 rpm and fractions were collected. The supernatant corresponded to the soluble fraction while the pellet corresponded to the insoluble fraction.

2.2.6. SDS-Page

SDS-Page (polyacrylamide gel) is widely used in molecular biology to separate biological macromolecules such as proteins, according to their electrophoretic mobility. Proteins can acquire many different conformations, different charges and lengths. The use of an anionic detergent (SDS) transforms the molecule into an unstructured linear chain whose mobility depends only on its length and mass-to-charge ratio. The binding of SDS to the proteins chains imparts an even distribution of charge per unit mass, resulting bands give to us the approximately size comparing to the ladder marker[63].

After preparation, samples were loaded and run at 20 mV in a SDS-PAGE gel with 12.5% concentration (Table VI). The Precision Plus Protein All Blue Standard molecular weight marker, which contains sharp bands between 10 and 250 kD, was applied in each SDS-PAGE gel to be used as reference for the molecular weight of expressed crystallins.

Table VI - Composition of resolving and stacking gel in SDS-Page. Volumes described are for one gel of 1.5mm thickness

<i>Acrylamide (%)</i>	<i>Stacking gel 4%</i>	<i>Resolving gel 12.5%</i>
Acrylamide/Bis-acrylamide (30%/0.8%(w/v)) (Biorad)	0.7mL	4.125 mL
0.5 M Tris-HCl (pH 6.8)	1.3mL	-
1.5 M Tris-HCl (pH 8.8)	-	2.5mL
10% SDS	50uL	0.1mL
Water	2.92mL	3.22mL
*10%(w/v) APS	37.5uL	75uL
*TEMED (Biorad)	10uL	10uL

* Added before gelification

2.2.6.1. Protein Polyacrylamide Gel Electrophoresis Staining

Following electrophoresis, the gel was stained with Coomassie Brilliant Blue for visualization of the separated proteins. The protein gels were immersed 45 min in a solution of 0.125% (w/v) Coomassie Brilliant Blue R-250, 40% (v/v) methanol and 10% (v/v) acetic acid with constant agitation.

After incubation, the staining solution was removed and gels were rinsed 3 times with tap water. The gels were then immersed in destaining solution (40% (v/v) methanol and 10% (v/v) acetic acid) for 1 h. After 1 h the destaining solution was removed and gels were immersed in water and visualized.

2.2.7. Protein Purification

Protein purification by magnetic particles are getting a considerable importance in recent years because they have a good purification yield and they are easy to work with. [64] Nickel beads (Biotools®) were used to purify the recombinant crystallins which had a His-Tag on the N'-terminal. The purification of CrysWt and CrysMut recombinant proteins was done according to manufacturer's instructions (Figure 16).

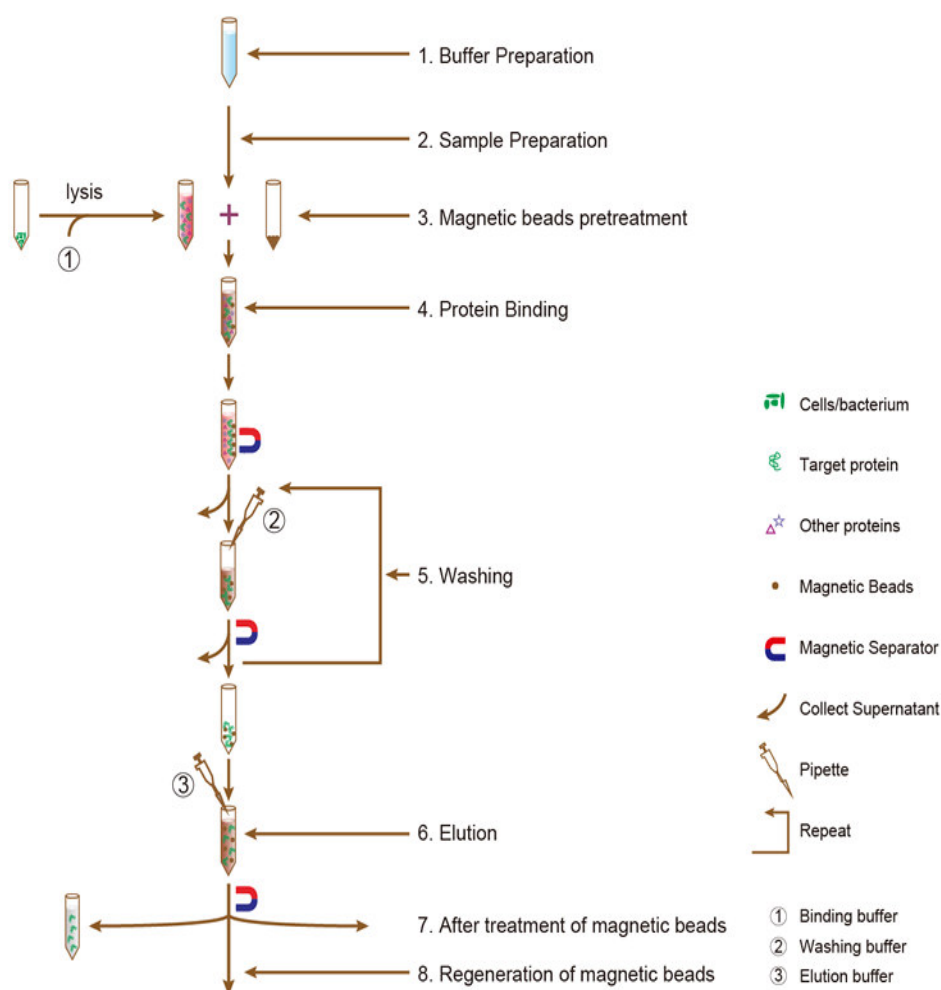


Figure 16: Schematic representation of protein purification by Magnetic Beads (Biotool).

2.2.8. Determination of crystallins molecular weight by MALDI-TOF

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules and large organic molecules like polymers, which tend to be fragile and fragmented when ionized. MALDI is a three-step process. First, the sample is mixed with a suitable matrix material and applied to a metal plate. Second, a pulsed laser irradiates the sample, activating ablation and desorption of the sample. Finally, the analyte molecules are ionized by being protonated or deprotonated in the hot plume of ablated gases, and mass spectrometer is used to analyse them.[65]

Lyophilized protein samples were dissolved in TA 30 solvent (30:70 [v/v] acetonitrile:0.1% TFA in water). About 5µL of matrix, sinapinic acid in 70% acetonitrile and 0.1% trifluoroacetic acid was added to the MALDI plate with 5µL of cold water dissolved samples. The plate was air dried and the mass spectrum was obtained in a MALDI-TOF Bruker Autoflex whit nitrogen laser with the following parameters: source 19.5 kV, extraction 18.3 kV, reflector 1.89 kV.

The equipment was calibrated with a peptide mixture: Bradykinin 1-7 (757Da), Angiotensin II (1047Da), Angiotensin I (1297Da), Substance P (1347Da), Bombesin (1620Da), Renin Substrate (1760Da), ACTH clip 1-17 (2094Da) and ACTH clip 18-39 (2466Da).

Chapter 3: Results and Discussion

3.1. Keratin-based particles

The keratin-based particles were prepared by high pressure homogenization (HPH). This technique is a high-energy method where nanoemulsions are obtained by continuous disruption of vegetable oil droplets. Eight different formulations of keratin-based particles were obtained. The particles had the general formula:



where $x = 80, 90, 95$ and 100% ; $y = 0, 5, 10$ and 20% ; $z = 0$ and 5% (w/v). The solutions were prepared in PBS 1x pH 7.4 to a final protein concentration of 10 mg/mL with 0.5% (v/v) of vegetable oil and 0.05% of poloxamer 407 when present.

The amount of vegetable oil ratio used as a cross-link agent was previously optimized in the research group and was used in all formulations. [66] Silk fibroin and poloxamer 407 were added to improve the properties of the particles. Silk fibroin is rich in beta secondary structures, and could improve nanoparticles strength and rigidity contributing for the maintenance of particles structure for longer times. Poloxamer 407 (Figure 17) is a tri-block polymer molecule with ethylene (PEO) and propylene (POP) polyoxides as backbone ($\text{PEO}_A\text{-POP}_B\text{-PEO}_A$). Poloxamer 407 shows a high bio-adhesion increasing the local retention time of formulations and also, due its hydrophilicity, modifies surface charge and stabilizes the nanosystems. [67,68]

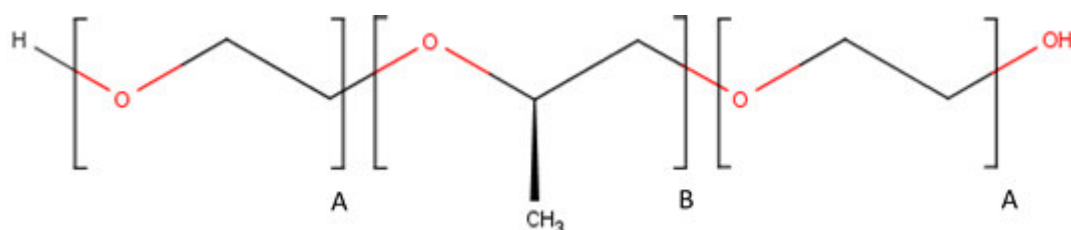


Figure 17 –Polymeric structure of Poloxamer 407

3.1.1. Particle Formation Efficiency

After the synthesis of the keratin-based nanoparticles the efficiency of particle formation was determined by the quantification of free protein. The particle formation efficiency was determined using the formula:

$$\text{Particle formation (\%)} = \frac{[Ci] - [Cf]}{[Ci]} \times 100$$

where Ci and Cf are the initial protein concentration and the free protein, respectively. The results for particle formation efficiency are shown in Table VI.

Table VI – Particle formation efficiency determined by Lowry's method

Particle	Particles Formation efficiency (%)
K ₁₀₀	98.5
K ₁₀₀ P	99.8
K ₉₅ SF ₅	97.1
K ₉₅ SF ₅ P	92.5
K ₉₀ SF ₁₀	98.4
K ₉₀ SF ₁₀ P	98.2
K ₈₀ SF ₂₀	97,3
K ₈₀ SF ₂₀ P	97,1

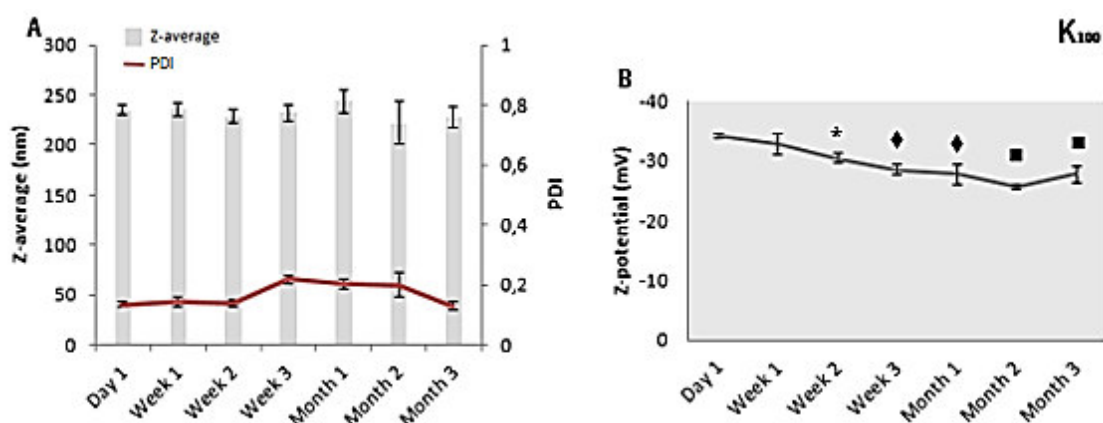
The efficiency of particle formation ranged from 97 to 100 %, which clearly demonstrates that the proteins from the formulations were able to form particles during the HPH process. The results were in line with other results using the same methodology. [66,69]

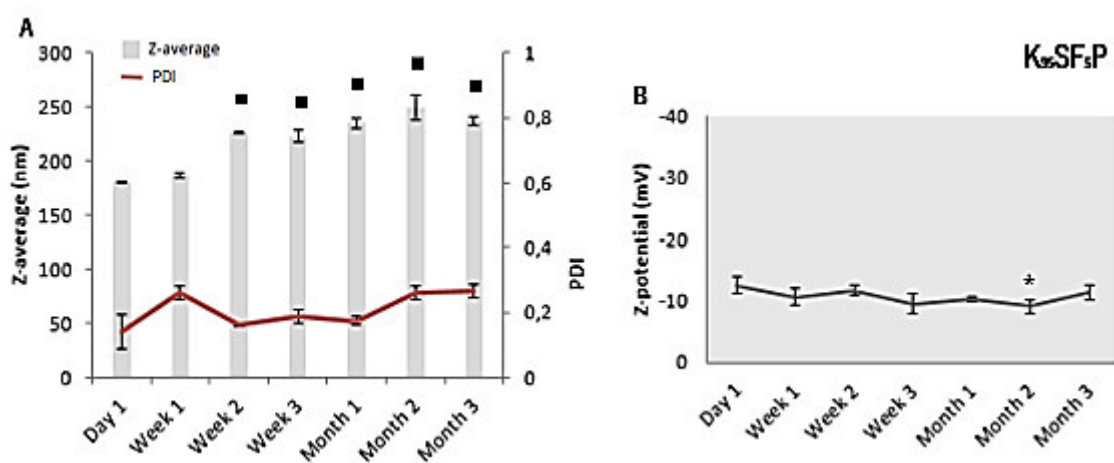
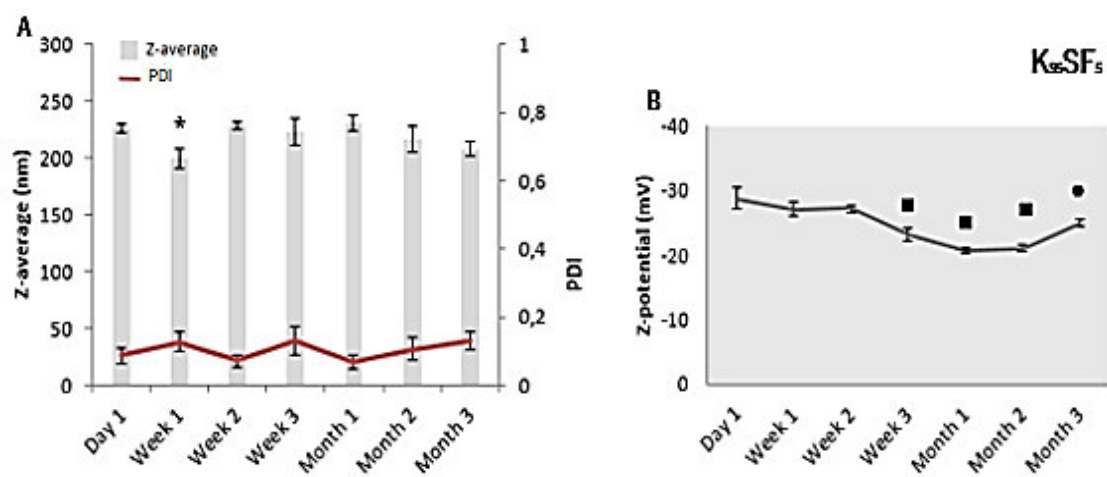
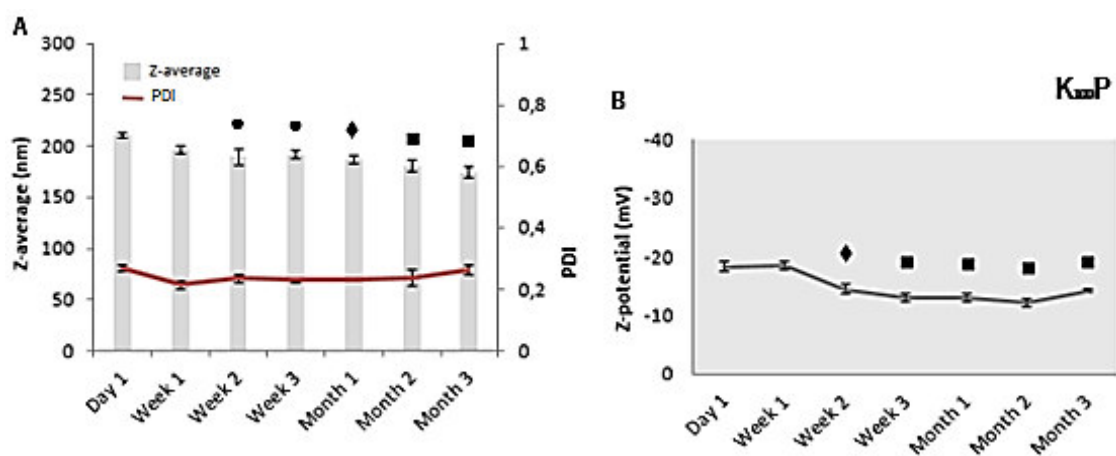
3.1.2. Particles Physicochemical Characterisation

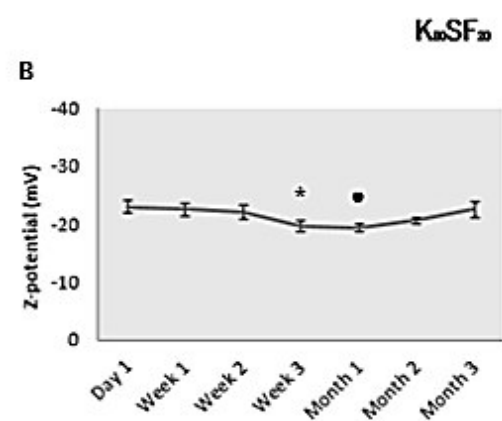
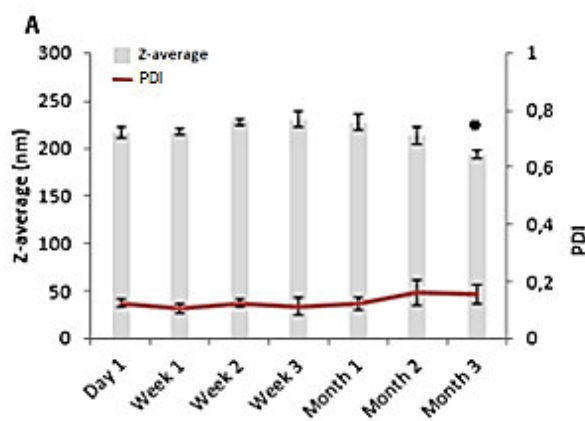
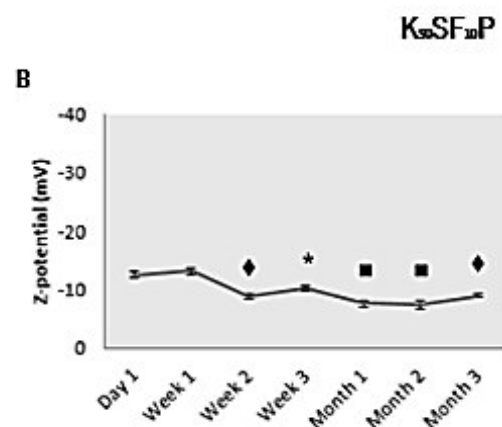
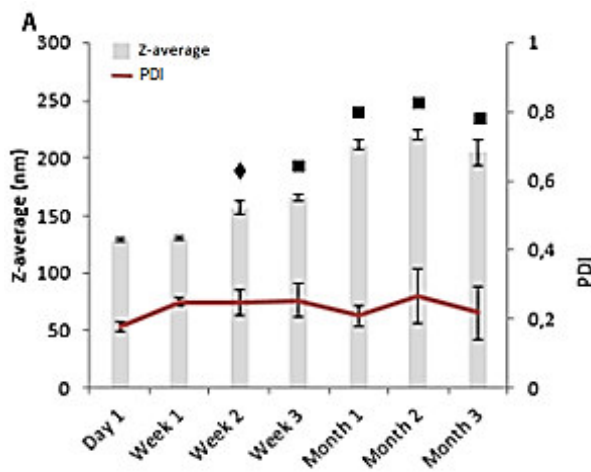
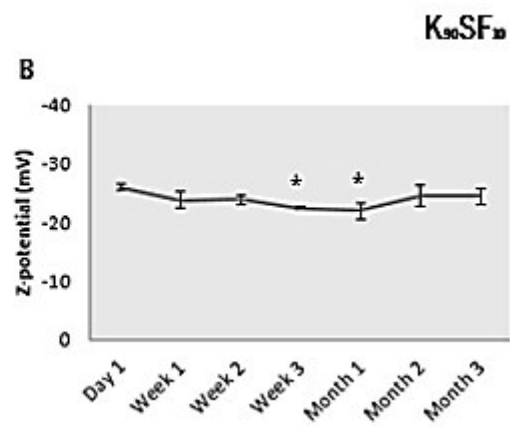
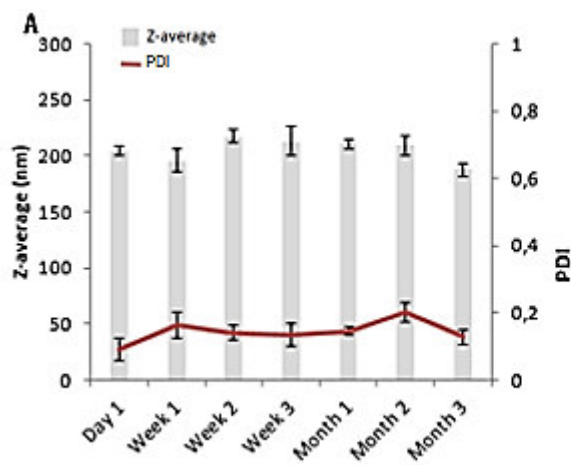
3.1.2.1. Dynamic Light Scattering (DLS)

DLS is a widespread method to study the size distribution, polydispersity index (PDI) and surface charge (Zeta-Potential) of particles. The study of these properties along time allows to characterize the stability of particles, which is a key parameter during the development of formulations. [69–73] The particle size is defined as the size of a hypothetical hard sphere that diffuses in the same way as that of the particles being measured. The result is reported as a mean particle size and homogeneity of size distribution (PDI). A PDI value from 0.1 to 0.25 indicates a narrow size distribution, and a PDI value greater than 0.5 corresponds to a broad size distribution. Surface charge, expressed as Zeta-Potential, critically influences the interaction of a particle with its environment. Usually, particles with ζ -potential more positive than +30 mV or more negative than -30 mV have colloidal stability maintained by electrostatic repulsion. [74]

The Z-average and PDI of particles were assessed by Photon Correlation Spectroscopy (PCS) whereas laser Doppler Anemometry was employed for Zeta-Potential. The particles physical stability was evaluated over 3 months, by weekly measurements of Z-average, PDI and Zeta-Potential of the particles stored at 4 °C. The physical stability of the keratin-based particles is represented on Figure 18. The values for Z-average, PDI and Zeta-Potential after 3 months of storage are represented in Table VII.







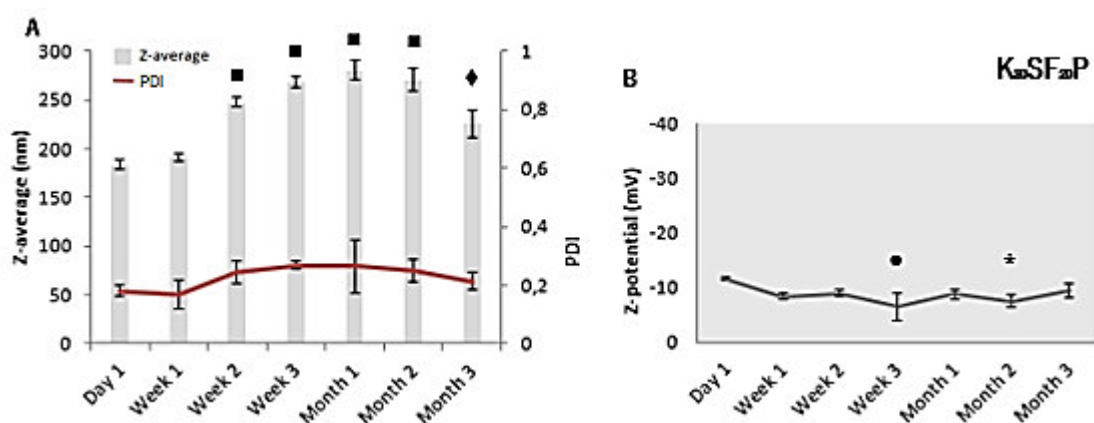


Figure 18 - Characterization of keratin-based particles, during storage at 4 °C: A) Z-average and PDI; B) Zeta-Potential. Data were analysed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤ 0.001; ■ p-value≤0.0001, compared to day 1.

Table VII – Values of Z-Average, PDI and Zeta-Potential for keratin-based particles after 3 months of storage at 4 °C. Data were analysed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001 compared to K₁₀₀ particles.

Particles	Z-Average (nm)	PDI	Z-Potential (mV)
K ₁₀₀	227.5 ± 11.3	0.162 ± 0.018	-30.3 ± 1.0
K ₁₀₀ P	188.9 ± 4.9 [■]	0.239 ± 0.015 [■]	-15.4 ± 0.7 [■]
K ₉₅ SF ₅	217.9 ± 7.3 [■]	0.104 ± 0.027 [■]	-25.1 ± 0.8 [■]
K ₉₅ SF ₅ P	204.4 ± 7.2 [■]	0.139 ± 0.025	-24.5 ± 1.1 [■]
K ₉₀ SF ₁₀	220.3 ± 5.9 [●]	0.124 ± 0.025 [♦]	-21.1 ± 1.0 [■]
K ₉₀ SF ₁₀ P	216.7 ± 3.9 [■]	0.192 ± 0.026 [●]	-11.2 ± 1.1 [■]
K ₈₀ SF ₂₀	167.5 ± 5.1 [■]	0.225 ± 0.039 [■]	-10.3 ± 0.7 [■]
K ₈₀ SF ₂₀ P	230.3 ± 7.2	0.225 ± 0.038 [■]	-8.9 ± 1.1 [■]

The results depicted on figure 18 and on Table VII demonstrate that the keratin-based particles were stable along storage at 4 °C. The keratin-based particles presented narrow size distribution between 150-250 nm and a net negative surface Zeta-Potential. Considering

composition and time, some statistical significant differences were identified when analysing the properties of the keratin-based particles' PDI.

When the surfactant was added to the formulation, there was a general decrease on particle size except for the $K_{80}SF_{20}P$ particles. Surprisingly, this reduction on particle Z-average was not accompanied by a decrease on PDI. Poloxamer 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. This compound is used not only to control the particle size, particle size distribution and shape but also to prevent particles to aggregate and coalescence. Most likely the increase on PDI is related with the formation of particles of Poloxamer 407 during the homogenization process which will contribute to a more disperse population of particles. Regarding the Zeta-Potential of the particles, a reduction of the surface charge potential was seen after adding Poloxamer 407 to the formulations. This reduction could be explained by the coating of the particles by the nonionic surfactant Poloxamer 407, which shielded the surface charge making it less negative.[66]

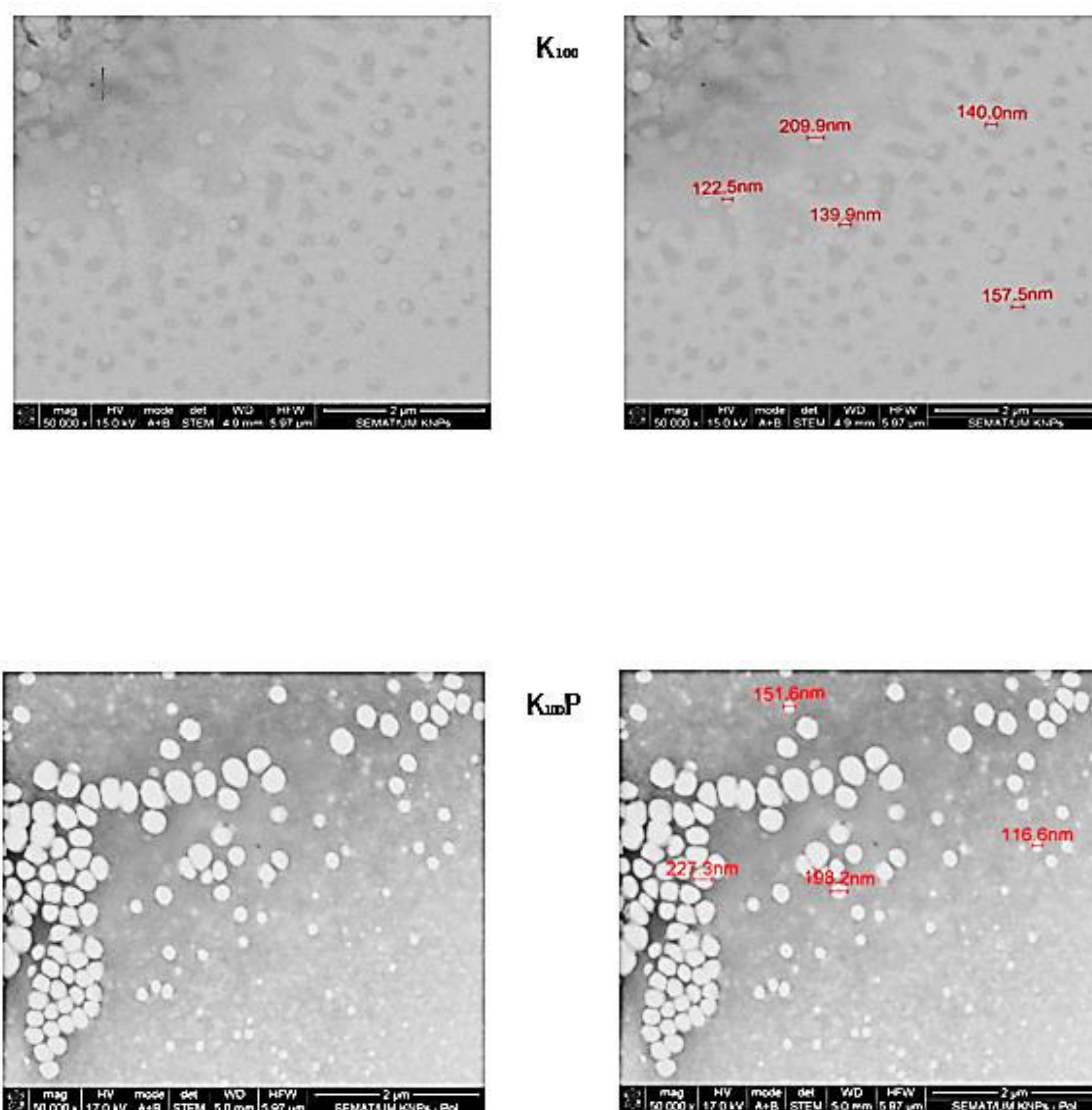
The $Ker_{80}SF_{20}P$ formulation did not follow the same trend regarding the variation of the Z-average upon addition of surfactant, which could be related with the higher concentration of silk fibroin. It is described in literature that Poloxamer 407 propylene oxide chains are not strong enough to be ideally absorbed in silk fibroin particles, so these results could be due the presence of this protein on particles' surface, interfering with the polymer effect.[75]

Even though adding silk fibroin and Poloxamer 407 almost did not change the particle Z-average and PDI, it had a strong influence on the Zeta-Potential. There was a decrease on the net negative charge of the particles when the silk fibroin and the Poloxamer 407 were added, separately or together. Keratin side chains are negatively-charged ($R-SO_3^-$) and, with the addition of silk fibroin and Poloxamer 407, were masked resulting in a decrease on the Zeta-Potential to less negative values. [76]

3.1.2.2. Particles Morphology

To study the morphology of the keratin-based particles obtained by HPH, the developed formulations were visualized by STEM. HPH is a method that usually produces spherical particles conferring a better area/volume ratio which is directly related with nanoparticle stability.[77]

The size and structure of the keratin-based particles is observable in the STEM micrographs in Figure 19.



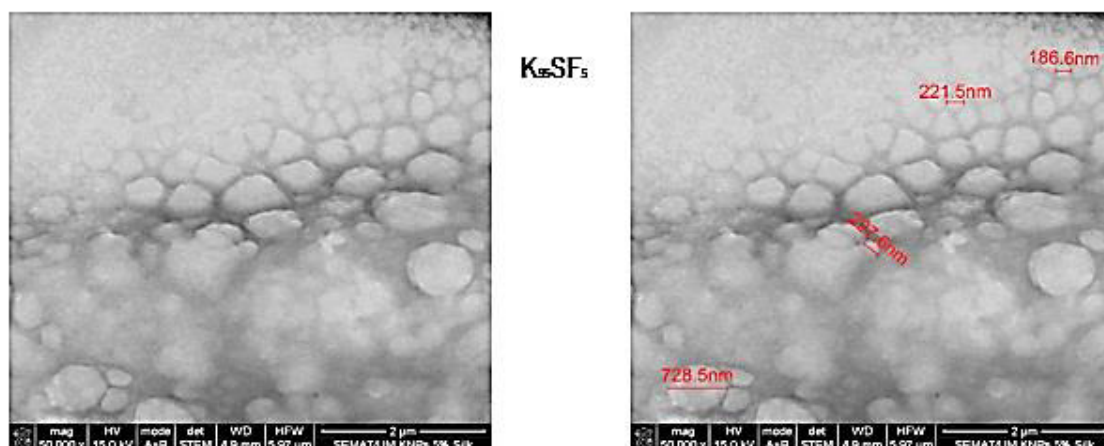


Figure 19 - STEM microphotographs (50000x magnification) of keratin-based particles obtained by high pressure homogenization.

All particles were spherical and the sizes were in accordance with the results from DLS. It was also evident the effect of the Poloxamer 407 and silk fibroin on the particles surface and structure. The $K_{100}P$ particles presented a dense coat surrounding the particles' shell, highlighted by a red arrow. This coat was constituted by Poloxamer 407, a hydrophilic surfactant which tends to accumulate on the interface of oil/water systems contributing for their stabilization.

The addition of silk fibroin to the particles formulation affected the smoothness of the particles. While K_{100} and $K_{100}P$ particles presented smooth surfaces, the $K_{95}SF_5$ particles had more irregular surfaces. The accommodation of silk fibroin to the water/oil interface tends to promote self-assembly and β -sheet formation which was reflected on the structure of the particles.

Also, during the preparation of particle samples for STEM analysis, there was a dehydration step which could induce some structural changes on particle surfaces. This step removes water from the environment exposing the carboxyl and amine groups, leading to the reaction between these two groups in different amino acids and thus some structural changes and artefacts may be observed. [78]

3.1.2.3. Particles' proteins secondary structure

FTIR is a technique used to analyse protein secondary structure. Infrared (IR) spectroscopy is based in the fact that each molecule absorbs distinct wavelengths, causing molecular vibration, which can be detected.[79,80]

The ATR method uses a thin protein film deposited on an internal reflection element (IRE), for the keratin-based particles potassium bromide (KBr) was used as IRE. Then IR radiation passes through IRE material and interacts with material at the interface of these two compounds, sample and IRE.[81]

The keratin-based particles were produced by high-energy emulsification using high-pressure homogenization (HPH). Briefly, in this process, oil-in-water samples were submitted to disruptive forces, such as hydraulic shear, intense turbulence and cavitation[82]. When high shear forces are applied to the biphasic system of water/oil, the proteins tend to adapt their structure and migrate to the interface. Depending on the protein structure, its adaptation may include conformational changes of the proteins. [83–85]

To study if and to what extent the emulsification process modified the particle's proteins secondary structure, FTIR spectra were acquired (Figures 20 - 24). To assess the type of secondary structures present, a deconvolution of Amide I ($1600\text{-}1700\text{ cm}^{-1}$) region [80,86] was performed and the results are presented on Tables VIII and IX in percentages.

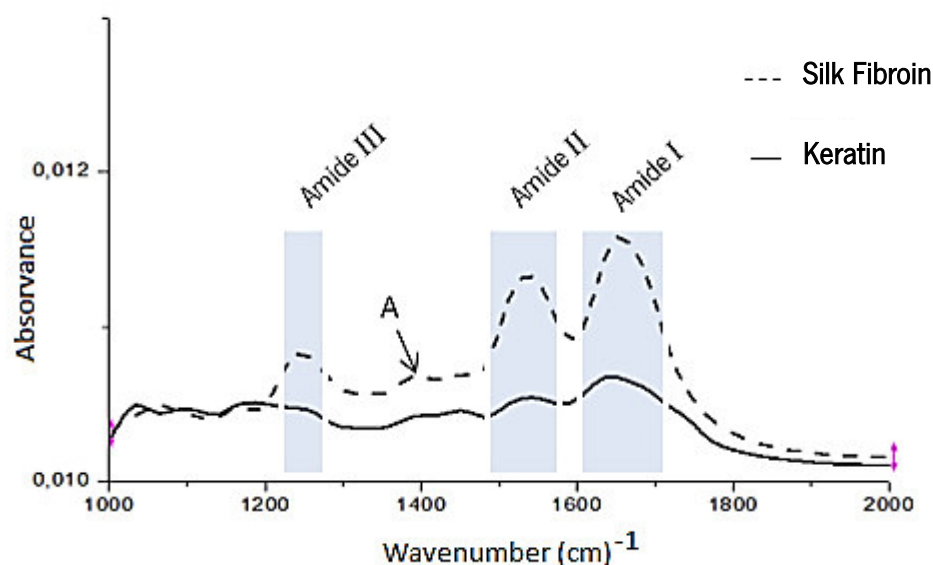


Figure 20 - FTIR spectra of keratin (solid line) and silk fibroin (dash line). The three amides regions are highlight: Amide I ($1600\text{-}1700\text{ cm}^{-1}$) C=O stretch; Amide II ($1510\text{-}1580\text{ cm}^{-1}$) N-H bending; Amide III ($1230\text{-}1270\text{ cm}^{-1}$) C-O stretch; as well the peak at 1400 cm^{-1} peak (A). [80]

Analysing the FTIR spectrum of keratin and silk fibroin (Figure 20), the silk fibroin presents higher absorbance than the keratin for all amide regions. Fibroin with higher molecular weight can explain this increase. Also, the absorbance for the peak at 1400cm^{-1} , which corresponds to the COO^- protein side chain, was superior in the fibroin.[87] This could be the evidence of silk fibroin instability in aqueous solution, tending to gelify with side chain – side chain interactions.[88]

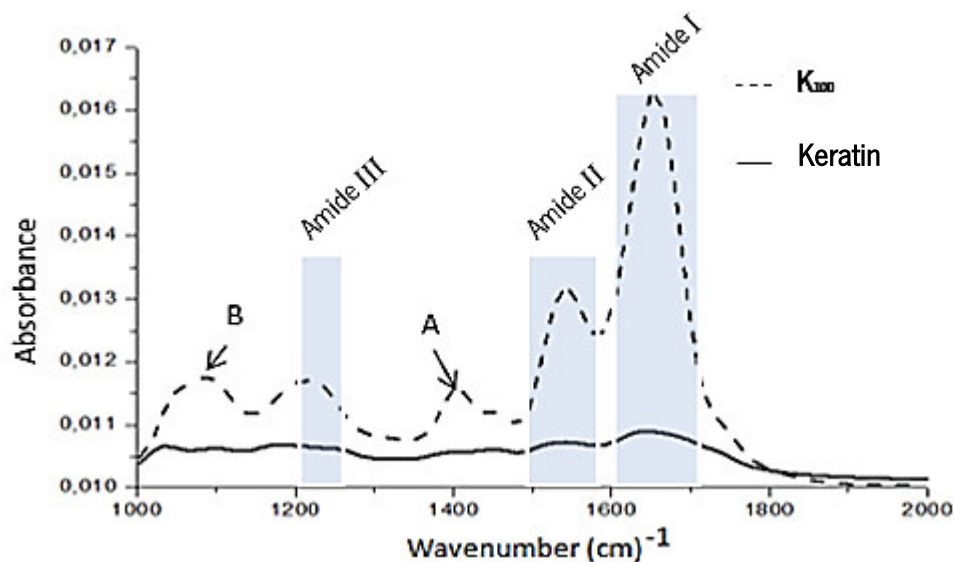


Figure 21 - FTIR spectra of extracted keratin (solid line) and K_{100} particle (dash line). The three amides regions are highlight: Amide I ($1600\text{-}1700\text{ cm}^{-1}$) $\text{C}=\text{O}$ stretch; Amide II ($1510\text{-}1580\text{ cm}^{-1}$) N-H bending; Amide III ($1230\text{-}1270\text{cm}^{-1}$) C-O stretch; as well the 1095cm^{-1} and 1400 cm^{-1} peaks, B and A respectively. [80]

When the proteins were subjected to cycles of high pressure homogenization, some structural modifications were detected in the keratin particles (K_{100}). The K_{100} particles showed higher absorbance for the analysed amides, which is related with chemical-bonds concentration, when compared with the keratin solution (Figure 21). The appearance of a peak at 1095 cm^{-1} (B) corresponds to C-O stretching vibrations of carboxyl groups on K_{100} samples, due to the hydrogen bonds present in keratin structures (α -helix and β -strands). [87,89]

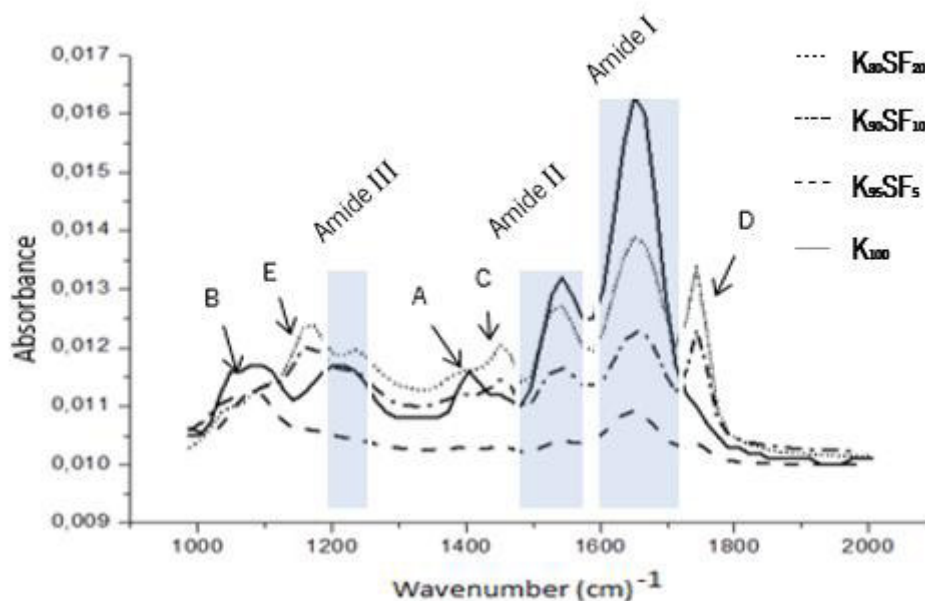


Figure 22 - FTIR spectra of K_{100} particles (solid line), $K_{95}SF_5$ particles (dash line), $K_{90}SF_{10}$ particles (dash-dot line) and $K_{80}SF_{20}$ particles (dot line). The three amides regions are highlight: Amide I (1600-1700 cm^{-1}) C=O stretch; Amide II (1510-1580 cm^{-1}) N-H bending; Amide III (1230-1270 cm^{-1}) C-O stretch; as well the 1095 cm^{-1} , 1168 cm^{-1} , 1400 cm^{-1} , 1465 cm^{-1} and 1738 cm^{-1} peaks, B, E, A, C and D respectively. [80]

An increase on silk fibroin content lead to an increment on the absorbance of Amide I and Amide II regions, yet the absorbance for the K_{100} particles for these Amides was higher when compared with $K_{95}SF_5$, $K_{90}SF_{10}$ and $K_{80}SF_{20}$ particles (Figure 22). The addiction of fibroin to the particles formulation contributed to a vibrational decrease of C=O, N-H and C-O. This could be related with the interference of silk fibroin in these bondings which was more evident for the lowest fibroin concentration. The interference of silk fibroin in $K_{90}SF_{10}$ and $K_{80}SF_{20}$ particles was not as evident as for the $K_{95}SF_5$ particles. Also 1095 cm^{-1} (B) assignment corresponding to C-O stretching vibrations of carboxyl groups, 1168 cm^{-1} (E) indicated stretching vibration of the C-O-C group, [89] as well as 1465 cm^{-1} (C) and 1738 cm^{-1} (D) peaks which match with β -sheet structures and their aggregate, due CH_2 and C=O vibration of fibroin β -strands . [90]

The variations of the FTIR spectra could indicate conformational changes on the proteins and the achievement of complex particles. The results presented in Table III summarize the structural alterations of the proteins throughout the HPH process. The band assignment was performed according to Kong [80] where the deconvolution of Amide I allowed to analyze the corresponding structural assignments.

Amide I deconvolution results were obtained and variations in conformations in keratin-based particles were verified. For controls, the α -helix/ Random coil conformation, formed by winding the polypeptide backbone into a tightly packed right-handed helix, was predominant. After application of HPH to the particles formulations there was an increase in the percentage of β -strands (sheets and turns). Concomitantly, there was a decrease on 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 on protein's crystallinity, mostly due to the formation of β -strand structures[51,82,91].

The incorporation of silk fibroin on particles formulation contributed to an increase in the percentage of β -sheets. This contribution was already described [92,93]. Silk fibroin self-assembly process is predominantly composed by this conformation which increases encapsulation efficiency and enables control of drug release kinetics. The increase of β -strands on keratin-based particles obtained by HPH could lead to a novel drug delivery system using bio-recoverable materials, as their self-assembly behaviour is similar to the self-assembly behaviour described for silk fibroin. [93,94]

Table VIII – Secondary structural assignments on keratin solution and on K_{100} , $K_{95}SF_5$, $K_{90}SF_{10}$ and $K_{80}SF_{20}$ 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					
	<i>Keratin Control</i>	<i>Silk Fibroin Control</i>	K_{100}	$K_{95}SF_5$	$K_{90}SF_{10}$	$K_{80}SF_{20}$
β-sheet	27	28.2	29	34.8	32.4	33.4
β-turn	33.7	39.2	40.3	39.2	41.76	39.4
α-helix/ Random coil	39.3	32.6	30.7	25.9	25.84	27.2
FWHM*	14.2	15.9	14.4	14.46	16.23	14.5

*Full width half maximum

The effect of Poloxamer 407 on the particles' conformation was also studied and FTIR spectra were collected as shown in Figures 23 and 24.

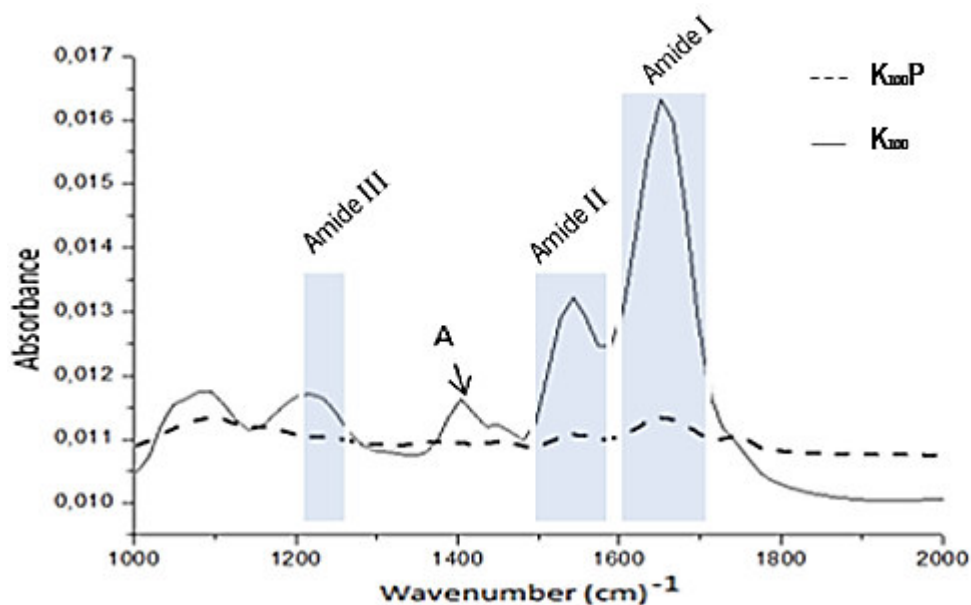


Figure 23- FTIR spectra of K_{100} (solid line) and $K_{100}P$ (dash line) particles. The three amides regions are highlight: Amide I ($1600\text{-}1700\text{ cm}^{-1}$) C=O stretch; Amide II ($1510\text{-}1580\text{ cm}^{-1}$) N-H bending; Amide III ($1230\text{-}1270\text{ cm}^{-1}$) C-O stretch; as well the peak at 1400 cm^{-1} peak (A). [80]

Poloxamer was incorporated into nanoformulations to stabilize and modify the particles surface potential. The non-absorption at 1400 cm^{-1} (A), relative to proteins' side chain COO^- , could explain the more positive Zeta-potential of the particles with poloxamer which was directly related with the coating/shielding properties of poloxamer. This effect was verified for all particles with a Zeta-Potential less negative than the K_{100} particles. For these particles, the absorption at 1400 cm^{-1} was lower than for K_{100} particles.

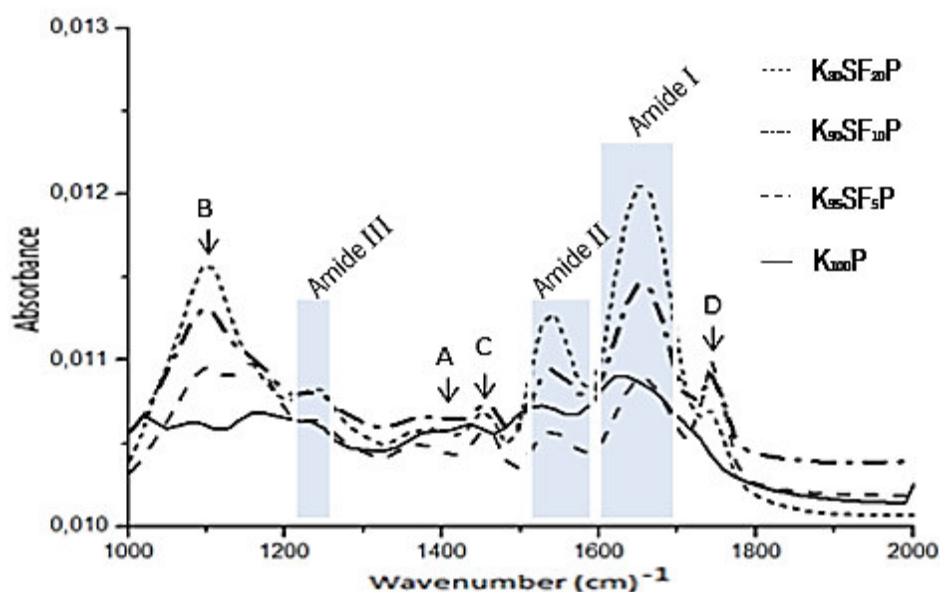


Figure 24 - FTIR spectra of $K_{100}P$ particles (solid line), $K_{95}SF_5P$ particles (dash line), $K_{90}SF_{10}P$ particles (dash-dot line) and $K_{80}SF_{20}P$ particles (dot line). The three amides regions are highlight: Amide I (1600-1700 cm^{-1}) C=O stretch; Amide II (1510-1580 cm^{-1}) N-H bending; Amide III (1230-1270 cm^{-1}) C-O stretch; as well the 1095 cm^{-1} , 1400 cm^{-1} , 1465 cm^{-1} and 1738 cm^{-1} peaks, B, A, C and D respectively. [80]

When compared with the K_{100} particles, the addiction of silk fibroin and Poloxamer 407 to the formulations lead to a decrease on the absorbance on the regions corresponding to the Amides I, II and III. These changes evidence the chemical modifications on the developed formulations when other components were added. Comparing the results obtained in Tables VIII and IX, the full width at half maximum (FWHM) also confirmed the heterogenicity of the particles when the silk fibroin and the poloxamer were incorporated, which revealed the formation of complex formulations.

Analysing Table IX was evident the effect of silk fibroin and poloxamer on particles conformation triggering the formation β -strands in special β -turn, during the HPH treatment. An increase on these conformations may lead 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 particles resistance and rigidity. [95,96]

Table IX – Secondary structural assignments on keratin solution and on K₁₀₀, K₁₀₀P, K₉₅SF₅P, K₉₀SF₁₀P and K₈₀SF₂₀P 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						
	Keratin Control	Silk Fibroin Control	K ₁₀₀	K ₁₀₀ P	K ₉₅ SF ₅ Ps	K ₉₀ SF ₁₀ P	K ₈₀ SF ₂₀ P
β-sheet	27	28.2	29	31	30.8	30.1	34.2
β-turn	33.7	39.2	40.3	38.4	42.7	39.6	38.7
α-helix/ Random coil	39.3	32.6	30.7	29.6	26.4	30.3	27.1
FWHM*	14.2	15.9	14.4	16.2	16.3	16.3	16.4

*Full width half maximum

3.1.3. *In vitro* evaluation of keratin-based particles cytotoxic effect

When developing new products that will contact with skin is extremely important to understand how the products will potentially react in and with it. Ultimately, the development of new cosmetic products depends on the product's safety when applied to humans.

The use of cell lines is well established for the evaluation of the potential cytotoxicity of cosmetic products. The term cytotoxicity illustrates the effect of chemical agents demonstrated by altered cellular morphology, failure of the cell to attach to surfaces, changes in the rate of cell growth, cell death, and cell disintegration. Cytotoxicity tests are rapid, standardized and sensitive means to determine if the cosmetic product is biologically harmful to the cells, providing predictive evidence of its safety to end-users. [97]

Compared to animal studies, cellular testing is more ethically correct, is easier to reproduce and control, and cheaper. In the case of cytotoxicity, it is important to recognize that cell cultures are sensitive to changes in their environment such as fluctuations in temperature, pH, and nutrient and waste concentrations, in addition to the concentration of the potentially toxic agent being tested. Therefore, controlling the experimental conditions is crucial to ensure that the measured cell death corresponds to the toxicity of the added nanoparticles [98]

Commonly, when assessing the cytotoxicity of a given product, colorimetric methods are employed to evaluate their effect at several levels: on the cytoplasmic membrane, on the mitochondrial activity or in the nucleus of cells. For this study, the MTS assay, which evaluates the cellular metabolic activity, was chosen. [59]

As the keratin-based particles are to be applied on human hair and would therefore come into contact with the scalp, and even be adsorbed through it, the cytocompatibility was evaluated using human keratinocytes.

The NCTC-2544 human keratinocytes is a cell line derived from NCTC 2414 and NCTC 109 cells. This cell line is a reliable method to prevent animal skin tests for cytotoxicity studies and contact of potential allergens.[99–102] Thus, the NCTC-2544 keratinocytes were chosen to evaluate the potential cytotoxic effect of keratin-based particles.

To assess the effect of the keratin-based particles on cells, six concentrations (0.01, 0.05, 0.1, 0.25, 0.5 and 1 mg/mL) were incubated with the NCTC-2544 cell line. After 24 and 48 h of incubation, the metabolic activity of the cells was evaluated by the MTS assay (Figure 25, 26 and 27).

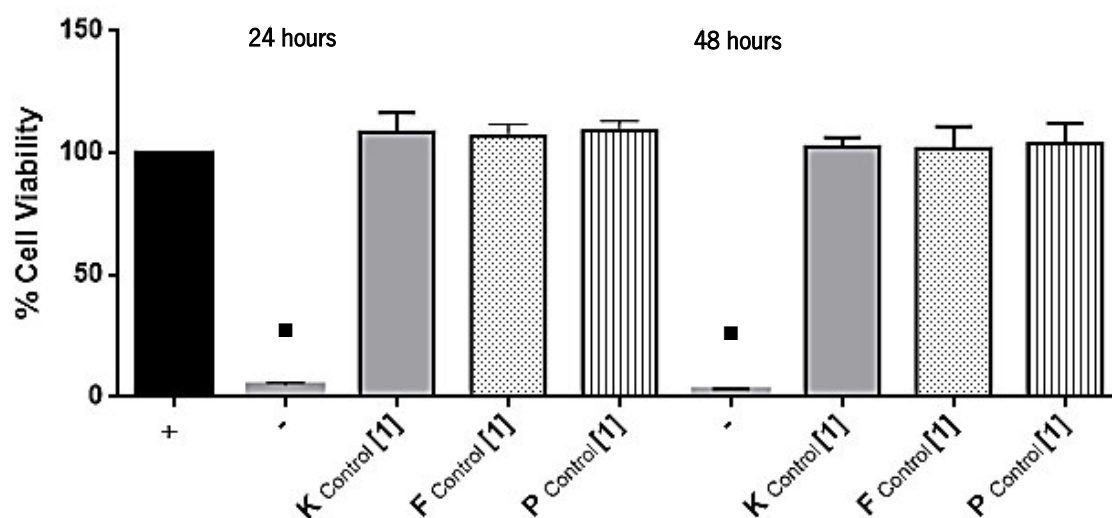
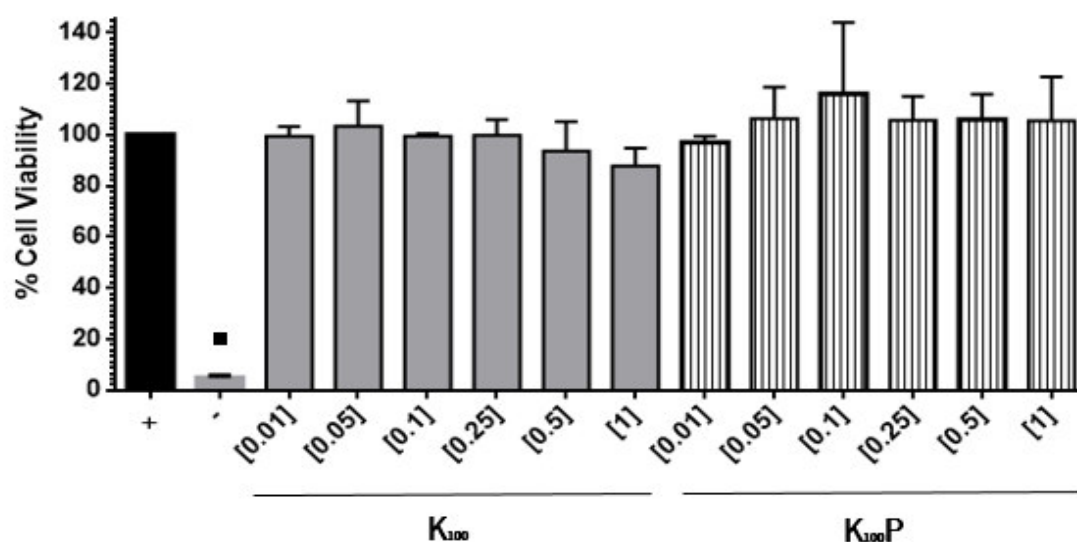


Figure 25 – Relative viability of NCTC-2544 human keratinocytes (evaluated by the MTS assay) after 24 h and 48 h incubation in medium containing control solutions (1mg/mL) of keratin, fibron and poloxamer. Cells only incubated with medium were used as life control (+) and cells incubated with 30 % DMSO as control of death (-). Data were determined in relation to the life control. Results are the mean $\pm$ SD of triplicate of three independent experiments. Statistical significant differences from the life control are indicated: ■ p-value $\leq$ 0.001.



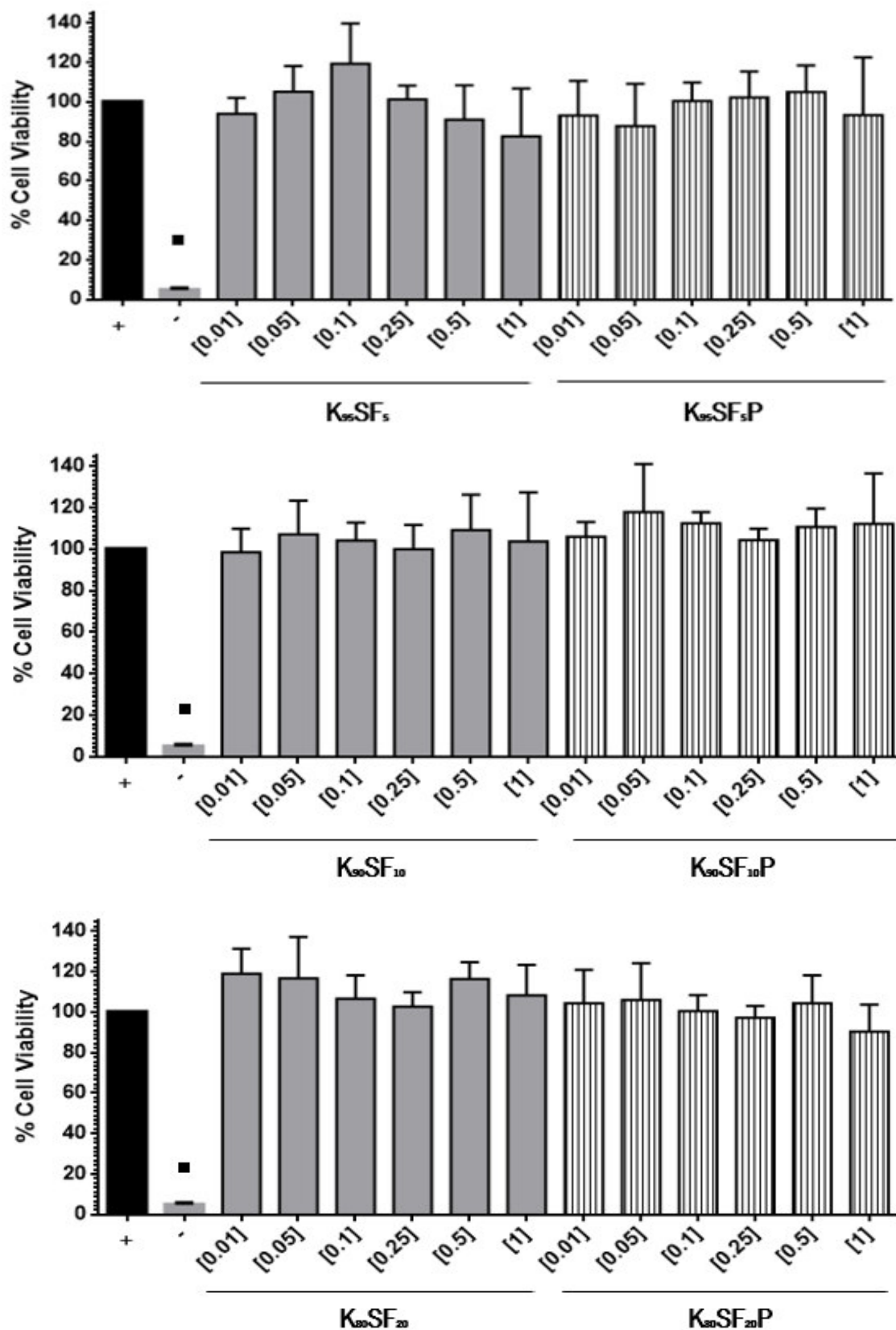
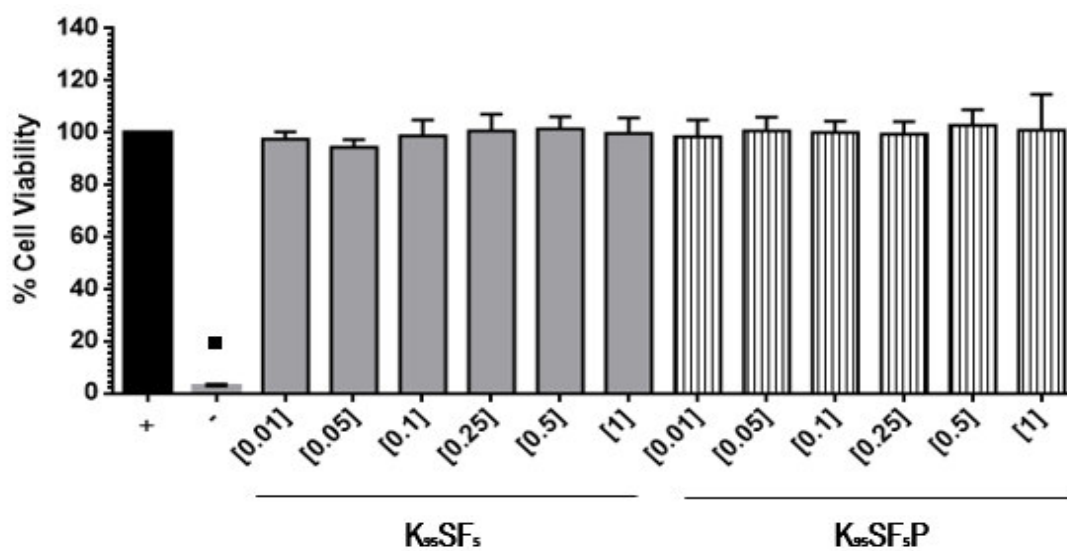
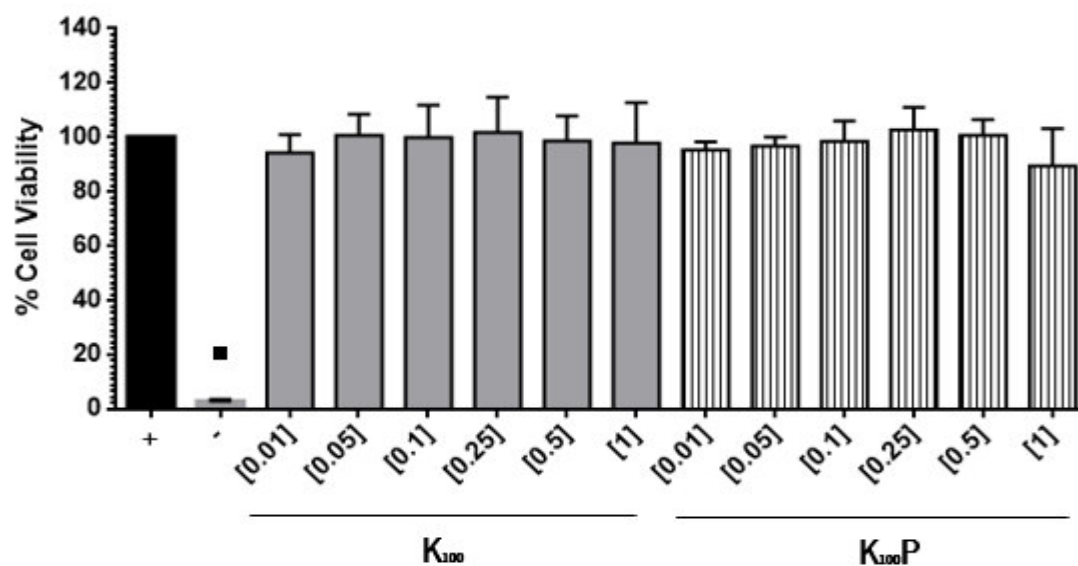


Figure 26 – Relative viability of NCTC-2544 human keratinocytes (evaluated by the MTS assay) after 24 h incubation in medium containing K_{100} , $K_{100}P$, $K_{95}SF_5$, $K_{95}SF_5P$, $K_{90}SF_{10}$, $K_{90}SF_{10}P$, $K_{80}SF_{20}$ and $K_{80}SF_{20}P$ particles. Cells only incubated with medium were used as life control (+) and cells

incubated with 30 % DMSO as control of death (-). Data were determined in relation to the life control. Results are the mean $\pm$ SD of triplicate of three independent experiments. Statistical significant differences from the life control are indicated as: ■ p-value $\leq$ 0.001.



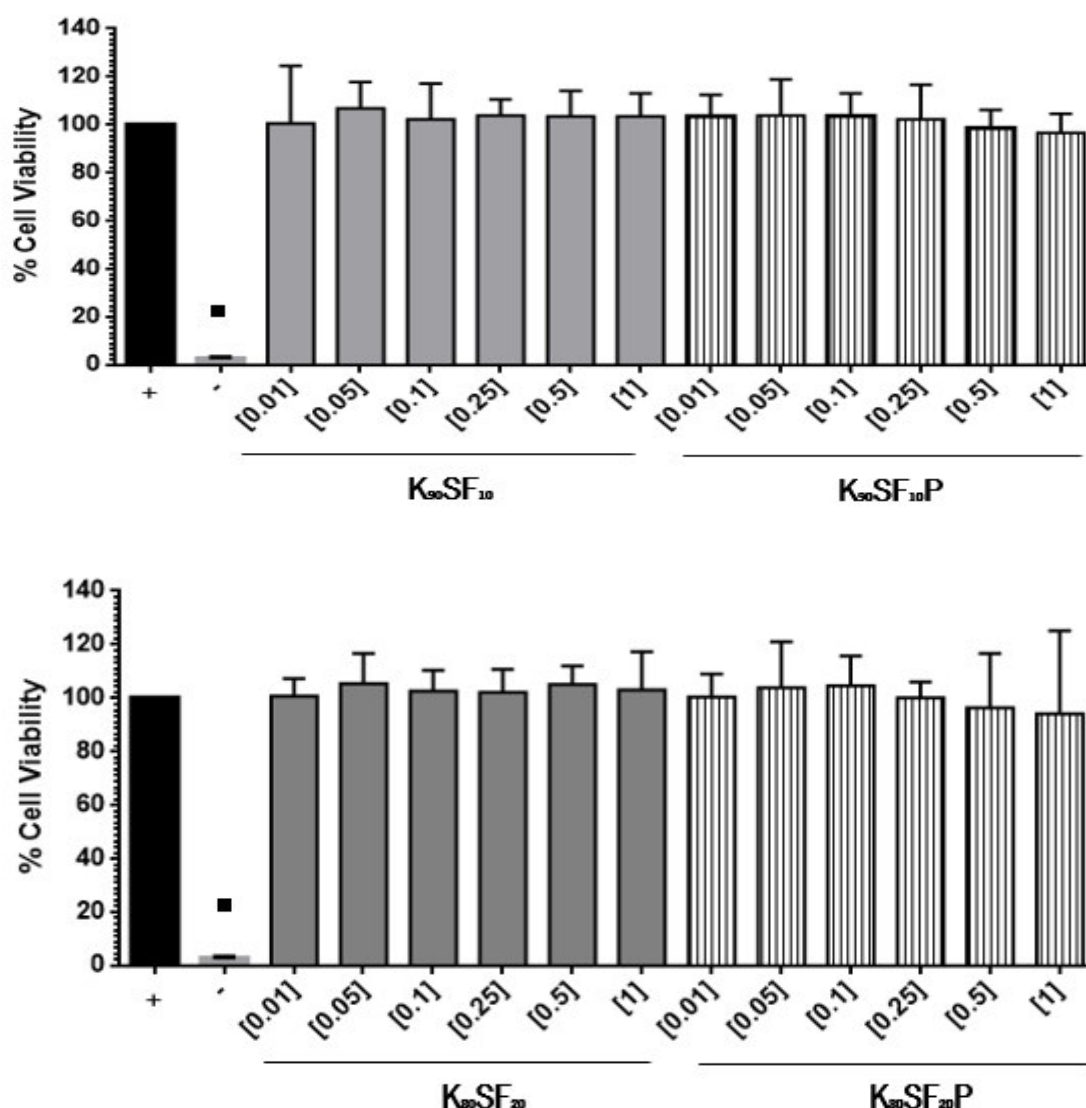


Figure 27 – Relative viability of NCTC-2544 human keratinocytes (evaluated by the MTS assay) after 48 h incubation in medium containing K₁₀₀, K₁₀₀P, K₉₅SF₅, K₉₅SF₅P, K₉₀SF₁₀, K₉₀SF₁₀P, K₈₀SF₂₀ and K₈₀SF₂₀P particles. Cells only incubated with medium were used as life control (+) and cells incubated with 30 % DMSO as control of death (-). Data were determined in relation to the life control. Results are the mean $\pm$ SD of triplicate of three independent experiments. Statistical significant differences from the life control are indicated as: ■ p-value $\leq$ 0.001.

Analysing Figures 26 and 27, it was possible to conclude about the cytotoxicity of the keratin-based particles on NCTC-2544 cells over time. Particles components such as keratin, silk fibroin and Poloxamer 407 did not induce any cell toxicity for both time points tested (Figure 25).

Although some slight variations in cell viability were observed at the end of 48 hours of incubation, the variations were not statistically significant. For both time points, the keratin-based

particles did not cause a dose-dependent cytotoxicity as no significant differences could be pointed out between the tested concentrations.

By comparing all cells incubated with the formulations with cells incubated with culture media (life control), it is possible to conclude that there were not significant differences and all nanoformulations showed a cell viability higher than 82.5%. Indeed, for some concentrations of particles (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. The proliferative effect of keratin-based materials, like hydrogels, scaffolds and films was already described in cell adhesion and proliferation studies.

The uniformity of the viability results at the end of 48 hours of incubation, and comparing with the 24 hours, indicated the ability of the keratinocytes to adapt to the presence of the keratin-based particles which supported the safety of these particles when tested on human keratinocytes. Finally, the addition of Poloxamer407 and silk fibroin to the particle formulation did not significantly affect the viability of keratinocytes.

3.1.4. Application of keratin-based particles to hair

The demand for cosmetic products that improve the look and feel of hair has created a massive and highly rentable industry based on the development of several strategies and application products for its care. Beauty care technology has advanced the cleaning, protection and restoration of desirable and specific hair properties by altering the hair fibre surface.[2]

After proving the safety of the keratin-based particles in keratinocytes, virgin and over-bleached (8xB) Asian hair were treated with 0.5 mg/ml of each particle for 1 h. The amount of particles used for the treatment was based on the particles cytotoxic profiles studied by MTS. However, there was no significant cytotoxic effect for any tested concentration after 24 and 48 h of exposition. 0.5 mg/ml of particles was the highest concentration where no decrease on cellular viability was noted for all keratin-based particles tested.

3.1.4.1. Keratin-based particles binding to hair

When developing a new particle-based formulation for hair care, the particle ability to bind the hair fibre must be studied. This is crucial and ultimately will determine the formulation success.

After the hair treatments, roughly bound keratin-based particles were washed off, and the remaining tightly bound particles (at the hair surface and/or cortex) were visualized. Figures 28 and 29 show the high-magnification SEM images of virgin, overbleached and treated Asian hair.

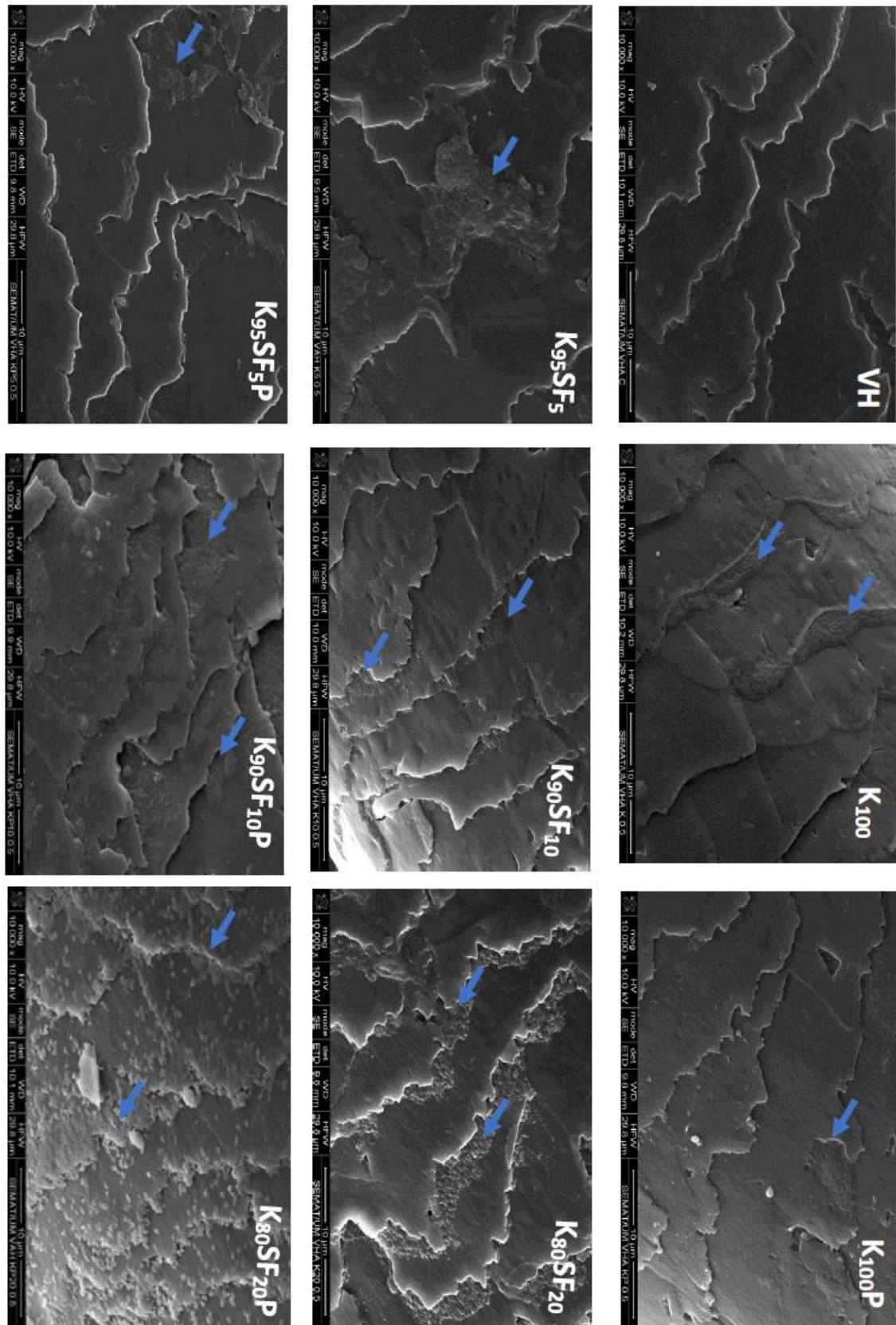


Figure 28 – SEM micrographs of normal Asian virgin hair after treatment with the keratin-based particles.

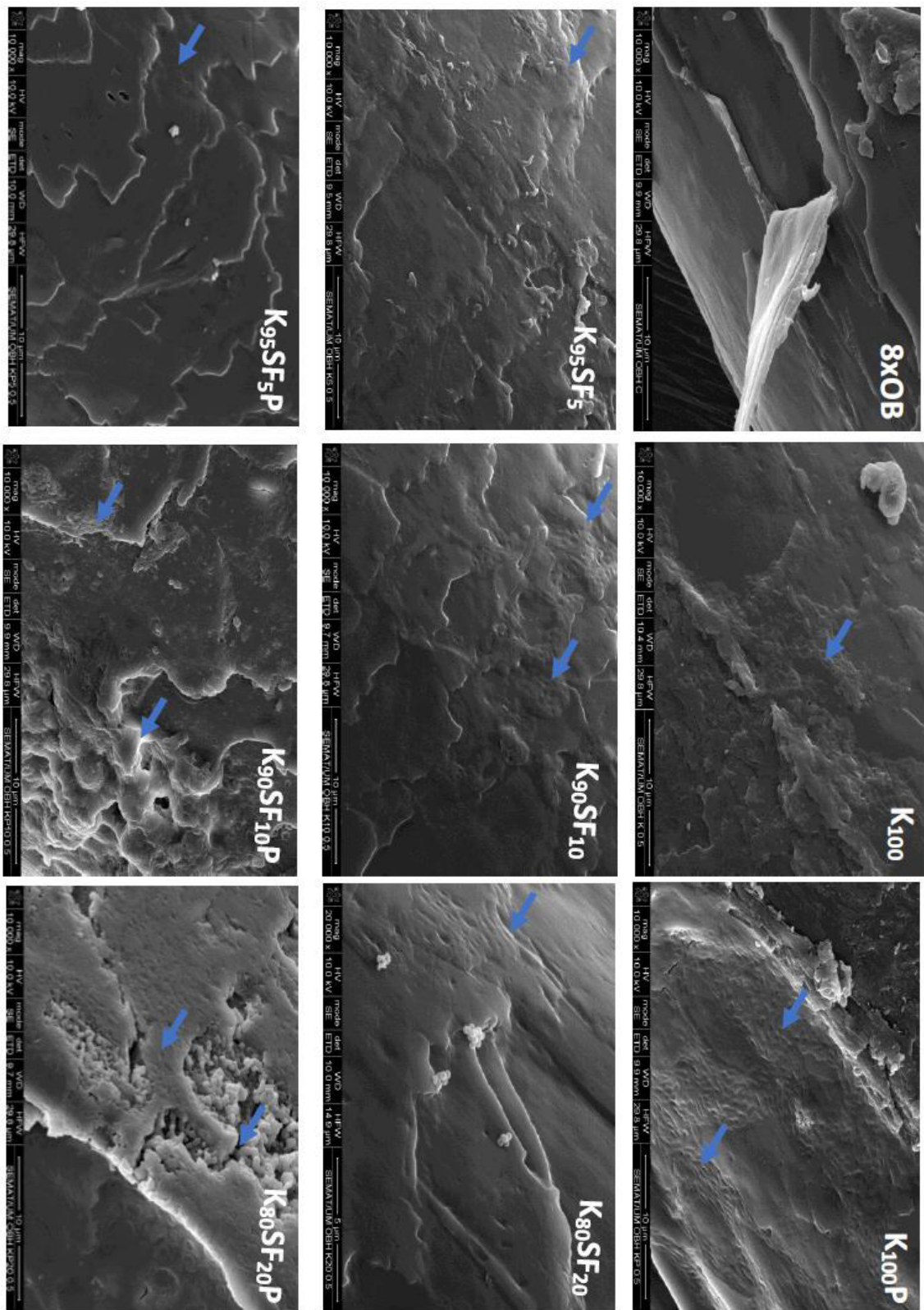


Figure 29 – SEM micrographs of 8x overbleached Asian hair after treatment with the keratin-based particles.

Comparing the micrographs of virgin and over bleached hair with the micrographs of hair treated with the keratin-based particles, it becomes evident, in all samples, the deposition of the particles on top of the hair fibres (blue arrows).

Despite the deposition occurring mostly in the inter-layer zone of the cuticle for the virgin hair, which can be observed by the formation of a rough surface in the edges of the cuticles; the deposition of the particles on the over bleached hair was more uniform. The differences on pattern deposition were directly related with the overbleaching process which affected cuticle structure and surface chemistry. The appearance of cysteic acid termination SO_3^- after bleaching resulted in an increase on the net negative charge and surface hydrophilicity of over bleached hair improving the electric attraction to keratin-based particles.[103] The formation of a film-like deposition in the $\text{K}_{80}\text{SF}_{20}\text{P}$ particles (Figure 29) on the over bleached hair resulted from a stronger interaction between the particles and the negatively charged hair. These particles had a Zeta-Potential close to 0 mV, the less negative between all keratin-based particles.

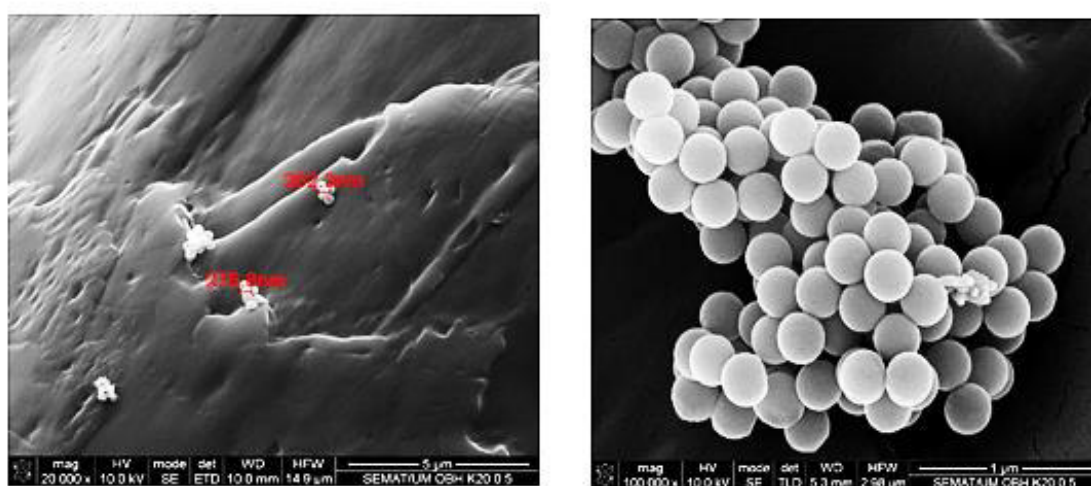


Figure 30 – SEM micrographs of overbleached Asian virgin hair treated with $\text{K}_{80}\text{SF}_{20}$ particles.

The micrographs were also show the effect of silk fibroin and Poloxamer 407 on particle structure. The stabilizing effect of poloxamer together with the structural effect of silk fibroin yielded particles with harder structures. Increasing the amount of silk fibroin in the formulation causes the particles to become more rigid while maintain their shape and size when the electric forces were applied during SEM analysis (Figure 30). Figure 30 illustrates the spherical shape of the $\text{K}_{80}\text{SF}_{20}$ particles. Moreover, the measured size was in line with the DLS measures for day 1 (Figure 18).

3.1.4.2. Effect of keratin-based particles treatment on hair's mechanical properties

Due to its intricate and unique structure, hair has remarkable mechanical properties, and the measurement of these is one of the simplest means of assessing the integrity and attributes of the fibre. Indeed, the slightest modification in hair chemical composition or structure may greatly alter its mechanical properties.[104]

After proving the affinity of keratin-based particles towards virgin and overbleached Asian hair, the effect of particle treatment on hair's mechanical properties was studied by tensile tests. These mechanical tests are widely spread among manufacturers of hair products allowing to measure the effect of a given product has on a hair fibre. Hair fibres are normally very thin with some variation between hair shafts of the same individual, which carries many challenges to the study of hair's mechanical properties. Thus it is of the outmost importance to test large number of samples, $n \geq 20$, to obtain reliable results.[105]

Hair fibre cortex is responsible for maintaining the shape and confer resistance to fibres when external forces are applied. This ability relies on the presence of long keratin chains with high molecular flexibility. If there are variations on hair's mechanical properties after treatment with a cosmetic formulation this will depend on how and to what extent the formulation components interact with the hair cortex and cuticles. [106]

When an external force is applied, material deformation occurs and the capability to return to a normal state when removed is called elastic deformation. A stiff material needs more force to deform compared to a soft material, and an infinite force would be needed to deform a perfectly rigid material, implying that it would have an infinite Young's modulus.[107]

To evaluate the effect of keratin-based particles in virgin and overbleached Asian hair fibres stiffness, tensile tests were performed and Young's modulus was determined. Young's modulus measures the stiffness of a solid material through a relationship between stress (force per unit area) and strain (proportional deformation). The higher the value of the Young's modulus (mPa), the more resistant the material is, because a higher load force is needed to lead to a permanent deformation.[108]

Tensile tests were performed in virgin and overbleached Asian hair. Sample populations of twenty hair shafts treated with the several keratin-based particles were used for the measurements.

Young's modulus (values expressed in MPa) of treated and untreated hair samples are represented in Figures 31 and 32.

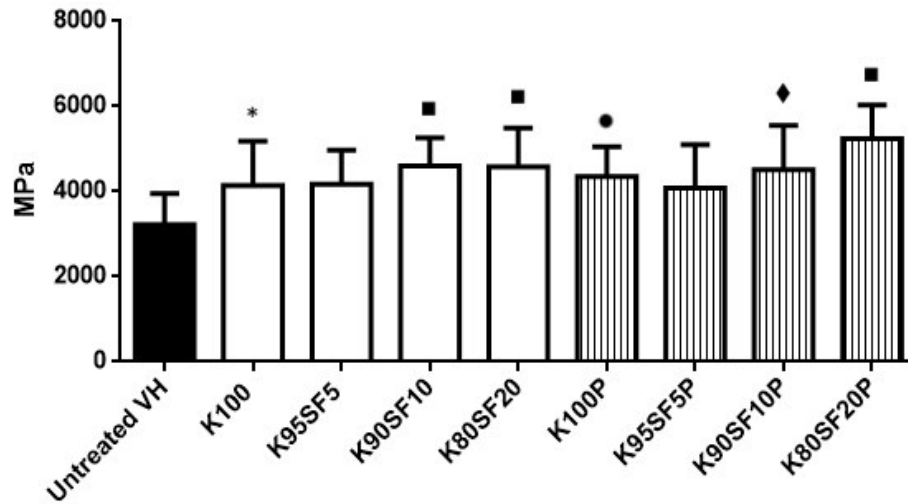


Figure 31 – Mechanical resistance parameters shown by Young's Modulus. Values are mean of $\pm$ SD of thirty independent measurements. Statistical significant differences from the control (untreated virgin hair) are indicated as: * p-value ≤ 0.05 , ● p-value ≤ 0.01 ; ◆ p-value ≤ 0.001 ; ■ p-value ≤ 0.0001 .

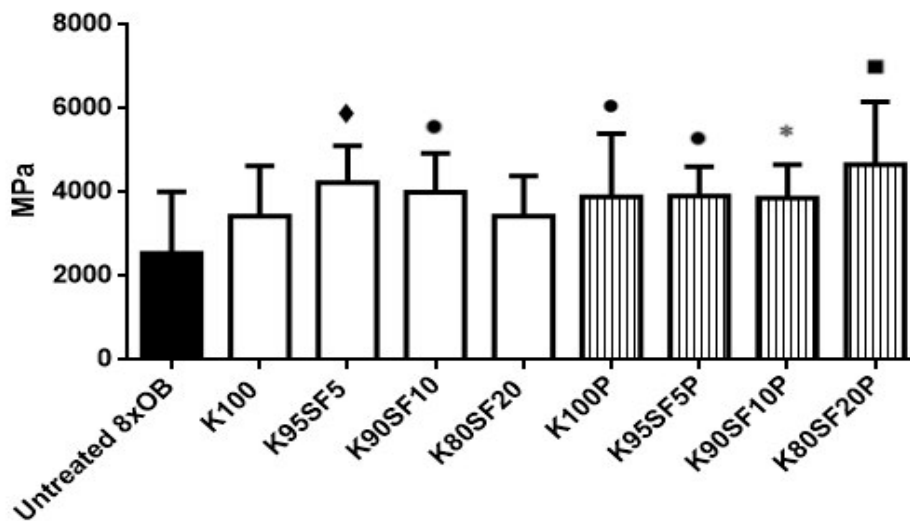


Figure 32 – Mechanical resistance parameters shown by Young's Modulus. Values are mean of $\pm$ SD of thirty independent measurements. Statistical significant differences from the control (untreated overbleached hair) are indicated as: * p-value ≤ 0.05 , ● p-value ≤ 0.01 ; ◆ p-value ≤ 0.001 ; ■ p-value ≤ 0.0001 .

Application of keratin-based particles resulted in the improvement of virgin and overbleached hair's mechanical properties in a magnitude up to 62.7 and 83.4%, in terms of Young's modulus for the K₈₀SF₂₀P particle (Table X). The repeated chemical bleaching treatments induced 21.2% of resistance loss when compared with the virgin, unaltered hair.

The improvement and recovery of hair's mechanical properties was directly related with the number of particles attached to the hair surface. Hair samples with more particles in their surface, K₁₀₀P, K₉₀SF₁₀ and K₈₀SF₂₀P (Figures 31 and 32), had the highest and more statistical significant values for Young's modulus. Despite the relation between the amount of particles at hair surface and the improvement of mechanical properties, the increase on Young's modulus could also be related with the ability of the keratin-based particles to penetrate hair's cuticles into the hair cortex contributing for the stabilization of keratins' α -helix, giving this structure a higher resistance to unfold during stretching.

The particles composition also affected the improvement of hair's mechanical properties. Generally, there was an increase on Young's modulus values increasing the amount of silk fibroin on particles' formulation (Figures 31 and 32, Table X). This relation was directly related with the secondary structure of silk fibroin. This protein is rich in β -strands which are associated with higher resistance and stiffness profiles.[95,109]

Table X – Improvement (I) and recovery (R) of hair's mechanical properties measured in terms of Young's modulus and expressed in percentage (%), treated with keratin-based particles, compared with untreated virgin and overbleached hairs.

Formulation	K ₁₀₀	K ₉₅ SF ₅	K ₉₀ SF ₁₀	K ₈₀ SF ₂₀	K ₁₀₀ P	K ₉₅ SF ₅ P	K ₉₀ SF ₁₀ P	K ₈₀ SF ₂₀ P
<u>Virgin hair</u> (% I)	28.6	29.5	42.8	42.4	35.1	26.7	40.1	62.7
<u>Overbleached hair</u> (% R + I)	35.0	66.5	57.5	35.1	53.2	53.9	52.0	83.4

3.2. Recombinant Crystallins

Human eye γ D-Crystallin is structurally characterized by the Greek key motif which is described to be associated on the coating of structures like spores and hair. [39] Exploring the filming properties of crystallins, two new recombinant crystallins (CrysWt and CrysMut) were designed, obtained and characterized to be used in the development of new functional cosmetic products for the treatment of hair.

Wild type crystallin (Crys~~WT~~) displayed the same amino acidic sequence as human eye γ D-Crystallin (Protein Data Bank ID: 1HK0), whereas the mutant (Crys~~Mut~~) results from site-directed mutagenesis of the ~~wt~~ form by substitution of three arginine residues (R) at positions 15, 37 and 80 by cysteine residues (C). The presence of cysteines induces an interunit crosslink in a network of mutant crystallins. This also creates possible points of anchorage to hair keratins that are naturally rich in cysteine residues. Thus, the mutant crystallin, with three additional cysteines (ARG15CYS, ARG37CYS and ARG80CYS) on its sequence, was used in order to fully exploit these possibilities.

The ability of both crystallins to bind to hair for enhancing its physical properties was previously described.[2] Combining crystallins with the sequence of a keratin peptide like (KP), the new protein will have improved affinity towards hair fibres. The KP peptide, based on a fragment of hair keratin type II cuticular protein, was previously used to improve hair properties. This peptide relies on its amino acidic sequence to link to hair fibres and even to penetrate into hair cortex.[1]

The recombinant proteins resulted from the association of KP peptide and the crystallins. To assure however that the multifunctional proteins display the properties given by KP peptide and crystallins, a linker constituted by five repetitions of glycine and alanine residues ((GA)₅) was included between the peptide and the crystallin sequences. This linker endows the two components with internal molecular mobility, essential for protein function.

The sequences for CrysWT and CrysMut are described on Table X and correspond to KP-(GA)₅-CrysWT and KP-(GA)₅-CrysMut for wild-type and mutant crystallin, respectively.

Table XI – Recombinant wild-type crystallin (Crys ^{WT}) and mutant crystallin (Crys ^{Mut}) amino acidic sequences. Characters in bold correspond to the replacement of arginine residues by cysteines in the mutant form. Characters in dark blue and light correspond to the His-Tag and trombine cleavage site sequences, respectively. Characters in Green correspond to the KP peptide sequence and characters in red correspond to the linker sequence.

Crys^{WT}	MGSSHHHHHHSSGLVPRGSHMGGVCGSPSPCITTGAGAGAGAGAMGKITLYEDRGFQGRHYECSS DHPNLQPYLSRCNSARVDSGCWMLYEQPNYSGLQYFLRRGDYADHQQWMGLSDSVRSCRLIPHSG SHRIRLYEREDYRGQMIEFTEDCSCLQDRFRFNEIHSLNVLEGSWWLYELSNYRGRQYLLMPGDYRRY QDWGATNARVGSLRRVIDFS
Crys^{Mut}	MGSSHHHHHHSSGLVPRGSHMGGVCGSPSPCITTGAGAGAGAGAMGKITLYEDRGFQGCCHYECSS DHPNLQPYLSRCNSACVDSGCWMLYEQPNYSGLQYFLRRGDYADHQQWMGLSDSVRSCCLIPHSG SHRIRLYEREDYRGQMIEFTEDCSCLQDRFRFNEIHSLNVLEGSWWLYELSNYRGRQYLLMPGDYRRY QDWGATNARVGSLRRVIDFS

3.2.1. Plasmid analyses

The crystallins sequences cloned pet-28a (+) expression vector were purchased and two *E. coli* strains XL1-Blue (for plasmid maintenance) and BL21(DE3) (for protein expression) were transformed by TSS method.

3.2.1.1. Plasmids Concentration

XL1-Blue transformants harbouring the two plasmids were grown overnight in LB medium with kanamycin and cells were collected for plasmid extraction using the GeneJET Plasmid Miniprep from ThermoFisher Scientific kit. This is a rapid purification method to get high-yield

plasmid DNA from 1-2mL of culture. However, plasmid yield depends on a number of factors such as vector copy number. [143]

The plasmid concentration and purity of samples were determined using Nanodrop and results are shown on Table XII.

Table XII – Concentration and purity in terms of $\angle 260/280$ and $\angle 260/230$ ratio of pet-28a (+)::KP-(GA)₅-CrysWt and pet-28a (+)::KP-(GA)₅-CrysMut plasmids.

	Concentration (ng/ μ mL)	$\angle 260/280$	$\angle 260/230$
pet-28a (+)::KP-(GA) ₅ -CrysWt	98.1	1.86	1.60
pet-28a (+)::KP-(GA) ₅ -CrysMut	112.0	1.83	1.78

The results evidenced the success of plasmid purification protocol. The two plasmids respected purity parameters. Nevertheless, $\angle 260/230$ ratio presented a lower result than that would be expected which was related with carbohydrates or phenols residues coupled to DNA. [144,145]

3.2.1.2. Plasmids Restriction

Diagnostic plasmid digestion using endonucleases is a fast technique to verify the size of inserts cloned in a specific plasmid. The pure pet-28a (+)::KP-(GA)₅-CrysWt and pet-28a (+)::KP-(GA)₅-CrysMut plasmids were digested using the *Nde*I and *Not*I endonucleases. These restriction enzymes recognize nucleotide sequences flanking the crystalline inserts and their action resulted in the vector backbone plasmid and the inserts.

The results of restriction with the pair of endonucleases were analysed in an agarose gel (1%) using a DNA marker (100bp DNA ladder Plus). The result of restriction ran were analyzed and compared with a simulated restriction made using the same conditions in the Snapgene software (Figure 33).

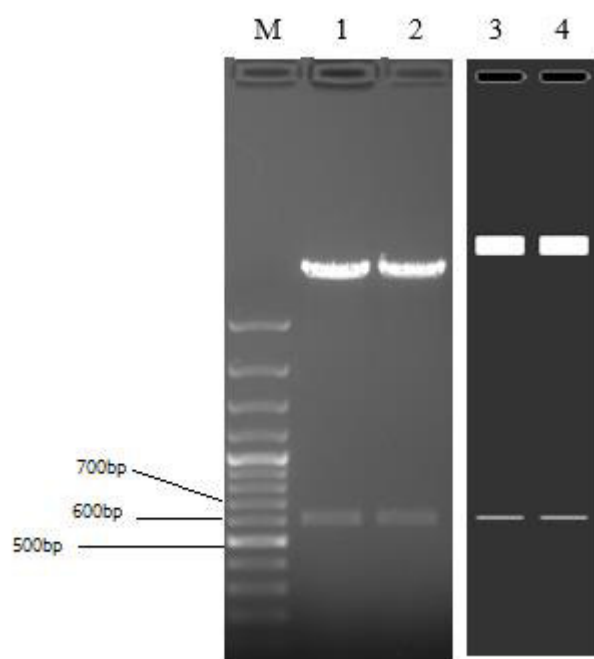
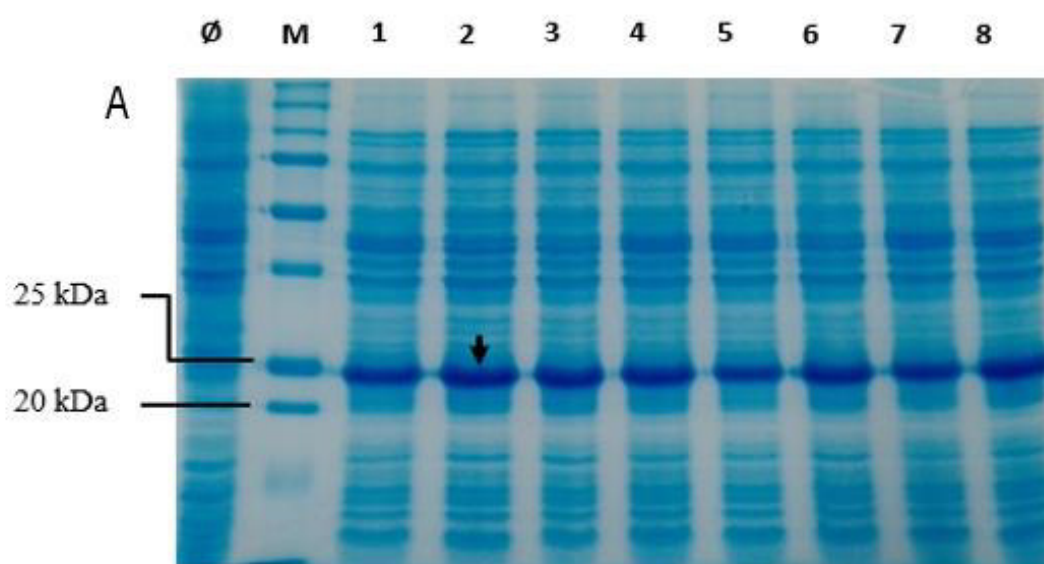


Figure 33 – Plasmid digestion of pet-28a (+)::KP-(GA)₅-CrysWt and pet-28a (+)::KP-(GA)₅-CrysMut using *Nde*I and *Not*I restriction enzymes. Agarose Gel 1% in the left: M – 100bp DNA ladder Plus; 1 – KP-(GA)₅-CrysWt; 2 – KP-(GA)₅-CrysMut. SnapGene simulated gel in the right: 3 – KP-(GA)₅-CrysWt; 4 – KP-(GA)₅-CrysMut

As expected, the digestion of the plasmids with *Nde*I and *Not*I restriction enzymes originated two bands which corresponded to the linearized pet-28a (+) vector and the crystallin inserts. The bands corresponding to the crystallins were near the 600 bp band of the DNA molecular weight marker, which corresponded to the size of crystallin inserts (591 bp) plus the extra residues from the vector (12 bp). Both restriction profiles corresponded to the theoretical profile simulated on Snapgene software.

3.2.2. Expression of recombinant crystallins

After verification of pet-28a (+)::KP-(GA)₅-CrysWt and pet-28a (+)::KP-(GA)₅-CrysMut constructs, the *E. coli*/BL21(DE3) expression strain was transformed with the plasmids. Eight single positive colonies were selected from each construct and were plated in fresh LB-Agar medium with kanamycin antibiotic for colonies maintenance. To check which transformant yielded the highest crystallin expression, the transformants were grown overnight in auto inducible media (TB-AIM) at 37 °C with constant agitation (200rpm) and the total protein extracts were analysed on a SDS-PAGE (12.5%) (Figure 34). BL21 cells transformed with the pet-28a (+) plasmid were used as control of protein expression (negative control).



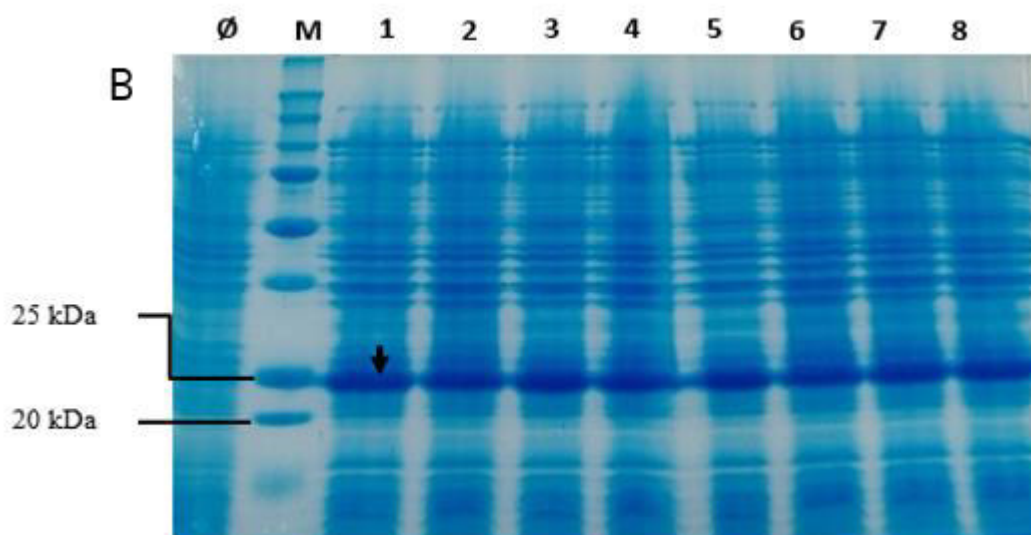


Figure 34 – A - Total protein fractions analysis of CrysWt producing colonies. B - Total protein fractions analysis of CrysMut producing colonies. M – Protein Marker; Lanes 1 to 8 – total protein fraction of eight B21 (DE3) producing transformants after overnight induction in AIM medium; Lane Ø - negative control of protein expression.

The analysis of total protein fractions were made with standardized culture media volumes according to the cell number estimated by absorbance at 600nm. The numbers on the right side of the image correspond to the size in kDa of protein marker reference bands, the CrysWt (24.8 kDa) and CrysMut band (24.7 kDa) are highlighted by a black arrow. When analysing the electrophoretic images of both screening assays, it was evident that all transformants were able to express the two crystallins. Also, there was no significant variability between transformants. Despite the uniformity among the transformants for each crystallin, the transformants for the CrysWt had higher expression levels when compared with the CrysMut transformants.

One transformant from each crystallin was selected and protein expression was optimized. For the optimization of crystallins expression several culture media were tested: LB, TB, TB-AIM, LB-IPTG and TB-IPTG (1mM). After expression the total protein extracts were analysed on a SDS-PAGE (12.5%) (Figure 35).

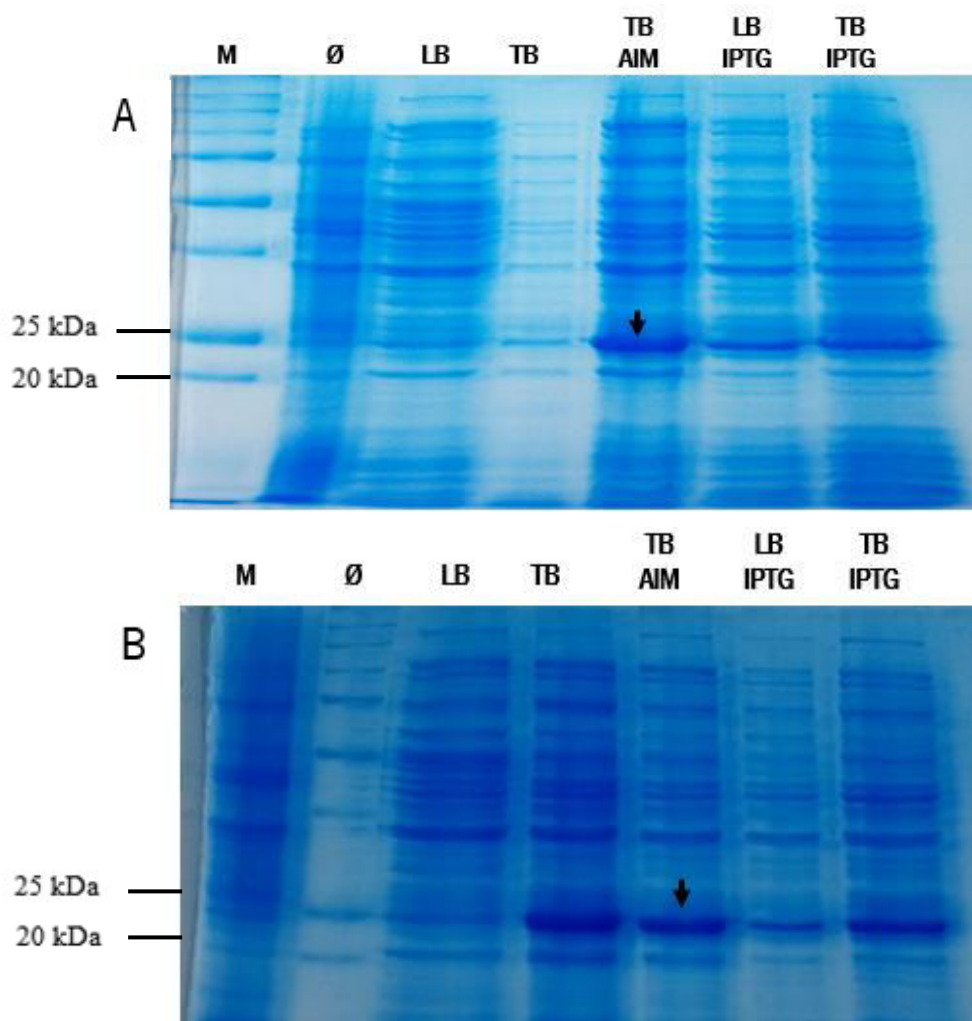


Figure 35 – A - Total protein fractions analysis of CrysWt expression. B - Total protein fractions analysis of CrysMut expression. M – Protein Marker; Lanes LB, TB, TB-AIM, LB IPTG and TB IPTG – total protein fraction of best producing transformant B21 (DE3) after induction in the corresponding culture media; Lane Ø - negative control of protein expression performed in TB-AIM.

TB-AIM media was selected as expression medium for both crystallins. Broader bands are the result of a superior protein expression and this could be due to the complex composition of TB-AIM powder. Moreover, this medium provides a non-monitorizing cell density in contrast with other media, making it easier and better for 24h cell expression.[110]

3.2.2. Purification of recombinant crystallins

Purification of the crystallins was achieved taking advantage of the His-tag present on the protein's N' terminal. Prior to purification, the presence of the two crystallins in the soluble fraction was confirmed. The presence of proteins in this fraction is essential for the purification process but

also indicates that proteins had the correct conformation. After protein expression samples were processed (2.2.5.3.) and fractions were analysed in a SDS-PAGE (12.5%) (Figure 36).

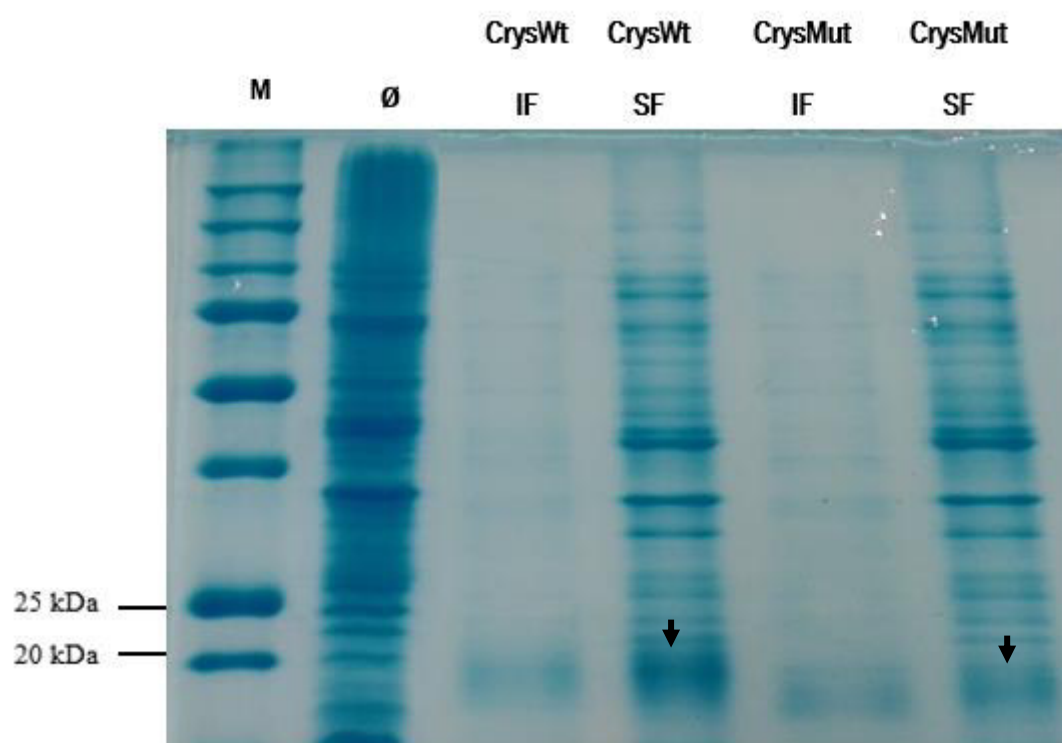


Figure 36 – Fraction analysis for expressed crystallins. M – Protein Marker; Lanes SF: Soluble Fraction; Lanes IF: Insoluble Fraction; Lane Ø - negative control of protein expression performed in TB-AIM.

Despite the detection of some of the proteins of interest in the insoluble fraction, the majority of the crystallins were on the soluble fraction (black arrow). The presence of some amount of crystallins in the insoluble fraction could be related with the sonication protocol which was not effective in disrupting all bacterial cells.

The crystallins' soluble fractions were purified using magnetic beads. These systems have affinity for the protein's His-tag sequences and after a series of incubation and washed with increasing concentrations of imidazole (from 10 to 500 mM) the pure crystallins were obtained. For purification six concentrations of imidazole were tested for binding, washing and elution of the crystallins (10, 50, 80, 100, 250 and 500 mM). Figure 37 show the electrophoretic separation in polyacrylamide gel of the crystallins purification.

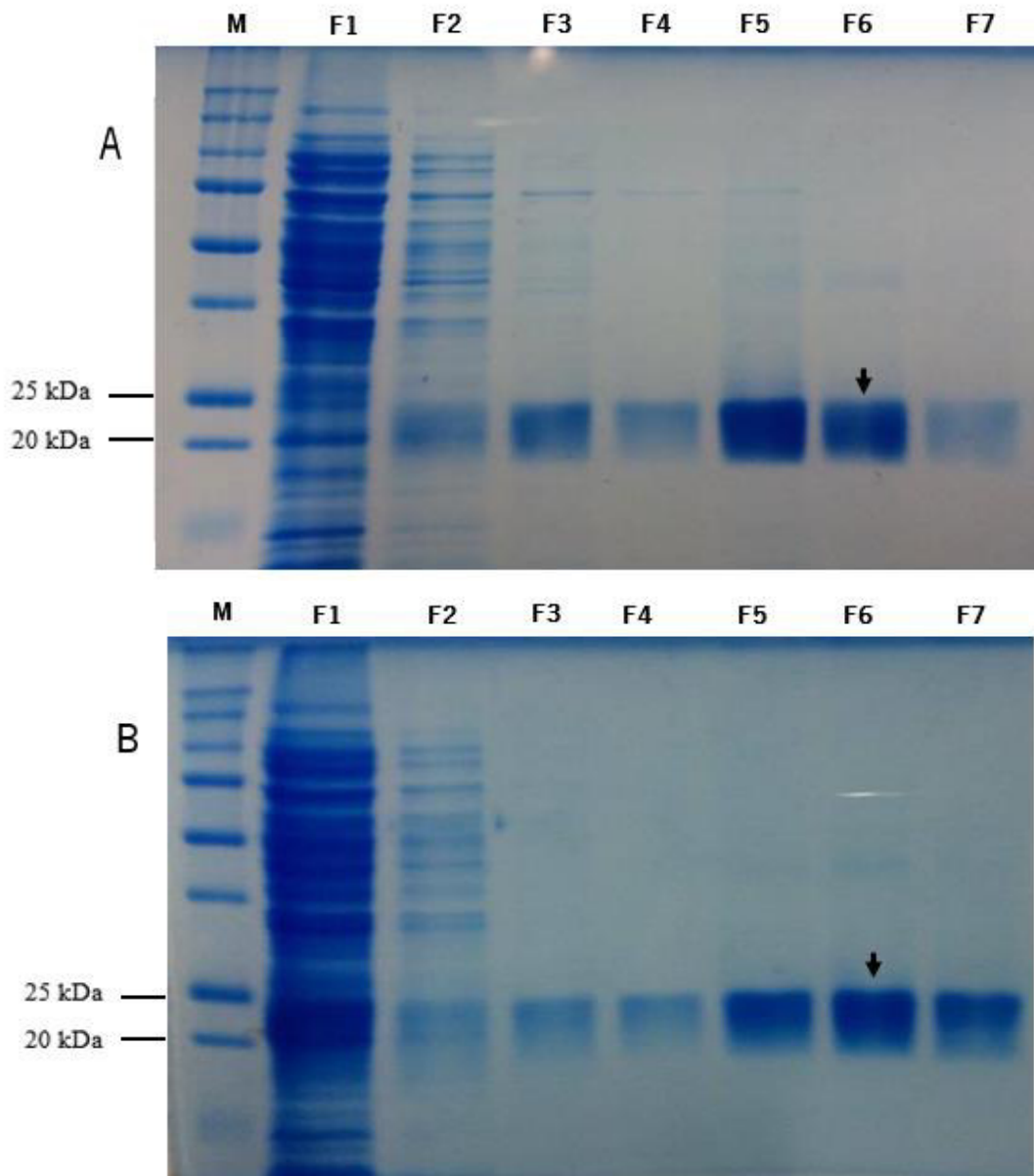


Figure 37 – A – Purification fractions of CrysWt; B – Purification fractions of CrysMut. M: Protein Marker ; Lane F1 – Binding fraction (10 mM imidazole); Lane F2 – Washing fraction 1 (50 mM imidazole); Lane F3 – Washing fraction 2 (80 mM imidazole); Lane F4 – Elution fraction 1 (100 mM imidazole); Lane F5 – Elution fraction 2 (250 mM imidazole); Lane F6 – Elution fraction 3 (500 mM imidazole); Lane F7 – Elution fraction 4 (500 mM imidazole).

Both crystallins were successfully purified using the magnetic beads. The elution fractions (Lanes F5, F6 and F7 in SDS-PAGE) were collected and dialysed for 5 days in distilled water using a dialysis membrane with a 14 kDa cut-off. After dialysis, sample of pure CrysWt and CrysMut were ran in a SDS-PAGE (12.5%) to confirm purity (Figure 38).

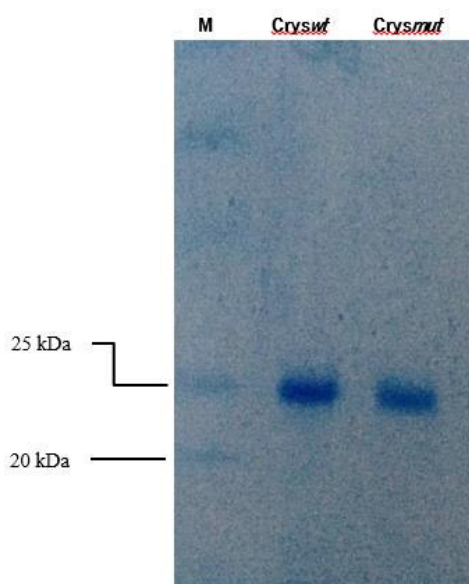


Figure 38 – SDS-PAGE of pure crystallins. M – Protein marker; Lane 1 – CrysWt and Lane 2 – CrysMut.

3.2.4. Crystallins characterization

3.2.4.1. Analyses of recombinant crystallins by MALDI-TOF Mass Spectrometry

MALDI-TOF analyses were performed on samples of pure CrysWt and CrysMut to confirm the correctness of the expressed recombinant proteins. The methods gives information regarding the protein molecular weight and the theoretical value, based on the amino acidic sequence, should

be close enough to the value determined by MALDI-TOF and inside the experimental error. On figure 39 are present the spectra of purified CrysWt and CrysMut.

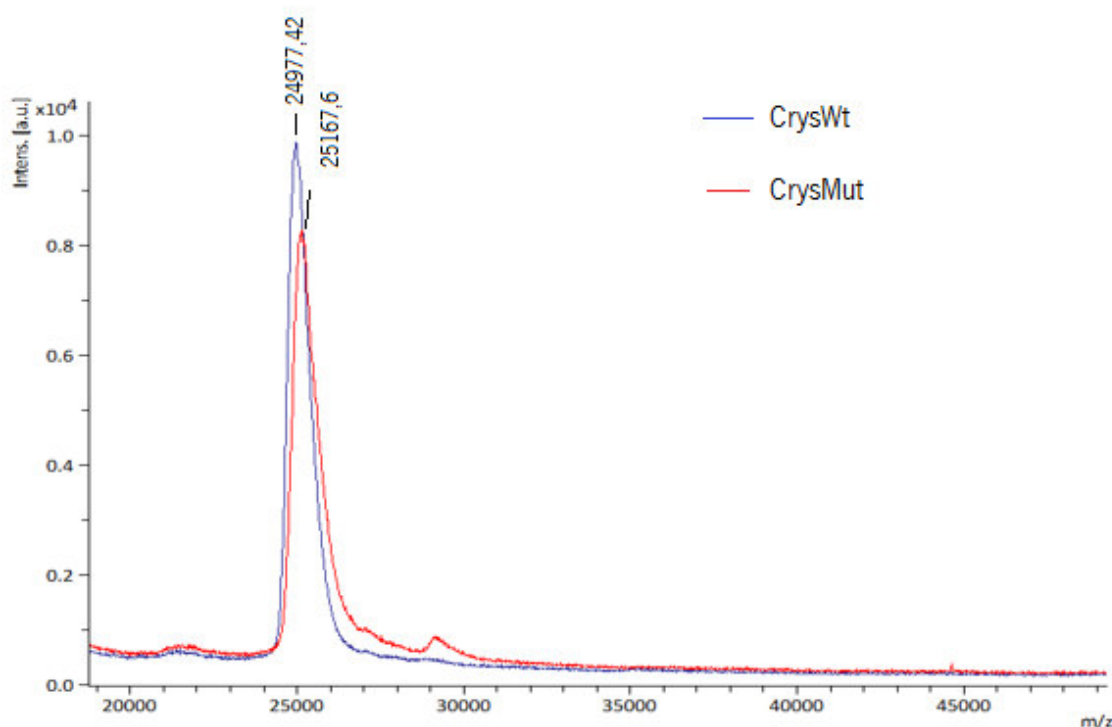


Figure 39 – Determination of CrysWt (A) and CrysMut (B) molecular weight by MALDI-TOF mass spectrometry.

The MALDI-TOF confirmed the monodisperse character of the purified CrysWt and CrysMut. The differences between the theoretical molecular weight, 24843.66 Da for CrysWt and 24684.52 Da for the CrysMut, and the experimental value, 24977,42 Da for CrysWt and 25167,60 Da for the CrysMut, were within the experimental error associated with the technique.

3.2.4.2. Analyses of recombinant crystallins secondary structure

Recombinant proteins were analysed by FTIR to get an insight into their secondary structure. The type of analysis performed was similar to the performed for the keratin-based particles (3.1.2.3.).

To study the effect of KP peptide and GA linker on the structure of crystallins, the results obtained were compared with the information available on UniProt website (UniProtKB - P07320). FTIR spectra acquired for CrysWt and CrysMut are depicted in Figure 40. To access the type of

secondary structures a deconvolution of Amide I ($1600\text{-}1700\text{ cm}^{-1}$) region [80,86] was performed and the results are presented on Table VII.

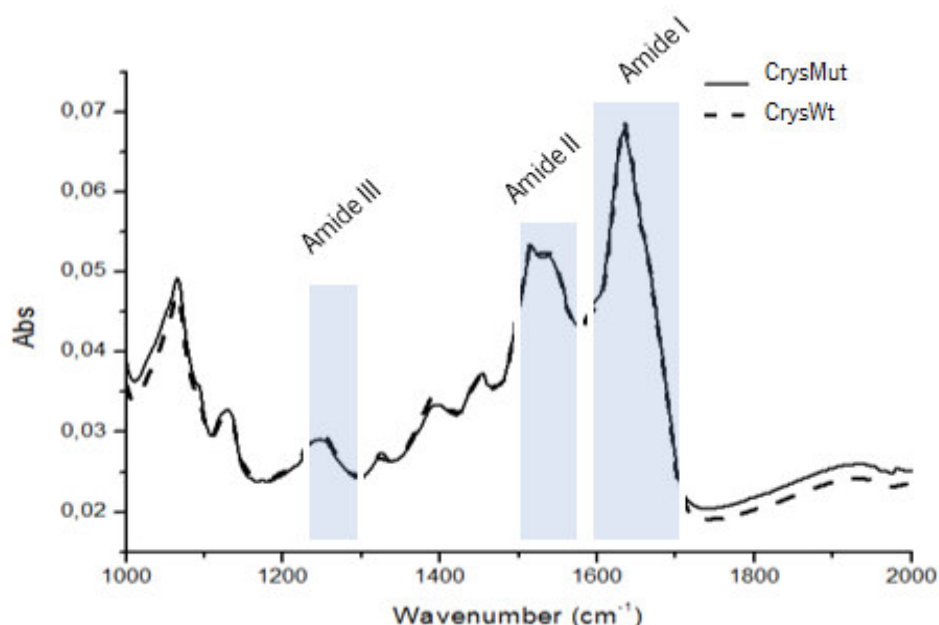


Figure 40 - FTIR spectra of recombinant Crystallins: CrysWt (dash line) and CrysMut (solid line). The three amides are highlight: Amide I ($1600\text{-}1700\text{ cm}^{-1}$) C=O stretch; Amide II ($1510\text{-}1580\text{ cm}^{-1}$) N-H bending; Amide III ($1230\text{-}1270\text{ cm}^{-1}$) C-O stretch.[80]

The FTIR spectra of CrysWt and CrysMut suggests no significant variations between the two crystallins. However, when the deconvolution of Amide I peak was performed, some variations were found. The CrysMut protein displayed a higher percentage of β related structures than the CrysWt protein. Also, the mutant crystallin was less conformationally heterogeneous as it had lower FWHM values compared with wild type crystallin. [51] These differences on the structure of CrysMut could be related with presence of three extra cysteines that potentially contribute to higher protein polymerization and conformational rigidity.[111]

When comparing the structure of both crystallins (Table VII) with the structure of human γ D-crystallin from UniProt (UniProtKB - P07320) there was an agreement between structures. Like the human γ D-crystallin, the recombinant crystallins had mainly β strands on their secondary structure. The presence of this structures are directly related with the presence of the Greek key motif, which is rich in β -sheets interallied with β -Turns. [2,112] The presence of KP peptide and GA linker on crystallin's sequence did not affect the crystallins rich in β related structures as predicted by the presence of the Greek key motif.

Table XIII - Secondary structural assignments on CrysWt and CrysMut proteins. The results of Amide I deconvolution, expressed in percentages, were analyzed using OriginPro 8.5 software.

Secondary Structure (%)		
Amide I deconvolution		
	CrysMut	CrysWt
β-sheet	47,10	47,00
β-turn	31,90	31,53
α-helix/ Random coil	21,0	21,47
FWHM*	4,83	5,23

*Full width half maximum

Chapter 4: Conclusion and Future perspectives

In this work two distinct strategies for the treatment of keratinaceous appendices were implemented.

The first part of this work was to produce keratin-based particles for the future development of cosmetics for the restoration of hair's mechanical properties.

Keratin-based particles [Keratin, SilkFibroin, Poloxamer407, (K.SF,P.)] created by high pressure homogenization (HPH) were accomplished with particles efficiency between 97 and 100%. The particles Z-average was between 150 and 250 nm, determined by DLS; with a net negative surface Zeta-potential. The incorporation of silk fibroin and mainly the Poloxamer 407 contributed for a shift of the particle's surface potential for less negative values. Despite some slight variations along time when compared with day one, the keratin-based particles were stable in PBS 1x up to 3 months when stored at 4°C.

Spherical keratin particles were observed in STEM micrographs as well as the coating effect of Poloxamer 407. The silk fibroin also affected the surface of the keratin-based particles, making them less smooth due to the crystalline β -strands structures characteristic of this protein.

Particles' proteins secondary structure studies show the α -helical and β -strand conformation of both extracted proteins, when in raw solution. After HPH, the disruptive forces prompt proteins to self-assemble into more crystalline structures. This effect was more evident in particles with silk fibroin.

Human skin keratinocytes (NCTC 2544) exposed to different concentrations of keratin-based particles did not show signals of significant cytotoxicity (% cell viability $\geq 82.5\%$) for any of the tested particle's concentration. The absence of significant cytotoxicity makes the keratin-based particles suitable for administration to hair.

Hair treatment with the keratin-based particles revealed to be very effective both for virgin and overbleached fibres of dark Asian hair. The deposition and binding of the particles on hair was confirmed by SEM micrographs taken after each treatment. While for virgin hair the particles preferentially settled in the inter-scalar zone of the cuticle, in the overbleached hair the distribution of the particles was more uniform.

The effect of overbleaching on the mechanical properties and surface of Asian hair was confirmed by SEM and by tensile tests, with a decrease on hairs stiffness of 21.2% measured in terms of Yong's modulus. Nevertheless, treatment with the keratin-based particles was able to recover and improve the properties of damaged hair and virgin hair up to 83.7% and 62.7%, respectively, when the hairs were incubated with the particles with the highest amount of silk fibroin

(20%) and Poloxamer 407 on its composition. The presence of β -strands and the coating properties of the surfactant increased the binding of the particles to hair surface and most likely improved their diffusion into hair cortex contributing for α -helical keratins stabilization giving these structures a higher resistance to unfold during stretching.

The inclusion of keratin-based particles in a cosmetic formulation for hair treatment could potentially reverse the negative effect of cosmetic colouring protocols like bleaching while improving hair strength and appearance.

In the second part of the work the expression of two recombinant crystallins, CrysWt and CrysMut, based on the sequence of natural human eye γ D-crystallin was achieved in BL21 *E. coli* strain using TB-AIM as culture media. Proteins purification was performed with magnetic beads taking advantage of the His-tag present on the crystallins amino acidic sequence. The purification method was effective as confirmed by SDS-PAGE and MALDI-TOF.

Both proteins were monodisperse and the theoretical molecular weights; 24.8 kDa for CrysWt and 24.6 kD for CrysMut; were within the experimental error of the values determined by MALDI-TOF analysis.

The study of crystallins' secondary structure revealed that the recombinant proteins preserved their structure, rich in β -sheets, when compared with the structure of human eye γ D-crystallin. The presence of the Greek key domain, rich in β -sheets and characteristic of the crystallin family of proteins, was confirmed. The mutant form displayed an increase in the percentage of β -sheets which could be related with the presence of three extra cysteine residues that contribute for higher degree of protein polymerization and molecular structure rigidity.

Regarding future perspectives, the potential of both keratin-based particles and recombinant crystallins could be explored in different applications such as:

- Study the potential of keratin-based particles to be used in the transdermal delivery of compounds. These studies could be extended to other structures such as nails.
- Encapsulation of dyes in the keratin-based particles for the development of new hair colouring methods.
- Study the potential of recombinant crystallins to form films with altered optical properties.
- Application of crystallins on hair for the improvement of hair fibre properties and appearance.

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