Universidade do Minho
Escola de Engenharia

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Protein Disulfide Isomerase-assisted functionalization of proteinaceous substrates

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Tese de Doutoramento em Engenharia Têxtil

Trabalho efectuado sob a orientação do
Professor Doutor Artur Manuel Cavaco-Paulo

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Ao meu pai

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Abstract

The formation of intramolecular disulphide bonds is critical in the process of protein folding and on the stabilization of protein tertiary structure. Their formation involves the oxidation of two thiol groups that should be correctly paired. The incorrect paring inhibit the protein folding into its native conformation. The rearrangement of incorrectly disulfide bonds on proteins is catalysed *in vivo* by the protein disulphide isomerase (PDI). This versatile enzyme is able to catalyse the oxidation, reduction and isomerisation of disulfide bonds in a broad range of protein substrates. This dissertation successfully presents the use of PDI for functionalization of cysteine-containing (CC) proteinaceous substrates such as keratin fibres and RNase A microspheres. These approaches take advantage of the presence of thiol moieties and disulphide bonds in these substrates. It was shown that the type of reaction catalysed by PDI can be predicted, by controlling the redox environment. When the active site of PDI is in its oxidized state due to is characteristic potential redox (E^o = -180 mV) an oxidation reaction is catalyzed. When the active site of PDI is transformed to its reduced state (ΔE = - 260 mV), the isomerisation of disulfide bonds is promoted.

PDI was able to incorporate CC functional molecules on wool and hair trough disulfide bonds, as suggested by matrix-assisted laser desorption and ionization time-of-flight results (MALDI-TOF) analysis. Similarly, PDI increased the affinity of a synthesised keratin-based peptide (KP) towards hair and facilitated the penetration into its cortex. Targeting a biomedical approach, Ribonuclease A (RNase A) was oxidatively attached to the wool surface through disulphide bonds, and PDI was shown to induce its release.

Aiming a cosmetic application, KP and other synthesized surfactant-based peptide (SPB) were applied in over-bleached, damaged hair. Both peptides induced an improvement on its mechanical properties and thermal stability. Thus, recovering of the fibre integrity loss during the hair bleaching process was achieved. In the presence of PDI, peptides were linked by disulphide bonds and the thermal stability increased at higher levels.

Due to the great properties developed on over-bleached hair after KP application, in the absence of PDI, other type of hair (relaxed hair) was treated with this peptide and the same properties were measured. The relaxing treatment, commonly applied on excessively curly hair, often result on the weakening of the fibre. KP was then applied to this weakened

hair and its ability to recover its mechanical and thermal properties was proved. Two different peptide formulations were evaluated. In one formulation KP was dissolved in aqueous solution (WF) while in the other KP was dissolved in organic solvent solution (OF). The last imparted better mechanical and thermal properties to the hair, however, the safety assessment showed that OF is potentially cytotoxic, inhibit cell growth, and genotoxic. The KP itself did not inhibit the cell growth and was found to be non-cytotoxic and non-genotoxic, hence suitable for the application on cosmetic formulations at concentrations up to 0.5 g/L.

Showing its ability to act on a broad range of cysteine-containing compounds, PDI was also able to oxidize the thiol groups found in sphere-like particles, recovering their biological function, at the conditions that lead PDI's active site on its oxidized state. Using a reducing environment, PDI promoted the released of native RNase A from protein-based microspheres.

The research presented in this thesis shows the versatility of PDI to promote diverse functionalization on proteinaceous substrates, resulting in a wide applicability in different areas such as cosmetic, textile and biotechnology. The work was carried out partially in collaboration with a cosmetic company; hence the research included promising biotools-based strategies for hair-care product development, especially for the application on several types of damaged hair.

Resumo

As pontes dissulfídicas são uma característica muito importante para a estabilização da estrutura terciária de proteínas. A formação das mesmas envolve a oxidação de dois grupos tiol, os quais devem estar correctamente ligados. Caso contrário, a proteína perde a sua actividade biológica característica, devido à formação de pontes dissulfídicas não-nativas, e desnatura. *In vivo*, aquando da formação das proteínas na célula, uma enzima tem uma função muito importante: promover o rearranjo das pontes dissulfídicas não-nativas e prevenir a agregação das proteínas desnaturadas. Esta enzima é a Protein Disulfide Isomerase (PDI), uma isomerase de pontes dissulfídicas que catalisa a oxidação, redução ou isomerisação de uma vasta gama de substratos contendo cisteína na sua constituição. Esta dissertação apresenta com sucesso a aplicação desta enzima na funcionalização substratos proteicos, tais como as fibras queratinosas ou microesferas de Ribonuclease A (RNase A). As pontes dissulfídicas dos substratos, acima mencionados, são as ligações-alvo nesta abordagem. Foi demonstrado que o potencial de redução do centro activo da PDI é uma ferramenta importante na pré-determinação das reacções que esta enzima pode catalisar. Quando o centro activo da PDI está no seu estado oxidado, devido ao seu característico potencial redox (E^o = -180 mV), uma reacção de oxidação é catalisada. Quando o centro activo da PDI é transformado para o seu estado reduzido (ΔE = - 260 mV), a isomerisação das pontes dissulfídicas é promovida.

A PDI catalisou a incorporação de moléculas contendo cisteína (CC) em lã e cabelo através de pontes dissulfídicas, sugerido pela análise de espectroscopia de massa (MALDI-TOF). A PDI facilitou também, a penetração de um péptido de queratina (KP) no córtex da fibra de cabelo e induziu a libertação de uma proteína modelo (Ribonuclease A) da superfície da lã.

Com o objectivo de uma aplicação cosmética, o KP e outro péptido, derivado de um surfactante humano (SPB) foram aplicados em cabelo danificado, previamente sujeito a vários ciclos de branqueamento oxidativo. Ambos os péptidos melhoraram as propriedades mecânicas e a estabilidade térmica do cabelo danificado, provando a sua capacidade para recuperar a integridade da fibra. Quando a PDI foi aplicada, os péptidos ligaram-se ao cabelo através de pontes dissulfídicas e a estabilidade térmica aumentou para valores ainda mais elevados.

Devido ao efeito renovador que o KP teve sobre o cabelo branqueado, outro tipo de cabelo (cabelo relaxado) foi tratado com este péptido e as mesmas propriedades foram

medidas. Os tratamentos de relaxamento em cabelo extremamente encaracolado resultam em enfraquecimento do mesmo. O KP foi por isso aplicado neste tipo de cabelo e a sua capacidade para o melhorar foi provado. Dois tipos de formulações peptídicas foram também testados. Numa formulação o KP foi diluído numa solução aquosa (WF), enquanto na outra o KP foi diluído numa solução contendo solventes orgânicos (OF). Esta última promoveu melhores resultados, contudo, revelou-se potencialmente cytotoxica, genotoxica e inibidora do crescimento celular. Demonstrou-se, todavia, que o péptido em solução aquosa pode ser aplicado em formulações cosméticas até à concentração de 0.5 g/L não manifestando citotóxicidade, genotóxicidade ou inibição do crescimento celular.

Demonstrando a sua aptidão para actuar em compostos contendo cisteína, a PDI foi também capaz de oxidar os grupos tiol presentes nas microesferas, recuperando a sua função biológica nas condições que promovem o estado oxidado do seu centro activo. Usando um ambiente mais redutor, promoveu a libertação da proteína nativa das microesferas proteicas.

Os resultados apresentados nesta tese demonstram a versatilidade da PDI para promover a funcionalização de substratos proteicos, resultando numa ampla aplicabilidade em áreas distintas como cosmética, têxtil e biotecnologia. O trabalho foi desenvolvido em parceria com uma empresa de cosmética, o que fomentou a procura de estratégias biológicas para o desenvolvimento de novos produtos para cabelo, especialmente para a aplicação em vários tipos de cabelo danificado.

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Abbreviation list

ΔE: Redox potential created by Nernst equation

5(6)-TAMRA: 5(6)-carboxytetramethyl-rhodamine, succinimidyl ester

8xB: Over-bleached hair

A

AB: Alamar blue

ATR: Attenuated Total Reflectance

B

BCC: Business Communications Company

BSA: Bovine Serum Albumin

C

C&T: cosmetic and toiletries

CCC: Cysteine-containing compound

CC: Cysteine-containing

CC-dye: Cysteine-containing dye

CC-peptide: Cysteine-containing peptide

CC-protein: Cysteine-containing protein

CC-substrate: Cysteine-containing substrate

Cys: Cysteine

cCMP: cyclic 2'3'-monophosphate

D

DCFH-DA: 2',7'-dichlorodihydrofluorescin-diacetate

DE: Dynamic Extraction

DHB: 2,5-dihydrobenzoic acid

DMEM: Dulbecco's BJ5TA Modified Eagle's Medium

DNA: Deoxyribonucleic acid

DSC: Differential Scanning Calorimetry

DTNB: 5,5'-dithiobis(2-nitrobenzoic acid)

DTT: dithiothreitol

E

E^o: Standard redox potential

F

FBS: fetal bovine serum

FITC: Fluorescein 5(6)-isothiocyanate

FTIR: Fourier Transform-Infrared Spectroscopy

G

Gly: Glycine

GSH: Reduced glutathione

GSSG: Oxidized glutathione disulphide

H

HCCA: cyano-4-hydroxycinnamic acid

His: Histidine

I

IFAPs: Intermediate filament associated proteins

IFPs: Intermediate filament proteins

K

KIF: Keratin intermediate filaments

KP:Keratin peptide

L

LDA: Laser Doppler Anemometry

M

MALDI-TOF: Matrix-assisted laser desorption and ionization time-of-flight results

MIT: Massachusetts Institute of Technology

MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromid

Mw: Molecular weight

O

OF: Organic solvent formulation

P

PCS: Photon-correlation spectroscopy

PDI: Protein Disulphide Isomerase

pI: Isoelectric point

Pro: Proline

R

Rel: Relaxed hair

RH: Reduced hair

RNase A: Ribonuclease A

S

SEM: Scanning electron microscopy

SPB: Surfactant peptide

T

Tdw: Temperature of water removal

Tdα: Temperature of α-helix denaturation

Trx: Thioredoxin

V

VG: Virgin hair

W

WF: Water environment formulation

WH: Washed hair

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Objectives and Thesis Layout

The major objective of the thesis was to investigate the enzymatic functionalization of proteinaceous substrates such as hair, wool or microspheres using protein disulfide isomerase (PDI) enzyme, aiming cosmetic and biotechnological applications. This enzyme has the ability to act upon cystine/cysteine-containing compounds (CCC), promoting oxidation and/or isomerisation of disulfide bonds. Formation of new disulfide bonds between protein substrates and CCC by oxidation of free thiol groups or the rearrangement of non-native disulfide bonds that lead protein to its native conformation are functionalizations here presented. The influence of the state of the PDI active site in promoting these functionalizations is also discussed. In the quest to accomplish the general objective, the thesis is organized in 5 individual chapters each aiming to fulfil a specific goal

Chapter 1 presents a general introduction that reviews the PDI's natural functions and overall protein substrates used throughout of the work. An overview of the characteristics of keratin fibres, the state of the art related to their surface treatments and the possibility of being used as a PDI substrate is highlighted.

Chapter 2 focuses on the functionalization of human and animal hair fibres (hair and wool) using PDI for different applications: incorporation of a cysteine-containing peptide on hair for cosmetic purposes, grafting of a cysteine-containing dye on wool, and the release of a model protein from wool surface aiming biomedical applications.

Chapter 3 and 4 deals with the application of different cysteine-containing peptides on damaged hair fibres for cosmetic purposes. Chapter 3 focuses on the PDI-assisted grafting of peptides that were synthesized based on amino acid sequences from human hair keratin (KP) and human lung surfactant (SPB), on overbleached hair. The recovery of physical and chemical properties of hair fibre is also highlighted. Subsequently, Chapter 4 presents the application of KP on hair that was previously subjected to a relaxing treatment. Further investigation on the peptide risk assessment, including cytotoxicity and genotoxicity tests is presented.

Chapter 5 emphasize the ability of PDI to recover the partially lost enzymatic activity of RNase A after generation of enzyme microspheres by ultrasound application. In this chapter the PDI aptitude to catalyze the refolding of a protein substrate in the form of spheres is described for the first time.

Finally, **Chapter 6** is devoted to an integrative discussion focused on the main contributions of the present work to the understanding of PDI assisted reactions on protein fibres when the PDI active site is manipulated. Brief suggestions for future directions to complement our research are presented, together with a few general concluding remarks.

Chapter 1

General Introduction

1 General Introduction

The use of isomerases, e.g. protein disulfide isomerase (PDI) for functionalization of proteinaceous substrates has been central only to a few studies. The interest for exploiting PDI arises from its broad specificity and ability to catalyze three types of reactions: oxidation, reduction and isomerisation of disulfide bonds in proteinaceous substrates. They are responsible for the stabilization of protein tertiary structure (Wedemeyer et al. 2000) and in keratins act as a disulfide cross-linker holding the fibre cortical superstructure together (Plowman 2007).

Understanding the role of disulfide bonds in protein folding is important to recognize the ability of PDI for functionalization of proteinaceous substrates. For that reason, the present chapter starts with notions on these topics. Further, the structure and chemistry of keratin fibres and Ribonuclease A (RNase A), the substrates used throughout the work, is then highlighted in order to understand how the functionalization may occur. Human hair structure is particularly emphasized seeking new approaches for innovative hair-care products. Consequently, some concepts about cosmetic science, safety assessment and cosmetic product development are also reviewed.

1.1 Disulphide bonds and protein folding

The chemistry involved in the formation of disulfide bonds includes a two electron reaction between two thiol groups (-SH) that requires an oxidant or electron acceptor. For example, disulfide bonds are formed spontaneously *in vitro* by the loss of electrons from two cysteine thiols coupled with the gain of electrons by an available acceptor, such as molecular oxygen. *In vivo*, however, the most common mechanism for the disulfide bonds formation is a thiol-disulfide exchange reaction between a free and a disulfide-bonded species. This reaction may occur in the endoplasmic reticulum of eukaryotic cells or in the periplasmic space of prokaryotic cells, playing an important role in the protein proper folding and stability (Sevier et al. 2002). Disulfide bond formation is thus one of the key rate limiting steps in oxidative protein folding (Hatahet et al. 2009; Welker et al. 2001). The proximity, reactivity and accessibility of the disulfide bonds (S-S) and thiol groups (-

SH) has been identified as the three most important structural factors for proper folding of proteins (Wedemeyer et al. 2000).

The proximity of two reactive groups is defined as the probability of their sulphur atoms coming within the distance required for thiol/disulfide exchange. The reactivity of thiols and disulfide bonds are dependent on the pH of the solvent and the pKa values of the thiol groups involved and, more generally, on the electrostatic environment of the reactive groups. And finally, the most critical factor seems to be the accessibility of the thiol groups and disulfide bonds. Thiol disulfide exchange reactions can occur only when a thiol and a disulfide bond come into contact; hence, burial of the disulfide bond or the thiol prevents their contact and blocks the reaction (Wedemeyer et al. 2000). These conditions are of paramount importance for the protein proper folding and biological activity. In fact, numerous studies agree that disulfide bonds play one of the most important roles on folding process (Witt 2008). They are thought to control the conformational flexibility on unfolded proteins, decreasing its conformational entropy and then favouring the protein folded state (Bulaj 2005; Jungbauer et al. 2007).

However, the protein folding pathway is still case of many hypotheses and its understanding is one of the major challenges in genetic and protein engineering (Freedman 1995). One of the theories explaining this process relies on a funnel energy landscape concept (Bryngelson et al. 1995), that describes folding as the inevitable consequence of the requirement to lower the free energy (increased stability) as more native interactions are formed. In this landscape view of folding (Figure 1.1), the denatured state of the protein populates a higher energy state area in the funnel. The polypeptide chain may then fold by numerous pathways, potentially adopting multiple partially folded ensembles en route to the native state, thus lowering the structure energy (Onuchic et al. 2004). A protein can theoretically exist in a nearly infinite number of conformations along its energy landscape, but in reality they fold into a unique conformation that possess the lowest possible free energy (Anfinsen 1973). The ruggedness of the energy landscape arises from the fact that protein structures are stabilized by thousands of weak interactions that cannot all be satisfied simultaneously during folding (Bartlett et al. 2009). During the process, many metastable intermediates often include non-native interactions, which need to be overcome before the native state can be achieved (Hartl et al. 2002) (Figure 1.1).

Nevertheless, it is known that, *in vivo*, the proper protein folding is dependent on cellular catalysts that promote the formation of new disulfides (oxidation) and the rearrangement of non-native disulfide bonds (isomerisation) and on chaperones to inhibit

aggregation (Hartl et al. 2002; Kulp et al. 2006; Wang et al. 1998). Early in folding, disulfide formation is error-prone and the protein folding process may not occur properly (Wilkinson et al. 2004) due to wrong cysteines connected (Creighton 1979; Rothwarf et al. 1998), or the correct cysteines are paired but in a temporal order that inhibits folding (Creighton 1992a; Weissman et al. 1991). This could cause the accumulation of misfolded proteins in cell and lead to loss of its function. This phenomenon is observed in a number of diseases (Dobson 2001; Thomas et al. 1995; Koo et al. 1999), including Alzheimer's (Harper et al. 1997), goiter (Kim et al. 1996), emphysema (Cabral et al. 2001), and prion infections (DebBurman et al. 1997).The eukaryotic cell, however, uses a number of mechanisms to protect against protein misfolding. These include chaperones and folding catalyst that inhibit aggregation and stimulate folding, such as protein disulfide isomerase (PDI) and specialized redox environment, such as the endoplasmic reticulum (ER) environment (Hwang et al. 1992; Fassio et al. 2002).

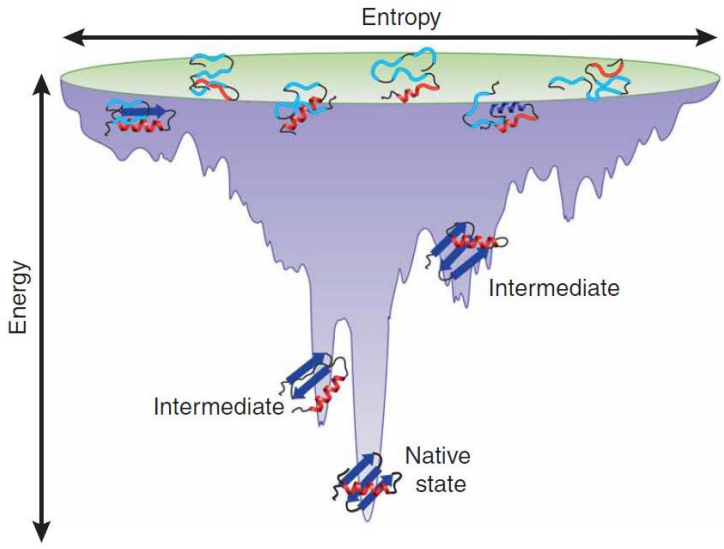


Figure 1.1 - Schematic representation of a protein folding funnel energy landscape, adapted from (Bartlett et al. 2009). Nascent chain polypeptides can initially explore a wide range of conformations. Certain conformations occupy a lower energy state and are more stable, representing intermediates in the folding pathway. The native state is usually the lowest energy and most stable state.

1.2 Protein Disulphide Isomerase (PDI)

First identified in 1963 and characterized based on its ability to catalyze the refolding of a protein with four disulfide bonds, the Ribonuclease A (RNase A)

(Goldberger et al. 1963; Venetianer 1963), PDI (E.C. 5.3.4.1) was later found as a multifunctional enzyme able to catalyze disulfide bond formation, breakage, and rearrangement. Playing a critical role in promoting native disulfide bond formation *in vivo*, PDI introduces disulfides into proteins (oxidase activity) and catalyzes the rearrangement of incorrect disulfides (isomerase activity). It recognizes a large number of cysteine-containing substrates including a multitude of native like, partially unfolded and non native states (Wilkinson et al. 2004; Lyles et al. 1991; Gilbert et al. 1991; Freedman 1995), and is thought to induce the state of lower energy on misfolded proteins, promoting their proper folding.

1.2.1 PDI structure: domain organization and thioredoxin fold

PDI was first sequenced by Edman and co-workers (1985) from rat liver, that found that this mature polypeptide in mammals possess approximately 491 amino acids and a molecular weight of approximately 56 kDa. These achievements led to further analysis and definition of the modular structure of PDI. The analysis of its amino acid sequence, revealed the multidomain nature of PDI, in which four domains, denoted a, b, b' and a', were identified (Edman et al. 1985). These four structural domains were followed by a stretch of acidic residues at the C terminus (designated c) (Figure 1.2).

From the DNA sequence information of the enzyme, two segments with homology to thioredoxin, a small ubiquitous protein which catalyses reduction of protein disulfides, were identified (Holmgren 1985). These segments were defined as the catalytic active domains of PDI (a and a' domains, Figure 1.2), each possessing a Cys-Gly-His-Cys active site motif (Wilkinson et al. 2004).

Although the two active sites can function independently they do not have equivalent catalytic properties (Lyles et al. 1994). It has been reported that, at saturating concentration of substrate, the amino-terminal domain is capable of providing almost all of the catalytic activity for the oxidative refolding of reduced, denatured RNase, while the carboxy-terminal domain contributes more to the steady-state binding of the substrate. However, at lower substrate concentrations (near Km) both active sites contribute almost equally to catalysis (Walker et al. 1996).

The domains b and b' (Figure 1.2) are similar in sequence to each other but not to thioredoxin and therefore are considered inactive. Nevertheless, studies have shown that b' domain is important in the overall catalytic ability of PDI, providing the principal peptide

binding site of the enzyme (Klappa et al. 1998). With the combination with a and a' domains, b' domains were found to be essential for simple isomerisation reactions (Ellgaard et al. 2005). These studies indicated that a and a' domains are active domains responsible for PDI main activities towards non-native proteins, whereas the b' domain contains a higher affinity binding site by which PDI holds substrates during isomerisation reactions.

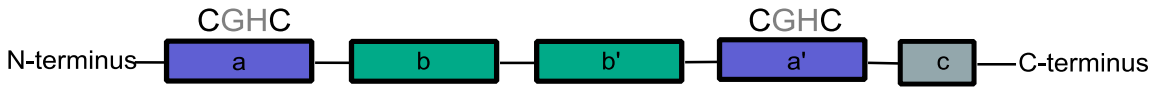


Figure 1.2 - Domain organization of PDI. Catalytic domains a and a'; and non-catalytic domains b, b' and c. The active site sequence of a and a' domains are also shown.

1.2.2 The thioredoxin superfamily

PDI is a member of a large family of dithiol/disulphide oxidoreductases, the thioredoxin superfamily. This family of thioredoxin-like enzymes includes prokaryotic enzymes, such as DsbA, DsbC, eukaryotic enzymes, such as Erp72, glutaredoxin, as well as the prototype of this family, thioredoxin (Ellgaard et al. 2005). These enzymes share the Cys-X1-X2-Cys active site motif and have a functional similarity: they are involved in sulphur based redox reactions in the cell. During catalysis of disulphide formation in its substrates, PDI has two catalytic active thioredoxin domains while other family members have one (DsbA), two (ERp57), three (ERp72) (Mazzarella et al. 1990), or four (ERdj5) (Cunnea et al. 2003). The residues between the two active site cysteines vary between each protein family and they are important in determining the active site potential and hence the physiological function of each enzyme. This can be modulated by mutations in the XX residues, which in part explains why enzymes with overall structural similarity can be reducing, such as thioredoxin (Cys-Gly-Pro-Cys), highly oxidizing, such as Dsba (Cys-Pro-His-Cys) or acting as isomerase such as PDI (Cys-Gly-His-Cys). PDI is the most versatile family member, capable of catalyzing oxidation, reduction and disulphide isomerisation (Wilkinson et al. 2004).

1.2.3 The catalytic domains of PDI- redox potential

The modulation of the redox potential of PDI is an important feature to understand the physiological relevance of the *in vitro* and *in vivo* reactions that PDI can catalyze. Each

active site in a and a' domains of PDI contain two cysteines in the sequence Cys-Gly-His-Cys that mediates all the activities of the enzyme. The cysteines in the form of vicinal thiol groups are essential for the catalysis of redox reactions and can either form an intramolecular disulfide (S-S) or exist in dithiol form (-SH).

The redox potential indicates the tendency of a chemical species to either gain or lose electrons when it is subject to a change by another molecule. It is an important characteristic that will define the propensity of two thiols to form a disulfide bond or of an existing disulfide bond to be broken (Gough et al. 2005a).The currently accepted redox potentials of various species, including a range of thioredoxin-superfamily members are shown in Figure 1.3 (Åslund et al. 1997). These enzymes' property is responsible for the highly reducing environment in the thioredoxin (E^o= -270 mV) (Moore et al. 1964), the quite oxidizing in DsbA (E^o= -90 to -110 mV) (Wunderlich et al. 1993) and the intermediate redox potential in PDI (E^o= -180 mV) (Wunderlich et al. 1993; Lundstroem et al. 1993).

Nevertheless, the redox potential of a species cannot be seen in isolation. By itself, the number is meaningless; it must always be seen in the context of reaction with another species and the fact that is usually involved in an equilibrium reaction in which the relative concentrations of the species present plays a major role in the net reaction. For example, PDI has a higher redox potential (E^o= -180 mV) than the redox potential of denatured proteins and peptides (E^o= -200 to 220 mV) and so it is expected to always promote the oxidation of the substrate, being reduced in the process (Hatahet et al. 2009). In this case PDI would act as a protein dithiol-disulfide oxidant toward the denatured protein (Figure 1.4 A). However, this is not always the case because of the mentioned concentration influence in the net reaction.

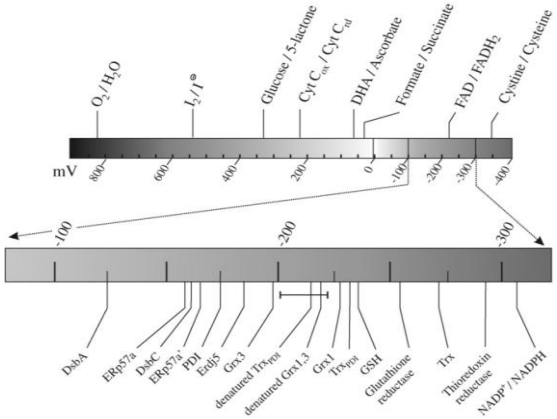


Figure 1.3 - Biochemical standard redox potentials, including thioredoxin-superfamily members, adapted from (Åslund et al. 1997)

The enzyme thioredoxin has the lowest redox potential of the thioredoxin superfamily members (i.e., it is the most reducing enzyme). Thermodynamically, it reduces disulfide bonds in proteins, and this is its primary physiological role *in vivo* (Lillig et al. 2007) because its redox potential is E^o= -270 mV, 50 mV lower than that of denatured proteins and peptides (Åslund et al. 1997). However, *in vitro*, it is able to catalyze the oxidation of dithiols to disulfides in folding proteins by using oxidized glutathione disulfide (GSSG) as the net electron acceptor (Figure 1.4 B). Because of this, all thioredoxin-superfamily members have the potential to act as catalysts of protein disulfide bond formation, reduction, and isomerisation.

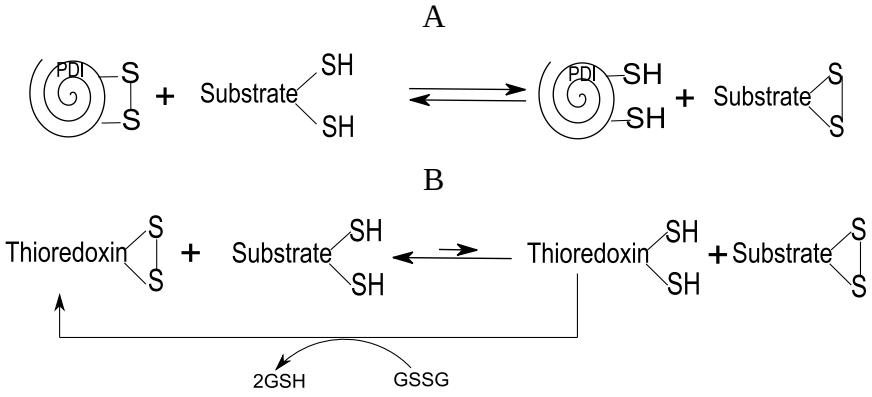


Figure 1.4 - Catalysis of protein dithiol oxidation by (A) PDI, based on its redox potential (-180 mV) and by (B) thioredoxin also based on its redox potential (-240 mV), and by using GSSG as the electron acceptor.

The redox potential of these enzymes depends on the stability of the reduced (-SH) and oxidized (S-S) state of the active site. Therefore, factors that stabilize the dithiol state of active site will increase the redox potential and thus make the enzyme a better oxidant: the enzyme is destabilized by having the active-site dithiol, and so it is preferential to donate it to a non-native protein substrate (i.e., to oxidize it). Similarly, factors that stabilize the disulfide state of PDI active site will lower the redox potential and will make the enzyme a better reductant, and so it is preferential to take it from non-native protein substrates (i.e., to reduce them) (Figure 1.5) (Wunderlich et al. 1993).

The catalytic role of PDI is based on the following: when PDI catalyses the oxidation of two cysteine thiol groups, it becomes reduced. Likewise, when PDI catalyses the reduction of a disulfide bond, it becomes oxidized. To complete both catalytic

activities, PDI must complete the cycle, i.e. it should be reoxidized when acts as an oxidase or re-reduced when acts as a reductase. For that, a redox generator such as glutathione should be present to act as an electron acceptor (GSSG-oxidase activity) or donor (GSH-reductase activity) (Hatahet et al. 2009) (Figure 1.5).

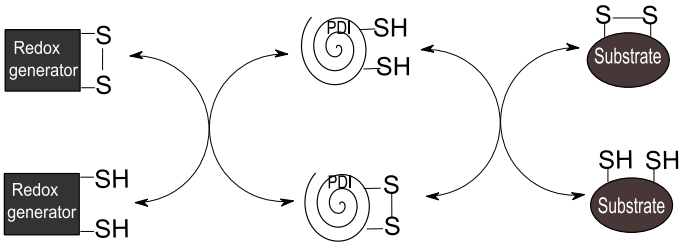


Figure 1.5 - Enzymatic reaction catalysed by PDI. The regeneration of the oxidoreductase can be accomplished by a small thiol buffer, such as glutathione**.**

The presence of these species is used as redox generators, to promote the active site redox balance, mimicking the ER environment *in vivo*. The occurrence of glutathione in ER provides a relatively reducing environment (GSH/GSSG=3-5) (Hwang et al. 1992; Taniyama et al. 1990; Bass et al. 2004) that allows disulfide formation (oxidation) while maintaining sufficient reducing power to break incorrect disulfides.

Besides the amino-acid sequence of the PDI active site in a and a' domains and the equilibrium between the active-site cysteines on stabilizing the disulfide (oxidized) or dithiol (reduced) state, the other important factor that influence the redox potential of thioredoxin-superfamily members is the pKa of the active sites cysteine. One domain is located near the amino terminus while the second is located near the C-terminus (Figure 1.2). The N-terminal active site cysteines are reported as having an unusually low pKa, ranging from 4.4 to 6.7, such that the active thiolate state ($-S^-$) predominates at physiological pH (Hawkins et al. 1991b; Kortemme et al. 1996). Thiols of low pKa leave more rapidly from a disulfide and are ionized more often, being therefore highly nucleophilic. Such an extremely low pKa value stabilizes the reduced state of PDI active site making the protein an excellent disulfide bond donor (Chivers et al. 1997). Therefore, when disulfides on active site are reduced to the dithiol state, the cysteine residues present at the N-terminal active site are predominantly in the thiolate ($-S^-$) state. These conditions provide a sufficiently higher redox potential that allows an efficiently protein dithiol

oxidation and to have efficient kinetics for the initial steps of catalysis of thiol-disulfide exchange. The C-terminal active site cysteines, however, are less reactive because they are buried and reacts only with the nucleophilic cysteine from N-terminal (Walker et al. 1996) and is usually cited as having a high pKa, such that the inactive thiol (-SH) predominates at physiological pH (Hawkins et al. 1991b).The pKa values of cysteine residues thus play a crucial role in PDI-assisted disulfide bond formation. While thiols are weak nucleophiles, thiolates are more potent, and hence the kinetics of thiol disulfide exchange is much faster once deprotonation of the cysteine side chain has occurred.

As summary, there are three prominent determinants of the redox potential of PDI to promote its enzymatic activity: the active site sequence; the presence/absence of redox generator that modulate state of N-terminal active site as disulfide (S-S) or dithiol (-SH) and the pKa of the active site cysteines that induce the presence of a reactive thiolate ($-S^-$). Therefore, PDI allows predictions to be made about the types of reaction it performs.

1.2.4 Catalysis of thiol disulphide exchange: isomerisation

PDI is one of the most abundant proteins in the ER and it has been reported to be mostly at its oxidized state (Frand et al. 1999), suggesting a determinant role for its oxidase activity. In addition to its oxidase activity, the evidences show that the most important activity *in vivo* arises from the reduced PDI active site: the isomerase activity (Pollard et al. 1998).Disulfide formation that occurs early during protein folding is often prone to error, resulting in the pairing of two cysteines that are not connected in the native structure or in the paring of correct cysteines but in a temporal order that impedes further folding (Wedemeyer et al. 2000). When incorrect disulfides are form, their isomerisation is required to provide correct protein folding (Figure 1.6). This involves the breakage of a substrate disulfide and its reformation with different cysteines connected. The efficient action of PDI as an isomerase arises, in part, from the unusually low pKa of the N-terminal active site cysteine residues (as above mentioned) that initiates isomerisation by attacking a substrate disulfide.

After the initial reaction, two different mechanisms could result in substrate isomerisation depending on how covalent PDI-substrate intermediate will react. In the first mechanism (Figure 1.6- black arrow) intramolecular rearrangement within the substrate may occur when the sulphydril in the substrate cysteine, generated by PDI reacts with another disulfide from the substrate. In this mechanism, PDI facilitates intramolecular

reactions between the thiols and disulfides of the substrate while the substrate is covalently bound to PDI. The intramolecular rearrangement is concluded with PDI displacement from the covalent complex and formation of another disulfide in the substrate together with the regeneration of reduced PDI for another round of catalysis. In the second mechanism (Figure 1.6 - dot arrow), PDI can resolve the covalent intermediate by reducing it. Cycles of substrate reduction and reoxidation in a different configuration lead to isomerisation, eventually leading to the native structure.

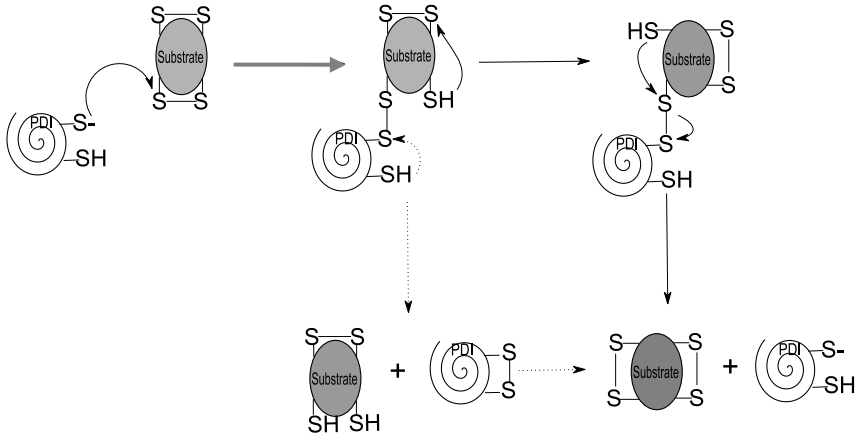


Figure 1.6 - Mechanisms of PDI-catalyzed disulphide isomerisation. Black arrow: in the intramolecular pathway, disulphide isomerisation occur through and intramolecular rearrangement. Dot arrow: the reduction oxidation pathway involves repeated cycling of substrate reduction followed by reoxidation in an alternative configuration.

1.2.5 PDI substrates and functions

Up to date, many proteins were identified as substrates for PDI *in vitro*, like soybean trypsin inhibitor (Steiner et al. 1965), insulin (Varandani et al. 1970), immunoglobulins (Murkofsky et al. 1979), vasopressin and oxytocin (Varandani et al. 1975), bovine serum albumin (Teale et al. 1976), cholera toxin (Moss et al. 1980), bovine pancreatic trypsin inhibitor (BPTI) (Creighton 1992b), ricin (Barbieri et al. 1982), and procollagen (Forster et al. 1984). Besides catalyzing the oxidation, reduction or isomerisation of disulfide bonds on a broad variety of substrates, PDI also possess chaperone activity inhibiting aggregation of misfolded proteins and stimulating the correct folding , thus preventing serious diseases that can occur on cell (Hartl et al. 2002).For example, it was found that PDI directly promotes tissue factor-dependent fibrin production during thrombus formation *in vivo* by, activating tissue factor via catalysis of thiol-

disulfide exchange and thus initiating the blood-clotting cascade at the site of wound damage (Reinhardt et al. 2008).

A conventional PDI substrate is RNase A, protein involved in the first studies of PDI ability to refold non-native proteins towards their native configuration (Goldberger et al. 1963; Venetianer 1963). The oxidative refolding of RNase A has been thoroughly investigated and led to the creation of the thermodynamic hypothesis of protein folding which claims that folded form of a protein represents the minimum of its free energy (Anfinsen 1973).

RNase A is a small enzyme consisting of 124 amino acids with a molecular weight 13.7 KDa (Berisio et al. 2002; Raines 1998). Native RNase A in aqueous solution is a folded, monomeric protein with three α-helices and six β-strands, which structure is stabilized by four disulfide bonds linking cysteines (Figure 1.7).

It is one of the classic model systems of protein science and was the third protein to have its structure solved (Wyckoff et al. 1967). With several high-affinity binding sites, RNase A is a possible target for many organic and inorganic molecules (Neira et al. 1999; Leonidas et al. 2003). Various forms of this protein, including its oligomers, have been demonstrated for antitumor and other biological activities (Soucek et al. 1999; Matousek et al. 2003; Fu et al. 2004).

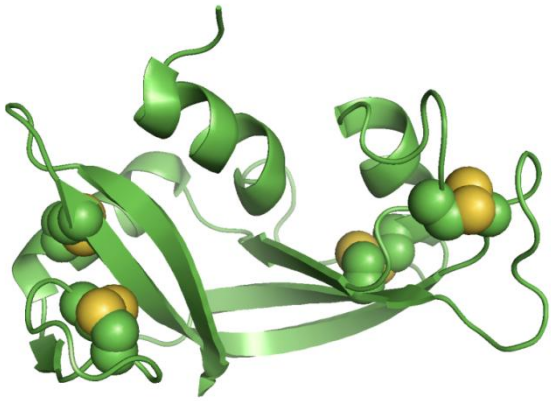


Figure 1.7 - RNase A structure showing the three α-helices, the six β-strands and the four disulphide bonds, represented by the yellow spheres, taken form protein data bank.

All the PDI substrates share one structural characteristic: the presence of cysteine residues. It is thought that PDI is able to interact with a broad range of cysteine-

containing compounds (CCC) to promote oxidation, reduction or isomerisation. Keratin fibres are the example of such compounds and their structure, properties and applications will be discussed in detail in the following section.

1.3 Keratin fibres

Keratins belong to the family of fibrous structural proteins being the basic building blocks of fibres such as hair and wool and the key structural materials of the outer layer of human skin and nails (Plowman 2007). Keratins are complex natural composites with a heterogeneous morphological structure, mostly proteinaceous in nature (95 % - 97 %) while structural lipids, pigment, and other materials represent only the remaining fractions (Heine 1995).

Being of biological origin, keratin fibres, unlike synthetic polymer fibres, are not continuous in their full length and are not chemically homogeneous. They rather result from compact groups of cells within the fibre follicle and from a complex mixture of widely different polypeptides (Rippon 1992). The individual polypeptide chains are held together by many different ways, from covalent bonds, such as disulfide and isopeptide crosslinks, to weaker interactions such as hydrogen bonds, coloumbic, Van der Walls and hydrophobic interactions. Keratins reactivity is thus complex and depends not only on the presence of reactive groups in the fibre, but also on their availability. The latter is significantly affected by fibre morphology and molecular structure (Wolfram 2003).

Three distinct varieties of cells are produced in the follicle base, which eventually form the three basic components of the fibre structure: the multicellular external cuticle sheath, the fibrous cortex and the medulla (Franbourg et al. 2003; Wolfram 2003). A single layer of cells gives rise to the cuticle, a protective layer covering the core of the fibres. It is mainly composed of β-keratins and displays a scaled structure with the cuticle edges pointing toward the tip of the fibre (Swift 1999; Koehn et al. 2010). The outer surface of the cuticle scale cells is coated by a thin membrane called the epicuticle, that covers the exocuticle, a constituent that contains most of the cysteine residues present in the scales (Feughelman 1997a) (Figure 1.8). The endocuticle, located at the interface of the cortex, is mainly composed by remaining cell organelles, and consists of proteins that, unlike those found in other parts of the keratin fibre, have very low sulphur content, thus poor in cysteine which causes the endocuticle of the scales to swell considerably more in water then the cysteine-rich exocuticle (Figure 1.8). This might explain the pronounced

projection of the scales and the tendency for wool felting in the presence of water (O'Connor et al. 1995).

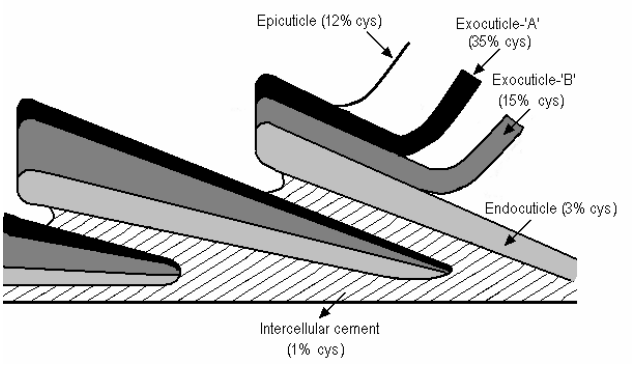


Figure 1.8 - Schematic scale structure of the cuticle showing the major components, adapted from (Feughelman 1997a).

The cuticle tightly encircles the cortex that forms the most voluminous part of the keratin fibre. The cortex is made of cortical cells which comprises the macrofibrils, long filaments oriented parallel to the axis of the fibre. Each macrofibril consists of proteins called the intermediate filament proteins (IFPs), known also as microfibrils, and the intermediate filament associated proteins (IFAPs), also known as matrix (Plowman 2007; Bhushan 2008) (Figure 1.9). It has been established that the molecules that aggregate to form the IFPs in keratin fibres are Type I and Type II keratin chains, arranged parallel to one another and in the axial register. After the formation of the α-helices, it is believed that the two types of chains associate to form a dimer that then aggregates with another dimer to form a tetramer. Finally, the formation of a pseudo-hexagonal structure (the IFP's structure) occurs by the association of seven or eight tetramers. Type I chains are net acidic, with pI values in the range of 4.5-5.5, while type II neutral-basic with pI around 6.5-7.5 (Smith et al. 2007; Zimek et al. 2006; Parry et al. 2006; Jones et al. 1997). The intermediate filaments are low in cystine (~6 %) while the matrix contains up to 20 % of total amino acid residues (Wysocki et al. 1954; Wilson et al. 1927; Block et al. 1939). The matrix proteins, i.e. IFAPs, are rich in cysteine and thus link through intermolecular disulfide bonds to IFPs, holding the cortical superstructure together and conferring high mechanical strength, inertness and rigidity to keratin fibres. High sulphur proteins, ultra-high sulphur proteins and high glycine-tyrosine proteins are present in matrix proteins (γ-

keratins), depending on their cysteine, tyrosine and glycine content (Figure 1.9) (Danciulescu et al. 2004; Feughelman 1997a; Plowman 2003; Franbourg et al. 2003). Vacuolated cells may also be present along the axis of coarser α-keratin fibres, forming the medulla. These cells generally constitute only a small percentage of the mass of keratin fibres and are believed to contribute negligibly to the mechanical properties of keratin fibres. Physically, the medulla forms the empty space of the fibre (Feughelman 1997a; Bhushan 2008).

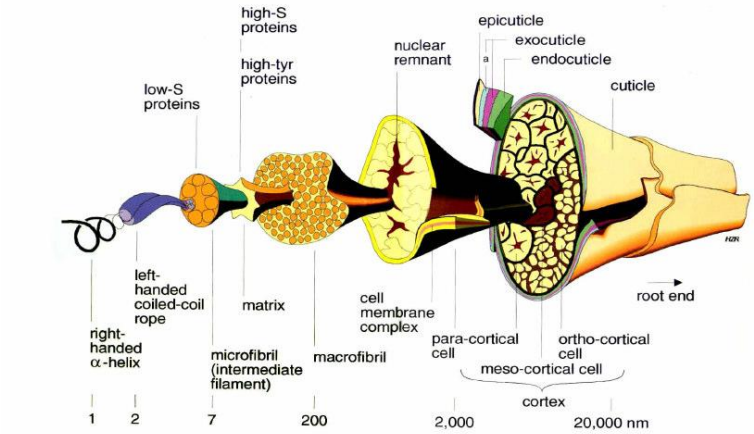


Figure 1.9 - Cross-section diagram of a keratin fibre showing the structure at progressive magnifications, according to Feughelman (1997).

To understand the properties of keratins it is essential to recognize their unique structural chemistry (Feughelman 1997b). The biological activity and biocompatibility of keratins have been explored in the development of keratin-based materials with applications in wound healing, drug delivery, tissue engineering, trauma and medical devices (Rouse et al. 2010; Li et al. 2007; Sierpinski et al. 2008). For example, the presence of high content of cysteine residues leads to a high rate of cross-linking through disulfide bonds (Franbourg et al. 2005), explaining the high stability of the macrostructure on keratin fibres, and imparting good mechanical, thermal and chemical properties (Wilson et al. 1927; Wysocki et al. 1954; Block et al. 1939). For this reason, keratin fibres are continuously being reported as novel biomaterials in the area of tissue engineering. Keratins can be isolated from human or animal hair by controlling the conditions which lead to disulfide bonds scission and reformation, and further fabricated into various designs, such as films (Vasconcelos et al. 2008; Tanabe et al. 2004; Yamauchi et al. 1996;

Fujii et al. 2004), microcapsules (Yamauchi et al. 1997), sponges and scaffolds (Katoh et al. 2004; Tachibana et al. 2005; Kurimoto et al. 2003).

Besides biomedical field, keratins have a multitude of other applications. The use of keratin from feather meal as a slow nitrogen release fertilizer, due to its slow decomposition rate, is well studied (Hadas et al. 1994; Riffel et al. 2002; Brandelli et al. 2009). Also, the possibility of using keratin as a foaming agent for fire extinguishers, taking advantage on the protein biomasses available as waste from textile industry and butchery, has been reported (Zoccola et al. 2009; Kittle 1998; Hoshino et al. 1978)

The knowledge of the chemical structure of keratins allows for the development of new technologies for the application in textile and cosmetic industries. Regarding cosmetics, besides understanding the hair surface as a substrate for functionalization, the application of new hair care products that contain keratin-based compounds, such as hydrolysate or fragments, are reported (Barba et al. 2009; Cavaco-Paulo et al. 2007; Pille et al. 1998; Negri et al. 1993), however, not sufficiently explored.

1.3.1 Enzymatic functionalization of keratin fibres

The structure of keratins from animal and human origin (wool and hair) is characterised by its complexity and the presence of a broad range of functional groups such as amine, amide, carboxyl, carboxyamide, hydroxyl, sulphydril, etc. (Plowman 2007; Block et al. 1939). This chemical versatility set keratin fibres as a suitable substrate for enzymatic modifications. It is, in fact, the surface morphology that plays a major role in the wool processing for textile industry (Araújo et al. 2009). Cuticle scales at the wool fibre surface are the reason for unwanted effects such as shrinkage, felting, and limited diffusion of dyes. Their removal results in an improved resistance to felting and shrinkage (Negri et al. 1993). The use of proteases to replace the conventionally used chlorine oxidation has been thoroughly investigated (Dybdal 2001; Mcdevitt et al. 1999). Improvements of protein physical and tactile properties as well as their dyeing characteristics are well reported. However, the enzymatic reactions need to be controlled to avoid undesirable effects, especially the enzyme diffusion into wool cortex and consequent fibre damage. These problems could be overcome by increasing the molecular weight of proteases in order to limit their action to the cuticle surface of wool fibre. Cross-linking the protease with glutaraldehyde, the attachment of polyethylene glycol to protease (Silva et al. 2005; Silva et al. 2006; Silva et al. 2004; Cavaco Paulo et al. 2006) or the development of a

biologically engineered subtilisin E fused with an elastin-like polymer (Araújo et al. 2009), are examples of successful application of modified proteases on wool. Transglutaminases have also been effectively tested on wool to improve the resistance to detergent damage and to reduce the propensity to shrink (Cortez et al. 2004; Cortez et al. 2005).

The enzymatic functionalization of human hair has been carried out as a safe alternative to the conventional chemical methods for shaping or dyeing of hair. Blount and co-workers (2002) have shown the development of new modes of benefit delivery in hair care, based on the application of an ingredient that may target an active endogenous hair fibre enzyme. Transglutaminases, proteases, lipases, steroid sulphatases, catalases and esterases are thought to be present in human hair as active endogenous enzymes, and therefore capable of interaction with exogenously supplied substrates.

Transglutaminases have been used to crosslink beneficial active compounds containing an amine moiety to glutamine residues in skin, hair or nails (Richardson et al. 1996). A method to gently and permanently relax or straighten hair was attained using a protease, kerA (Presti 2010). This enzyme was found to cleave inter-peptide bonds, allowing the hair fibre to be relaxed or straightened with less damage to the fibre than would have occurred using traditional or existing straightening methods.

However, the most characteristic structural feature of keratinous fibres - the high content of disulfide bonds in the form of cystine residues, has not been sufficiently explored for enzymatic modification. The high amount of cystine in protein fibres (Bradbury 1973) suggests that their surface would be susceptible to modification with enzymes active towards disulfide bonds, and more specifically with protein disulfide isomerase (PDI). PDI has been used for treatment of wool and hair fibres, which resulted in different patents. King and co-workers (1992) showed that PDI was able to restore part of the original properties on aged or harshly treated wool. The same enzyme was used by Brockway (Brockway 1992) to perform a curling, waving or straightening treatment safely under mild conditions. For the same purpose a thioredoxin-like compound (fragment of PDI) was used to efficiently treat hair with low amounts of thioglicolic acid, a commonly applied agent in relaxation treatments (Pigiet 1990).

1.3.2 Mammalian Hair: A unique physicochemical composite

Hair fibres are elongated structures composed of heavily cross-linked hard keratins found exclusively in mammals, enclosing both protective and cosmetic functions. Hair

protects the scalp from sunburn and mechanical abrasion providing thermoregulation, collection of sensory information, protection against environmental trauma, social communication and camouflage. Hair follicles, tens of thousands of which are deeply invaginated in the scalp tissue, are the essential growth structures of hair. At the base of each follicle, cells proliferate, and as they stream upward, the complex and intertwined processes of protein synthesis, structural alignment, and keratinisation transform the cytoplasm into the fibrous material known as hair (Rouse et al. 2010).

Keratinisation is a process on hair morphogenesis during which the cells gradually harden and finally die, giving rise to the hair fibre. The hair keratinises about one-third of the way up the follicle in a distinct keratinisation region that can be symmetric, wherein the process starts uniformly all round the fibre and proceeds concentrically inwards (Figure 1.10 A), or asymmetric, wherein cells begin to keratinise at a lower level in one side than the other, then spreading across the width of the cortex (Figure 1.10 B) (Ryder 1973; Chapman et al. 1971; Hashimoto 1988; Xu et al. 2011). As a result of keratinisation, two different cell types are found in the cortex of human hair: paracortical-like cells in which the macrofibrils are so closely packed that it is difficult to determine their boundaries and orthocortical-like with their discrete nearcircular macrofibrils (Swift 1997). Several studies have suggested that the difference between these two types of cells were due to the presence of higher quantity of cysteine residues in para-cortex in comparation with the ortho-cortex (Wortmann et al. 1998). However, histochemical results for human hair suggest that the two cell types contain approximately the same concentration of cysteine (Plowman et al. 2007).

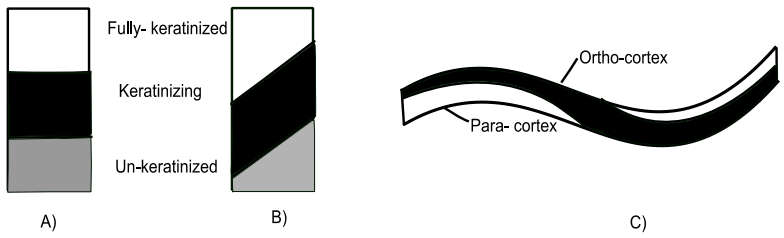


Figure 1.10 - Longitudinal section of a hair fibre through A) symmetric keratinization region and B) asymmetric keratinization region and C) bilateral nature of curly hair fibre.

In the fine wool, like merino wool, the ortho and para cortex form a bilateral structure in which the paracortex is always on the inside of a crimp wave and the orthocortex on the outside of the wave (Figure 1.10 C) (Feughelman 1997a). This

observation led to the belief that a relationship between these cells and the crimp or waviness of the fibre existed, in which the symmetric keratinisation would give rise to straight hair while asymmetric keratinisation would result in curly hair (Figure 1.10 A and B). Indeed, an interesting observation made by Swift (Swift 1997) is that the distribution of the two cell types appeared to relate to the racial origin of the hair. However, these facts have never been demonstrated to the degree of certainty (Plowman et al. 2007).

The hair follicle is one of the most complex mini-organs of the human body with the capacity to reconstitute itself, with a cyclic regeneration system comprised of actively migrating and differentiating stem cells responsible for the formation and growth of hair fibres (Araújo et al. 2010; Rouse et al. 2010). During postnatal life, hair follicles show patterns of cyclic activity with periods of proliferation and hair production (anagen phase), apoptosis-driven involution or regression (catagen phase) and relative resting (telogen phase), regulated by over thirty growth factors, cytokines and signalling molecules (Krause et al. 2006; Stenn et al. 1999; Alonso et al. 2006). Thus, the understanding of the hair follicle dynamics is important to define strategies to overcome the most common types of hair growth disorders, in which aberrant hair follicles cycling are the main reason. One such disorder is androgenetic alopecia, characterised by a shortening of the anagen phase and a prolongation of telogen, combined with miniaturisation of the hair follicle (Roberts 1997). Hirsutism, on the other hand, is defined as the presence of excess terminal hairs in females in an adult male typical pattern (Roberts 1997). To control the amount of hair produced for clinical purposes, the main strategy is to alter anagen duration either by shortening it in cases of hirsutism or by increasing it to correct alopecia.

1.3.3 Mechanical properties of hair

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 the chemical composition or structure of hair may greatly alter its mechanical properties (Franbourg et al. 2005). Moreover, the determination of the mechanical parameters also can be used to diagnose hair disorders (Franbourg et al. 2003).

The first comprehensive data, which laid the foundation for our understanding of the mechanical behaviour of wool fibres under differing conditions of temperature, time and moisture content were produced by Speakman (Speakman 1927). He demonstrated

that, in general, the longitudinal stress-strain relationship for a fibre equilibrated at a fixed relative humidity and at fixed temperature can be represented by three distinct regions of extension. When a keratin fibre is stretched, the load elongation curve shows three distinct regions as depicted in Figure 1.11 (Feughelman 1997a; Richard Beyak et al. 1969). In the pre-yield region, also referred to as the Hookean region, stress and strain are proportional, and elastic modulus can be found. In this region, there is the homogenous response of alpha keratin to stretching. The resistance is provided by hydrogen bonds that are present between turns and stabilize the α-helix of keratin. For example, by delicately handling a reasonably long hair it can easily be shown that it behaves like a piece of elastic; after extending slightly, it returns to its original length. Thus, for lengthening of up to 5 %, hair is elastic . The yield region represents transition of keratin from the alpha form to the beta form, the chains unfold without any resistance, and hence the stress does not vary with strain. The β configuration again resists stretching. When the stretching stops it returns to its initial form like a spring. Then the hair enters a condition known as flowing where, almost without effort, it can elongate by 25 % (Nikiforidis et al. 1992; Bhushan 2008). In the post-yield region, the stress again increases with strain until the fibre breaks. However, in this phase before breaking, the hair can still be elongated and it often breaks only after its length has actually doubled. The α to β transition of keratin is the reason for the unique shape of the stress strain curve of hair (Bhushan 2008) (Figure 1.1).

The hair fibre is a fibre of great strength. The load required to obtain breakage of a natural, healthy hair varies between 50 and 100 g. Thus, the average healthy head of hair (120000 hairs) may handle 12 metric tons (Franbourg et al. 2005).

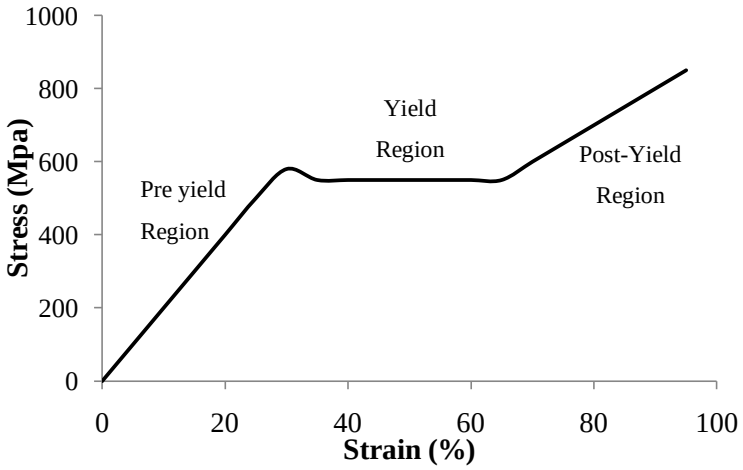


Figure 1.11 - Typical Stress-Strain curve for keratin fibres.

Two major properties of hair need to be taken into account while appraising the mechanical properties: the viscoelastic behaviour, which simultaneously shows time and strain dependence in the course of the finite duration of the experimental determination of the stress-strain curve (Erik et al. 2008); and the inhomogeneous structure, which is characterized by the three major compartments of hair: the medulla, the cortex and the cuticle (Franbourg et al. 2005).

1.3.4 Hair care products and processes

Human hair is the subject of remarkably wide range of scientific investigations. Its chemical, physical and biological properties are of importance to the cosmetic industry, forensic scientists and biomedical researchers, and as such it is a material for undergoing an infinite number of changes and treatments. Healthy and beautiful hair is desired and the need for products that improve the look and feel of hair surface has created a huge industry for hair care. Products such as shampoos and conditioners, along with damaging processes such as chemical dyeing and permanent wave or relaxing treatments, are daily applied on human hair (Gray 2001; Bolduc et al. 2001; Dawber 1996). These treatments are usually applied according to the specific need of each kind of hair, due to its ethnicity or aesthetic purposes. However, the chemical and mechanical damage imparted on hair by these treatments, which results in a weaker and more susceptible to breakage fibre after time, is one of the drawbacks of the commonly used cosmetic processes(Bhushan 2008). A variety of processes are applied everyday on hair to overcome these problems by improving the appearance, colour durability, robustness, softness and combability of hair, as well as for cleaning purposes (Bhushan 2008; Gray 2001).

Shampooing:

Shampoos are used primarily to clean the hair and scalp of dirt and other greasy residue that can accumulate after time. Modern shampoos include ingredients such as surfactants, lather boosters, conditioning agents, functional additives (to adjust pH and viscosity), preservatives (due to the possible microbial contamination) and aesthetic agents (fragrance, colorants, and pearlescent agents) (Bhushan 2008).

Surfactants are considered the most important ingredients on shampoos, providing foaming and detergent properties. Being amphiphilic substances, i.e., possess both hydrophilic (polar groups that have high affinity to water) and hydrophobic (nonpolar groups such as long hydrocarbon chain having a low affinity for water), they express very

different solubility characteristics and are able to disperse greasy materials in water. The non-polar groups (fat-soluble neutral components) bind to and surround dirt and sebum. The water soluble end faces outwards, and the hair, also negatively charged, repels the dirt/surfactant aggregate. Surfactants are classified according to their hydrophilic grouping, which may be ionic, i.e. carrying anionic or cationic charges or non-ionic (Beauquey 2005; Gray 2001).

Finally, shampoos confer increased mildness, conditioning benefits, anti-dandruff activity, volume enhancement, and even improved moisturisation (Gray 2001; C.Dubief et al. 2005).

Conditioning:

Conditioners are designed to deposit material onto the hair surface, particularly at the cuticular edges, to reduce grooming force and negative charges. In contrast to shampoos, conditioners are cationic systems that, for best effect, are presented as dispersions rather than solutions. Conditioners are usually oil/wax in water emulsions, with a cationic charge to encourage deposition with the net negative charge of the hair at areas of increased weathering (Gray 2001).

Unaltered human hair (virgin hair) has the isoelectric point near 3.67 (Regismond et al. 1999). Hence, during a normal hair shampooing procedure performed at neutral pH (pH>pI), the hair surface acquires negative charges. For this reason, most conditioning products are cationic polymers in order to counteract these negative charges, thereby improving hair texture and touch. Electrostatic interactions are believed to play a crucial role in the adsorption mechanism of such compounds (Regismond et al. 1999) and the negative charge of the hair is attracted to the positively charged conditioner molecules, which results in conditioner deposition on the hair. Thus, the ingredients of conditioners are mainly composed by cationic surfactants, fatty alcohols, silicones and water.

Permanent waving:

Permanent wave treatments underwent many advances in the beginning of the 20th century, but have not changed much after the invention of the Cold Wave around the turn of that century. Generally speaking, the Cold Wave uses mercaptans (typically thioglicolic acid) to break down disulfide bonds at low pHs and style the hair without much user interaction (at least in the period soon after the perm application). Rupture of ionic linkages or hydrogen bonds only leads to temporary hair shaping, namely the hair set. Cleavage of cystine links and their subsequent reforming in a new position is the process that affords permanent shaping (Zviak et al. 2005b). The chemical damage brought on by

the permanent wave is considerable and can increase dramatically when not performed with care.

Hair bleaching:

Besides albinos, all normal humans have melanin hair pigmentation, whatever the colour is. Dispersed throughout the structure of the cortex, in granular form, are the melanin pigment particles. The number, chemical characteristics, and distribution pattern of these cells determine the colour of the hair (Wolfram 2003). The actual shade of colour in each individual depends not only on which melanin is present but also its quantity and the site, number, and shape of pigment granules in the hair cortex (Borges et al. 2001).

Bleaching lightens the shade of the hair by oxidation of these hair pigments (eumelanin and pheomelanin) present in the hair cortex (Gray 2001). It has two main objectives: to give hair a lighter look or, more often, to prepare it for the application of a dye preparation, generally yielding a shade lighter or more vivid than the natural one (Zviak et al. 2005a). Hydrogen peroxide (H_2O_2) is used as the main ingredient for hair bleaching, present up to 12 % in alkaline solution. The alkaline solution is necessary since bleaching is favoured by alkaline pH; ammonia is the most commonly used as an alkali as it gives the best bleaching results. Thickeners are usually added to give a thicker, yet easy-to-spread consistency to the preparation (Brown 1997).

The process of melanin transformation by oxidants is not fully understood on a molecular level. It can be said, however, that depolymerisation occurs, giving rise to carboxylated derivatives, which are soluble in an alkaline environment and can therefore be eliminated by rinsing (Wolfram et al. 1970). Further, the chemical damage brought on by bleaching processes leads to high porosity and severe wear of the cuticle layer. The oxidation reaction destroys part of the disulfides within the keratin, leading to a weakening of the hair structure. Following bleaching, the hair is often different in texture, breaks more easily, and is more susceptible to humidity. To reduce these problems, a conditioner is often used either within the bleaching solution or after bleaching (Bolduc et al. 2001).

Hair colouring:

Today, people in ever-greater numbers alter their hair colour and appearance. However, the aggressiveness of the available techniques poses a big drawback in hair colouring. Commonly used hair dyeing compositions are driven by a mechanism of diffusion of small molecules into the hair fibre (Pille et al. 1998). There are three different types of hair colouring agents: permanent, semi-permanent and temporary colorants. The permanent hair dyes are constituted of small dye precursors, able to penetrate into hair,

which develop the colour within the hair shaft in the presence of high alkalinity and oxidative conditions. These dyes provide the best colouring results but cause significant hair damage. Due to the high levels of hydrogen peroxide used, severe chemical damage can ensue in the cuticle and cortex (Bhushan 2008; C.Dubief et al. 2005; Brown 1997). The semi-permanent and temporary dyes are molecules too large to diffuse into the hair, therefore acting on the exterior of the fibre, at the cuticle. This process does not harm the fibre due to the absence of alkaline oxidative conditions, but fails in terms of colour durability (Bolduc et al. 2001). For this reason, the development of a colouring agent that provides the durability of the permanent hair dyes without the use of oxidizing agents that damage hair is highly desired.

Hair relaxation:

Addressing the needs of the ethnic (i.e. African origin) hair, the hair-care market requires the understanding of the unique nature of the morphology of such hair. The main reason such hair is curly is due to the organisation of the disulfide bonds inside the fibres. The more disulfide bonds, the curlier is the hair. All hair has disulfide bonds but it is the shape of the hair strand itself which determines both how many and in what way the disulfide bonds are put together (Gray 2001).

Hair "relaxers" simply break these disulfide bonds and cap them so that they cannot chemically reform and will not go back to their original state. Relaxers usually consist of alkali metal hydroxides and guanidine as active straightening agents that cleave the disulfide bonds present in hair (Syed et al. 1998). The hair is then mechanically straightened using a comb to restructure the position of disulfide bonds between the new polypeptidic keratins. The high pH of the emulsion(between 12–14) swells the hair, helps opening the cuticle and allows the alkaline agent to penetrate the hair fibre and diffuse into the cortex (Gray 2001). Finally, these new bonds are consolidated using an oxidizing agent (Bolduc et al. 2001).

The breaking and reforming of bonds permanently rearranges the internal protein structure of curly hair. Although the reaction mechanism is not fully understood, it is well established that hair exposed to the relaxing agent transforms permanently one third of disulfide bonds into lanthionine bonds along with minor hydrolysis of peptide bonds (Figure 1.13). This results in a decrease of elasticity and tensile strength along with cuticular damage.

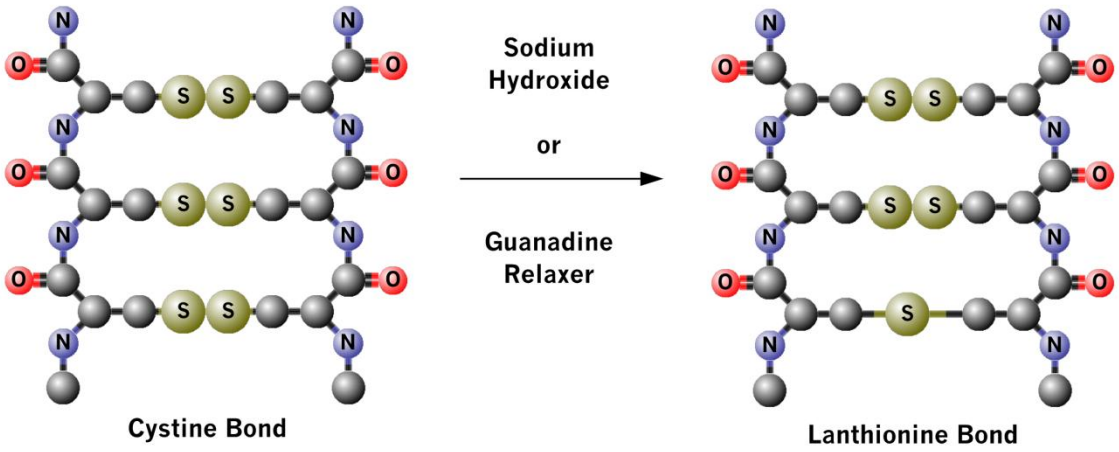


Figure 1.12 - Chemical structure of keratin showing disulphide bonds and lanthionine bond before and after relaxation treatment

Hair is very important and distinctive feature that plays a major role in self perception. It is one of the few physical features that we can easily change. Its length, colour, and shape can be modified to create a totally different style. However, the common chemical styling processes are also known to induce changes in the cuticle and cortex of hair and destroy part of its structure. Processes that replenish the cortex proteins of damaged hair and are meant to restore the fibres to a more healthy state are central to several investigations.

1.3.5 Research on hair surface modification

The main active ingredients used for formulating modern hair-care products are specifically designed to treat and improve hair conditions. However, commonly used processes are still aggressive, which impart damage to the fibre. For that reason, cosmetic industry is constantly seeking for new products to overcome these problems.

Penetration of compounds into the hair cortex is very important when new approaches for hair care applications are considered. However, the presence of the covalent isopeptide cross-links as well as the covalently attached lipids (predominantly 18-methyleicosanoic acid) on the hair surface constitute a diffusion barrier to chemicals and other treatment agents, thus impairing penetration (Heine 1995; Nolte et al. 1996). Being linked to the proteinaceous epicuticle via thioester linkage (Breakspear et al. 2005), this fatty acid forms a layer that can be removed by alkaline oxidative process, such as those

used in colouring and styling. In the case of wool, the process of removing the lipid layer bring advantages like enhancing the fibre wetability, dye uptake and polymer adhesion (Negri et al. 1993; Brack et al. 1999). When it comes to hair, however, these processes impart severe damage to the fibre. Products that could restore the damaged parts of cuticle and cortex are constantly under investigation.

The use of protein materials to improve shining, strength, softness and smoothness is well established. Several patents disclose compositions capable of restoring hair health. Application of proteins such as a water soluble compound derived from a vegetable protein derivate (Michiki 1998), non-naturally occurring keratin proteins (Ensley 2001), a mixture of a hydrolyzed protein and an amino acid with aliphatic side chain (Cornwell et al. 2003), and other hydrolyzed proteins (Ansmann 1985; Cannell et al. 1998) are also examples within this category.

The potential benefit effects of amino acids and peptides originated from keratin in hair care application were also explored in order to improve the structure of the keratin hair shaft (Roddick-Lanzilotta et al. 2007; Cochran et al. 2007; Oshimura et al. 2007). Another work describes the importance of peptide structure in the hair penetration using conventional fluorescent microscopy (Silva et al. 2007).

Polymer-containing compositions are one of the most common ingredients employed in cosmetic and personal care products. A diverse range of polymers are applied in this segment as conditioning and cleansing agents, film formers, fixatives, thickeners for hair dyes, emulsifiers, stimuli-responsive agents, foam stabilisers and destabilisers, skin feel beneficial agents and antimicrobials (Morgan et al. 2007). However, seeking for new polymer-based formulations for hair care products is still practice in cosmetic research science. Polymer compound combinations including vinyl pyrrolidone, vinyl caprolactam, among others were shown to confer improved hair-fixing properties (Birkel et al. 2002). Similarly, the polymerisation product of a monomer mixture comprising an acidic vinyl monomer and an associative monomer resulted in a product with good to excellent hair setting efficacy (Tamareselvy et al. 2006). Other polymer-based products were able to impart good flexibility and a good finish feeling to hair (Hiwatashi et al. 2002), or to impart colour to hair with excellent water resistance (Umeno 2009).

It is generally accepted that penetration of chemicals into hair occurs through intercellular diffusion, i.e. by adsorption onto keratin substrate. Faucher and Goddard (Faucher et al. 1976) have shown that the amount of polymer adsorbed on hair surface increased with decreasing molecular weight. Taking into consideration this study, low

molecular weight compounds might penetrate into the hair shaft where the diffusion process is greatly facilitated when hair is exposed to water. However, they are only retained while the hair is dry because further contact with water opens the cuticle scales facilitating their escaping. Recent solutions to this problem rely on the use of hair binding peptides coupled to dyes or pigments which are able to penetrate into the hair shaft, although they lack the required durability for long lasting dyeing effects (Fahnestock 2010). Huang and co-workers (2008) have tested a hair binding peptide coupled to carbon black and the use of chemically functionalised carbon nanotubes that provided enhanced interaction with the hair, resulting in a more durable hair colouring effect. Nevertheless, more durable hair colorants are still necessary. Another important factor when considering hair beauty and styling is air humidity, which affects hair form and structure at the level of hydrogen bonds. An increase of 30 % to 70 % on humidity will augment by 2-fold the water content of hair, thereby increasing its volume by more than 20 %. The influx of water eventually causes the hair fibres to swell, resulting in friction between fibres and an additional increase in volume and frizz, changing hair appearance (Schueller et al. 1998). To overcome this problem, scientists from Massachusetts Institute of Technology (MIT) developed a technology that reduces hair frizz using a polyfluoroester, a molecule smaller than the traditional ones used for frizz control. Due to its chemical nature, the formulation adheres tightly to the hair, promoting long-lasting resistance to moisture. Due to its low surface energy, this technology repels most other materials like water and oils. As an additional benefit, the low refractive index of the coating produces a unique, long-lasting shine and pop in the colour of the hair ((MIT) 2008).

There have been also attempts to enhance the binding of the cosmetic agents to hair by using enzymatic treatments. Richardson (Richardson 1994) described the covalent attachment of cosmetic agents to hair using transglutaminases, promoting the cross-linking between the amine containing cosmetic agent to the glutamine residues in hair. Similarly, Green (Green 1999) described the use of the enzyme lysine oxidase to covalently attach cosmetic agents to hair.

1.4 Cosmetic Science

While cosmetics can be fun to use, the behind-the-scenes work that goes in creating them involves serious, highly advanced science that always respond the specific consumer's needs.

In Europe, the most recent definition for a cosmetic product was published in the Sixth Amendment (Directive 93/35/EEC) (EU 1993) of the European directive 76/768/EEC (1976):

A "cosmetic product" shall mean any substance or mixture intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition.

This definition gives an indication of the target site of the application of a cosmetic product and on its functions, and does not restrict their functions to cleansing and make-up products. For that reason cosmetics are becoming of more importance in daily life. They are used regularly by increasing numbers of people and very large quantities are consumed each year. With people becoming increasingly older and desiring to feel more fulfilled, more emphasis is now being placed on the antiaging and psychological effects of cosmetics when considering their usefulness (Mitsui 1998). For that reason, cosmetics play an important role in society, by contributing not only to beauty but also to health in the widest sense, with its psychological and social implications.

The consequence of this is that cosmetic and toiletries (C&T) market is continually growing, with several high growth opportunities in the areas of e.g. colour cosmetics and hair care products. Business Communications Company (BCC) research showed a dynamic growth tendency of C&T market ingredients during the last years, where hair care segment was the biggest individual sector in performance (Figure 1.14) (Rajaram 2009).

There are many companies and specialist consultancies developing ideas for innovative products. The essence of product innovation is to create or establish something new. Since this process necessarily involves risk, innovating companies require a strategy not of risk avoidance, but of early diagnosis and management (Carbonell-Foulquié et al. 2004).

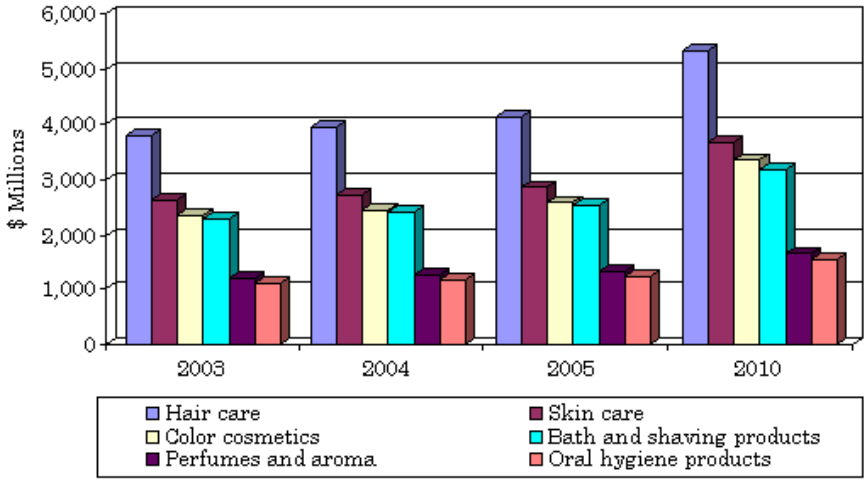


Figure 1.13 - Cosmetic and toiletries market performance by sector (Rajaram 2009)

Nevertheless, the creation of new cosmetics on C&T market has to pass through a strict regulatory product control. In recent decades, new regulatory requirements have arisen to protect our environment and reduce damage linked to human activities to a minimum. Hence, companies that place new cosmetic products on the market must also approach safety matters from another angle and evaluate the impact of these new products on the air, water, or soil, whether during manufacture, their usage or at the end of their shelf life (C. Hourseau et al. 2005).

Nevertheless, the consumer health protection aspect is the most important when a new product development is studied. Consequently, cosmetic product testing can be extremely challenging and complex (Romanowski 2001).

Taking into account the extent and frequency of human skin contact with cosmetics, their ingredients must be safe (Bednarz et al. 1997; Nohynek et al. 2004). The determination of risk assess is needed to ensure the safety of the ingredient in the context of the finished product.

1.4.1 Risk assessment and toxicology of hair-care products

Hair-care products, like all other cosmetics, must present the highest safety level both for the consumers who use them and for the professionals (hairdressers) who apply them. These products will be in contact with the scalp and skin and must not cause undesirable reactions locally. As skin maintains the body fluids and prevents the passage of foreign compounds into the circulation, it was considered as an impenetrable barrier for

topically applied chemicals. However, nearly all substances can penetrate the intact stratum corneum to some degree, and are then able to diffuse into the viable epidermis and the dermis, and finally to enter the blood or lymphatic vessels, i.e. to be absorbed. The degree of absorption will depend on the physicochemical properties of the substance and on the composition of the vehicle. Generally, the faster and greater penetrating compounds are the non-polar small molecules (Marty 2005).

For that reason, the hair care products must pass a highly restrictive regulation control, and it is of paramount importance that no side effects are observed. By contrast, pharmaceutical drugs for medical use are evaluated in consideration of a risk-benefit relationship and only used for short time periods to treat medical conditions. Their primary purpose is to cure illness, and they must be therapeutically effective and sometimes side effects of these drugs cannot be avoided (Mitsui 1998). Hair-care safety assessment is based on a virtual zero-risk situation, which rarely exists for any human activity or exposure to any natural or synthetic substance (Nohynek et al. 2004).

A large amount of legislation exists globally regarding the assessment of human safety hair-care products types, with the underlying principles of toxicological risk assessment prior to human exposure to chemicals. The evaluation of the potential risk to human health is performed for each product prior to marketing, taking into account the general toxicological profile of the ingredients present within the product, their chemical structure, the level of human exposure and the target group who will use the product (Bednarz et al. 1997). The use of hair products in general can induce four types of potential risk to health, called toxicological endpoints: skin irritation, eye irritation, allergy or contact hypersensitivity and systemic toxicity (C. Hourseau et al. 2005).

The risk of **skin irritation** results from direct contact between the product and the scalp; this reaction may occur as soon as the first contact is made, particularly if the user has an exceedingly sensitive scalp or a pre-existent dermatosis.

The risk of **eye irritation** is linked to the fact that many hair products must be rinsed off after application and can thereby come into contact with the eyes. Eye safety testing is therefore of particular importance.

The risk of **allergy** or contact hypersensitivity is the result of the recognition by the organism of a substance (allergen), to which the subject has become sensitised. Any further contact with this subject will trigger an inflammatory reaction on the site of application, capable of spreading to neighbouring skin areas. Contact hypersensitivity may also be pre-existent on application of the product, but equally it can be generated by repeated

applications of a product. Linked to the individual sensitivity of the user and only affecting a small proportion of them, the allergic reaction is harder to predict. The evaluation of this risk must take into account not only the specific data obtained on each of the ingredients in the formulation, but also the possible interactions between them in the finished product.

A risk of **systemic toxicity** results from penetration through the skin or mucosa, or inhalation of various constituents of the hair product. This type of risk is closely linked to the nature and toxicological profile of the ingredients on the one hand, and to their ability to cross the skin barrier in the course of application of the product on the other. Evaluation of this risk is based on toxicological data on the ingredients and on the level of exposure by the end user to these ingredients, taking into account their concentration in the finished product, the amount of the latter in contact with the scalp and the duration of contact.

The relationship between the prejudicial effects (toxicological endpoints) and the predicted exposure will determine the risk assessment for a consumer product. The process involves four distinct phases:

Exposure assessment: An assessment of the amount and frequency of human exposure to the chemical under normal and foreseeable misuse conditions.

Hazard identification: The intrinsic properties of the chemical under consideration, through the evaluation of the product potential to cause damage to human health.

Hazard characterization: The relationship between the toxic response and the levels of exposure to the chemical. In many cases, a No Observed Adverse Effect Level (NOAEL) is determined i.e., the highest tested dose of a chemical that does not cause toxicity.

Risk characterization: The assessment of the risk that the chemical in question in the proposed use scenario will have adverse effects on human health (Westmoreland et al. 2009; C. Hourseau et al. 2005).

Therefore, it is a basic requirement that new products are tested to assure they are safe under normal and reasonably foreseeable conditions of use.

1.4.2 *In vitro* toxicological tests

Within the European Union, the evaluation of potential risk to human health of new products or ingredients prior to human testing is performed *in vivo* on animals. However, ethical and financial objections to these methods have been raised in recent years, as well

as scientific drawbacks such as intra- and interlaboratory variability, good predictability only for strong irritants, and the fundamental difference between animal and human in morphological aspects of skin (Lee et al. 2000). As a consequence, recent changes in legislation (e.g. 7th amendment of the Cosmetics Directive and REACH) are driving researchers to develop and adopt non-animal alternative methods with which to assure human safety (Westmoreland et al. 2009).

Several *in vitro* alternatives to animal testing such as Draize rabbit skin tests (Draize et al. 1944), have recently been proposed to predict the *in vivo* skin irritancy potential of a variety of test agents (Osborne et al. 1994; Ponec 1992). These alternatives are based on recent advances in techniques for culture of human skin cells (epidermal keratinocytes or dermal fibroblasts). These tissue engineered 3D models of human skin are commercially available and consist of normal, human derived epidermal keratinocytes that have been cultured to form a multilayered, highly differentiated model of the human epidermis (Westmoreland et al. 2009). These models permit the production of a large number of cells, and large scale toxicity screening tests with many substances that can be applied in a broad concentration range, to predict dermal irritation (Ponec 1992).

Another approach to test the toxicity is to apply the chemicals in a monolayer of cells (epidermal keratinocytes or dermal fibroblasts) and cell viability is subsequently assessed using the MTT (a tetrazolium salt) assay (Mosmann 1983; Takeuchi et al. 1991) or Alamar Blue (AB) assay (Nakayama et al. 1997; Page et al. 1993; Slaughter et al. 1999). AB and MTT are thus indicators of cytotoxicity of various chemicals in cultured cells. Chemically-induced decreases in AB fluorescence and MTT absorbance are usually interpreted as indicative of mitochondrial damage and therefore an indicative of cytotoxicity.

The assessment of oxidative stress induction in cells exposed to the new products is also an accepted tool to measure its cytotoxicity. Endogenously produced reactive oxygen species (ROS) are essential to life, being involved in several biological functions (Suzuki et al. 1997; Babior 2000). However, when overproduced (e.g. due to exogenous stimulation), or when the levels of antioxidants become severely depleted, these reactive species become highly harmful, causing oxidative stress through the oxidation of biomolecules, leading to cellular damage that may become irreversible and cause cell death (Beckman et al. 1998; Vendemiale et al. 1999). It is therefore important to properly assay the propensity of new product to induce the overproduction of ROS in order to have a guideline for their proper use. A cell-based assay using 2',7'-dichlorodihydrofluorescin-

diacetate (DCFH-DA) as a substrate is a useful technique to quantitatively measure intracellular oxidant production (Girard-Lalancette et al. 2009; Gomes et al. 2005).

During the risk assessment process of new hair care products it is necessary and of major importance to pay attention to its genotoxic potential. The genetic toxicity tests are thought to be not only predictive for potential carcinogenic, but also mutagenic hazards, and are principally regarded as short-term screening studies for the prediction of the carcinogenic potential of substances. The strategies to evaluate the genotoxic hazard of a substance have been proposed by several authors and organisations, and are based on a combination of *in vitro* and *in vivo* tests (Ashby et al. 1996; Nohynek et al. 2004). Like this, the single cell gel electrophoresis (comet assay) is a sensitive, quick and simple method for evaluating DNA damage in single cells, and commonly used nowadays in genotoxic studies. It was first described by Singh and co-workers in 1988 and has since then gained in popularity as a standard technique for evaluation of DNA damage/repair, biomonitoring and genotoxicity testing. Damage to DNA have strong cellular implications and is involved in the stop of cell cycle, in the induction of cell death and/or in the increase of DNA mutation rate and carcinogenesis (Collins et al. 2008).

The *in vitro* tests carried out in this work are the example of a broad range of tests that can be performed to determine the safety of new hair care products. Even thought *in vitro* tests predicts with high accuracy the toxicity of a product, the complete insight in the product toxicity is only possible after *in vivo* testing.

Chapter 2

Protein Disulphide Isomerase-assisted functionalization of keratin-based matrices

2 Protein Disulphide Isomerase-assisted functionalization of keratin based matrices*

2.1 Abstract

In living systems, protein disulphide isomerase (PDI), regulates the formation of new disulphide bonds in proteins (oxidase activity) and catalyzes the rearrangement of non-native disulphide bonds (isomerase activity), leading proteins towards their native configuration. In this study, a fluorescent (5(6)-TAMRA)-labelled keratin peptide (KP) was incorporated into hair by using PDI. Similarly, PDI promoted the grafting of a cysteine-functionalized dye onto wool, as suggested matrix-assisted laser desorption and ionization time-of-flight results (MALDI-TOF). These reactions are thought to involve oxidation of disulphide bonds between cysteine-containing (CC) compounds and wool or hair cysteine residues, catalyzed by the oxidized PDI active site. On the other hand, PDI was demonstrated to enhance the migration of a disulphide bond-functionalized dye within the keratin matrix and trigger the release of RNase A from wool fibres' surface. These observations may indicate that an isomerisation reaction occurred, catalyzed by the reduced PDI active site, to achieve the thiol-disulphide exchange, i.e., the rearrangement of disulphide bonds between these cysteine containing compounds and keratin fibres. The present communication aims to highlight promising biotechnological applications of PDI, derived from its almost unique properties within the isomerase family.

***This chapter is based on the following publication:**

Margarida M Fernandes, Andreia C Gomes, Andreia Vasconcelos A, Florentina-Daniela Munteanu, Tzanko Tzanov, Maria S T Gonçalves, Nicole End, Kai-Uwe Schoening, Georg M Guebitz and Artur Cavaco-Paulo, *Protein Disulphide Isomerase-assisted functionalization of keratin-based matrices*, Applied Microbiology and Biotechnology, 2011, available online, 10.1007/s00253-011-3194-6.

2.2 Introduction

In vivo, correct protein folding is catalyzed by the enzyme protein disulphide isomerase (PDI). This multifunctional eukaryotic oxidoreductase is structurally characterized by the presence of functional thioredoxin-like (Trx) domains which are responsible for its catalytic properties (Appenzeller-Herzog et al. 2008; Wilkinson et al. 2004). PDI was first known as the catalyst of native disulphide bond formation from cysteine residues in the refolding of Ribonuclease A (RNase A) (Goldberger et al. 1963). Since then, several studies emphasized PDI as catalyst for reduction, oxidation and isomerisation in a wide range of cysteine-containing (CC) substrates (Kersteen et al. 2005; Walker et al. 1996; Xiao et al. 2005), where the type of reaction catalyzed is determined by the redox state of the PDI active site (Lyles et al. 1991). Thus, apart from its natural function of protein refolding, PDI could potentially catalyze the functionalization of cysteine-rich protein fibres using various functional cysteine-containing molecules.

Keratin is the basic building block of the complex morphological structure of human and animal hair fibres and constitutes an example of such cysteine-rich protein fibres. Their cysteine content varies from 7 % to 20 % of total amino acid residues, creating a stable disulphide bond (cystine) network (Wilson et al. 1927). Such high content of cystine suggests that keratinous fibres should be susceptible to modification with enzymes that are active towards disulphide bonds, and more specifically with PDI.

Several patents report on modification of keratin fibres using PDI for applications such as the restoration of original properties of harshly treated wool textiles (King et al. 1989), or for styling and colouring of human hair under mild conditions (Huang et al. 2008; Richardson et al. 1996; Green et al. 2001). However, the ability of PDI to catalyze incorporation of functional molecules onto keratinous substrates has not been reported so far.

Many current cosmetic hair treatments are based on the disulphide bond modulation, which alter many hair properties resulting on damage to the hair fibre (Schueller et al. 1998; Wolfram 2003; Gray 2001; Bolduc et al. 2001; Dawber 1996). The reversible and relatively stable nature of the disulphide bonds in keratin fibres (Block et al. 1939) makes them a good target for functionalization, as an alternative to chemical agents commonly used in cosmetics, or to take advantage of the strong linkage that can be created between hair and a cysteine-containing functional molecule.

The so mentioned high content of cysteine on keratinous fibres explains the stability of the fibre macrostructure, imparting those fibres' good mechanical, thermal, and chemical properties (Wilson et al. 1927; Wysocki et al. 1954; Block et al. 1939). For this reason, keratin fibres are continuously being reported as novel biocompatible materials in the area of tissue engineering. Keratins can be isolated from human or animal hair by controlling the conditions which lead to disulphide bonds scission and reformation, and further fabricated into various designs, such as films, microcapsules and sponges (Vasconcelos et al. 2008; Katoh et al. 2004; Sierpinski et al. 2008; Tachibana et al. 2005; Morgan et al. 2007; Tanabe et al. 2004). Therefore, the keratins' disulphide bonds are also thought to be ideal targets for the development of enzymatic systems for drug delivery. In fact, the versatility of disulphide bonds has been proposed as a general strategy for designing drug delivery systems (Saito et al. 2003). However, an approach based on PDI as an external switch-on agent for drug release has not been reported so far.

This study aims to broaden the application spectra of disulphide-rich keratinous fibres using enzymatic approaches. Three different PDI-assisted controlled reactions are described:

a) PDI-assisted incorporation of different CC-peptide and CC-dye into hair and wool substrates, respectively, in order to create new strong disulphide bonds between functionalized dyes and keratin fibres.

b) PDI-assisted promotion of the migration of a CC-dye, previously attached to keratin, along hair fibres, to avoid repetitive hair dyeing procedures.

b) PDI-induced controlled release of a reduced form of Ribonuclease A (RNase A) previously attached on wool matrices, as a new approach for drug release methodology.

2.3 Materials and methods

2.3.1 Materials

2.3.1.1 Enzymes

Stock solutions of PDI from bovine liver (Sigma) and Ribonuclease A from bovine pancreas (Sigma) were prepared in phosphate buffer pH 7.5 in the concentrations of 0.05 g/L and 1.5 g/L, respectively. The alkaline protease Esperase (E.C.3.4.21.62), a subtilisin with a broad specificity, was supplied by Novozyme and prepared in Tris-HCl buffer pH 8.5.

2.3.1.2 Keratins

Natural European human hair samples were provided by International Hair Importers & Products Inc. (New York) and prior to peptide treatment, two different pre treatments were performed. In one pre-treatment, the hair tresses were previously subjected to a washing procedure with a commercial shampoo. In the other pre-treatment, the hair was treated with ethanol (1:20 w/v) for 2 h to remove the external lipids followed by a reducing treatment in the presence of 0.01 M dithiothreitol (DTT) in Tris-HCl buffer 0.1 M pH 7.5, in a bath ratio of 1:100 for 1h. The reduction treatment was made to set the thiol groups available in hair for further PDI application. Woven 100 % merino wool fabrics were provided by Albano Antunes Morgado Lda (Portugal). The wool fabrics were washed with 1 g.L-1 non-ionic surfactant Lutensol AT-25 (BASF, Germany) at 0.1 M Na_2CO_3 / $NaHCO_3$ buffer pH 9.0, 40°C for 30 min in a Rota-wash laboratory machine (MKII Series 7227, Shirley Developments Ltd) at liquor to material ratio 20:1, and subsequently in distilled water and air-dried, prior to experiments.

2.3.1.3 Dyes

The cysteine-containing dyeing agents were provided by Ciba Specialty Chemicals (Switzerland). Figure 2.1 shows the structure of the dyes. Dye A contains one cysteine residue in reduced form, which amino group was used to attach the chromophoric group. Dye B contains two cysteine moieties oxidized to form disulphide bridge and the

chromophoric groups attached through carboxylic group. The stock solution of each dye (1.25 mM) was prepared in 0.1 M phosphate buffer pH 7.5.

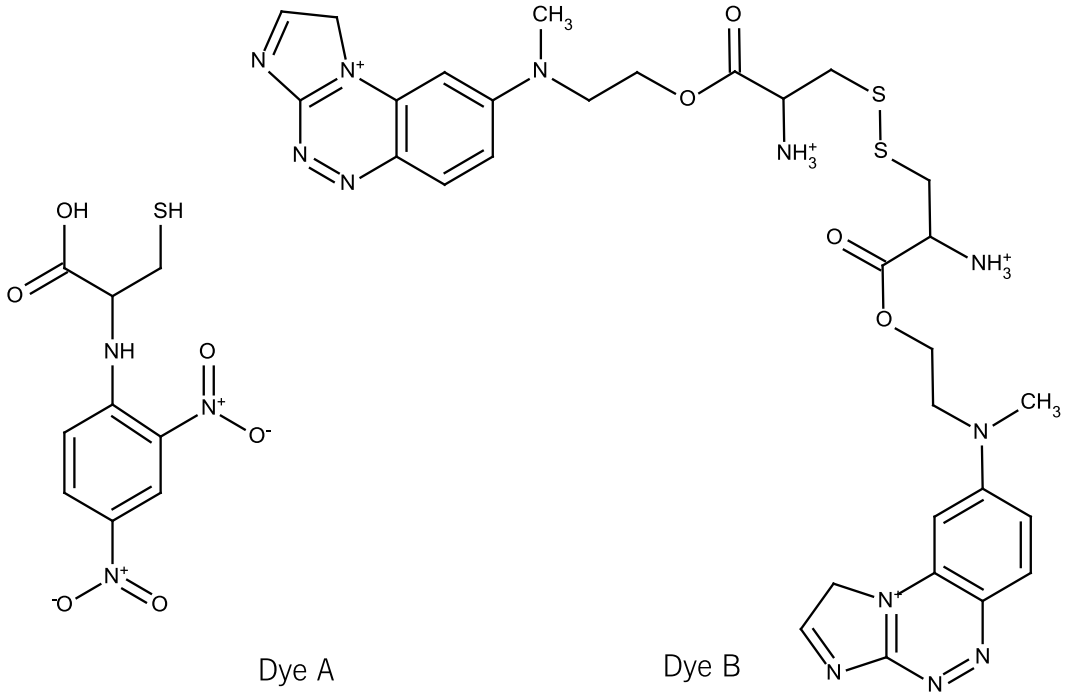


Figure 2.1 - Structure of the cysteine-containing dyes.

2.3.1.4 Peptide

A keratin peptide (KP) was specially designed for this work and thus synthesized by JPT Peptide Technologies GmbH (Germany). The peptide was developed based on the amino-acid sequence of cuticular keratin type II from human hair (Naeem et al. 2006) and possess a sequence with thirteen (13) amino-acids comprising two (2) cysteines residues (TAMRA- X_3**C**X_5**C**X_3-$CONH_2$) with a molecular weight of 1600 g/mol. The peptide was covalently linked by its N-terminal to a fluorescent dye 5(6)-carboxytetramethyl-rhodamine, succinimidyl ester (5(6)-TAMRA), λex=544 nm and λem=572 nm, to facilitate analysis of peptide penetration into keratin substrates. Stock solutions of 1 mg/mL were prepared in 0.1 M Tris-HCl buffer, pH 7.5.

2.3.2 Methods

2.3.2.1 PDI-assisted incorporation of cysteine-containing peptide on hair

2.3.2.1.1 Hair treatment with the peptide

Hair samples (0.1 g), both reduced (RH) and washed hair (WH), were treated with 3 mL 0.1M tris-HCl buffer pH 7.5 containing 200 µL of PDI stock solution and 600 µL of peptide stock solution (final concentration of 0.2 mg/mL), at 37 ºC in a bath with orbital agitation, 100 rpm for 1 h. After treatment, hair samples were washed with commercial shampoo and under running water. As control the hair was treated in the same way, omitting the peptide in the solution.

2.3.2.1.2 Fluorescence microscopy on hair cross-sections

The influence of PDI on peptide application was visualized by fluorescence microscopy on hair transversal cuts. The treated hair fibres were embedded into an epoxy resin and transversal cuts with 15 µm were prepared using a microtome (Microtome Leitz). Three cross sections per sample were made and analyzed on a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings using 40 × objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software. The fluorescence images were taken at same excitation conditions: the same filter was used for all the images collected as well as the brightness, time of exposure, and gain. The most representative images of different hair cross sections of the same hair sample were chosen.

2.3.2.1.3 Colour variation

The colour variation was measured directly from the hair-peptide solution before and after the treatment, at 555 nm. Since the colour of the solution comes from the peptide, from the differences in absorbance, the quantity of peptide that penetrates/attach into the hair was determined and expressed as colour variation. The % of colour variation was calculated based on the following Equation:

$$\text{Colour Variation} \quad (\%) = \frac{\text{Abs}_{\text{initial}} - \text{Abs}_{\text{final}}}{\text{Abs}_{\text{initial}}}$$

2.3.2.1.4 Zeta potential

The zeta potential values of the peptide solutions were measured by photon-correlation spectroscopy (PCS) using a Malvern zetasizer NS (Malvern Instruments) at 25.0 ± 0.1 ºC. The instrument was routinely calibrated with a ± 66 mV latex standard.

2.3.2.2 PDI-assisted incorporation of a cysteine-containing dye on wool

2.3.2.2.1 Wool coloration experiments

Wool samples (0.25 g each) were dyed at 20 °C in Ahiba Spectradye dyeing apparatus (Datacolor Int.) with bath containing 10 mL 0.1M tris-HCl pH7.5 buffer, 300 μL of the dye A stock solution (Dye A, Figure 2.1) and 20 μL of the PDI stock solution. After dyeing, all samples were washed extensively to remove the non-covalently bound dye using Lutensol AT-25 in a Rota-Wash machine, bath ratio 100:1, at 50°C, for 45 min according to BS 1006: 1990 CO2 (Liakopoulou-Kyriakides et al. 1998) The washing was repeated until complete removal of the unfixed dye.

2.3.2.2.2 Hydrolysis of wool samples

Hydrolysis of the wool samples (dyed and un-dyed) was performed after PDI-assisted dyeing prior to MALDI-TOF analysis. The treatment was carried out in a thermostated laboratory shaker at 100 rpm in liquor ratio 100:1 in the case of pre-treatment and 150:1 in the case of hydrolysis after dyeing. 10 mg of sample were treated with 7 U/mL Esperase (native form). The treatment was performed in 0.1 M Tris-HCl buffer, pH 8.5, at 37 °C for 24 h.

2.3.2.2.3 MALDI-TOF Mass Spectroscopy

Digested dyed wool samples were analyzed by MALDI TOF (matrix-assisted laser desorption and ionization time-of-flight) mass spectrometer (Thermo BioAnalysis MALDI TOF DYNAMO system) equipped with a pulsed nitrogen laser (332 nm, 2 ns pulse width) and Dynamic Extraction (DE, a method for optimizing the extraction and acceleration of ions from the sample) in negative ion reflector mode, using 2,5-dihydrobenzoic acid (DHB) as matrix solution. A volume of 1.5 μl of sample was mixed with 5 μl of cyano-4-hydroxycinnamic acid (HCCA) diluted (1:1) with 50:50 acetonitrile/nanopure water. Then, 1.5 μl of the resulting mixture was spotted on the stainless steel MALDI target and dried.

2.3.2.3 PDI-assisted migration of the cysteine-containing dye on hair

Hair samples (150 mg; UNA-Europ. Natural hair, colour white bleached from Fishbach&Miller) were dyed with or without PDI in a dye bath composed of 10 mL of 0.1 M Tris-HCl pH 7.5 buffer, 300 µL of the dye B stock solution (Dye B, Figure 2.1) and 20 µL of the PDI stock solution. The hair samples were only partially dyed to be possible to observe the colour migration on the un-dyed parts. The dyeing experiments were carried out in the Ahiba Spectradye dyeing apparatus (Datacolor Int.) at 20°C, pH 8.0, for 50 min. After dyeing, all hair samples were washed extensively to remove the unfixed dye in a Rota-Wash machine (MKII Series 7227, Shirley Developments Limited) using commercial shampoo at 50 °C during 45 min according to BS 1006: 1990 CO2 (Liakopoulou-Kyriakides et al. 1998) immediately after the dyeing procedure. The hair samples were dried at room temperature.

For the migration tests, the dyed hair was treated with a PDI formulation (0.05 g/L), containing a redox buffer GSH/GSSG (1 mM GSH, 0.2 mM GSSG, ΔE=-260mV), to set the redox state of the PDI active site to the reduced form, in an Ahiba Spectradye dyeing apparatus at 20ºC, pH 8.0, for 50 min.

2.3.2.4 PDI-assisted release of a cysteine-containing protein from wool

2.3.2.4.1 Incorporation of RNase A on wool

In order to incorporate RNase A into a keratin matrix, both the protein and the wool samples were reduced prior to the oxidation attachment. The wool samples were reduced in 0.1 M Tris-HCl buffer pH 7.5, in the presence of 50 mM dithiothreitol (DTT) and 3 M guanidine hydrochloride (GndHCl) (wool to liquid ratio 1:20), at 37 ºC for 2h. After reduction, the wool fabrics were extensively washed with tap water and 0.1 M citric acid/$Na_2HPO_4.2H_2O$ buffer, pH 4.0 to prevent its re-oxidation by air oxygen. The reduction of RNase A was performed according to Lyles (Lyles et al. 1991) in the presence of DTT and GndHCl. The oxidation (attachment) of the reduced RNase A (1.50 mg/mL) into the reduced wool (50 mg) was assessed in the presence of an oxidation buffer (2 mM GSH, 10 mM GSSG, ΔE=-228 mV) in 0.1 M tris-HCl buffer pH 7.5. The reduced wool was incubated with 50 µM reduced RNase A solution overnight at 25 ºC in a shaker bath at 90 rpm, in presence of redox buffer in order to promote its oxidation. The samples were then immersed in a washing buffer (0.1 M Tris-HCl buffer, pH 7.5) until no more protein

was released (measured by Lowry method). For control, samples prepared by the same procedure using phosphate buffer (pH 7.5) were used.

2.3.2.4.2 Fourier Transform-Infrared Spectroscopy (FTIR)

In order to evaluate the differences at the surface of wool treated fibres, measurements were performed in a Perkin Infrared Spectrophotometer using an Attenuated Total Reflectance (ATR) accessory. Before collecting the samples spectra, background scanning was performed. At least 16 scans were obtained to achieve an adequate signal to noise ratio. The spectral data was taken in the region of 375-4000 cm^{-1} with a resolution of 8 cm^{-1} at room temperature.

2.3.2.4.3 PDI induced release of RNase A from wool

The release of RNase A from wool surface was enzymatically catalyzed by using PDI, based on its ability to catalyze the isomerisation of disulphide bonds on misfolded proteins. The PDI was incubated for 25 min in GSH/GSSG buffer (1 mM GSH, 0.2 mM GSSG, ΔE=-260 mV) to set the redox state of the PDI active site (reduced form) to be able to isomerise the disulphide bonds between wool fabric and RNase A. The wool fabric with incorporated RNase A was immersed in 0.22 µM PDI in GSH/GSSG buffer for 1 h, in a shaker bath at room temperature. The biological activity of RNase A and protein release was assessed at different time points. For control, the RNase treated wool was incubated in the same buffer omitting PDI.

2.3.2.4.4 RNase A activity assay

The RNase A biological activity was assayed based on the PDI oxidative folding of RNase A (Lyles et al. 1991). RNase A activity was determined spectrophotometrically at 25 ºC using cytidine 2':3'-cyclic monophosphate (cCMP) as a substrate (Spencer et al. 2004). Assay mixtures were composed of 0.1 M Tris-HCl buffer, pH 7.5, 4.5 mM cCMP and 250 µL of released RNase A from sample solution. The reaction was followed at 296 nm in a quartz cuvette of 0.1 cm path length.

The slope, i.e. linear increase in absorbancy, was determined and activity was calculated using Beer Law, where ε is the substrate molar extinction coefficient (190 M^{-1} cm^{-1}) according to the following equation:

$$\text{Activity/L} = \text{Slope} \times V_{total} \times \frac{1}{\varepsilon} \times \frac{1}{V_{sample}} \times 10^3$$

2.3.2.4.5 Protein determination

The total protein concentration was determined by a modification of the micro Lowry method (Lowry et al. 1951) using bovine serum albumin as standard and using Sigma test kit nº P 5656, measured at 750 nm.

2.3.2.4.6 FITC linkage to RNase A

To enable to study the RNase A incorporation onto wool fabrics and the release in the presence of PDI, fluorescein 5(6)-isothiocyanate (FITC) was linked to the protein. A volume of 2 mL of 2 mg/mL solution of RNase A protein in a sodium carbonate buffer, pH 9.0 was incubated with 100 µL of a 1 g/L FITC solution at RT for 2 h. Unbound FITC was separated from the conjugate by passing the mixture through a desalting column PD10. The ratio of fluorescence to protein (Molar F/P= 0.79) was calculated in order to determine labelling efficiency. The estimated value was within the ideal limits (0.3 - 1.0) indicating efficient labelling.

2.3.2.4.7 Fluorescence microscopy on wool cross-sections

Two different wool samples were analyzed by fluorescence microscopy: wool fabric incorporated with FITC-RNase A and wool fabric incorporated with FITC-RNase A followed by the treatment with PDI in the refolding buffer. Therefore, several fibres of each sample were embedded into the epoxy resin and transversal cuts of the fibres with 15 µm were prepared using a microtome (Microtome Leitz). The fluorescence microscopy images were taken using the same conditions of the method for the analysis of hair fibre cross-sections, above mentioned. The most representative images among all were chosen.

2.3.3 Results and discussion

A novel strategy for functionalization of keratinous fibres based on the ability of PDI to catalyze formation and scission of disulphide bonds between substrates and functional molecules has been developed in this study. Figure 2.2 illustrates the mechanistic background of PDI reactions exploited for keratin functionalization. The figure illustrates two possible reactions based on the state of PDI active site. When the active site is in its oxidized form, PDI catalyses formation of disulphide bonds and cysteine-containing compounds (CCCs) are therefore incorporated on to keratins. The

release or migration of CCCs (disulphide scission), however, demands the PDI active site in its reduced form.

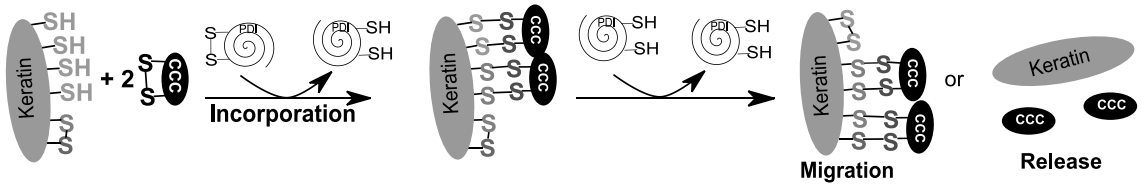


Figure 2.2 - Schematic illustration of the PDI assisted reactions of CCCs on keratins.

The peptide and dyes used in this study were engineered as PDI substrates, i.e. cysteine residues were introduced. Two dyes with general formulas R-SH and R-S-S-R' (Figure 2.1) containing cysteine and cystine, respectively, and the peptide (KP) comprising two cysteine residues, linked to a fluorescent dye, were synthesized.

2.3.3.1 PDI-assisted incorporation of cysteine-containing peptide on hair

A keratin intermediate filaments (KIF) protein was chosen for the KP development. The amino acid sequence of the keratin type II cuticular Hb5, a protein that in humans is encoded by the KRT85 gene (Rogers et al. 1997; Koehn et al. 2010), was chosen[1] and a fragment of 13 amino acids comprising two cysteines separated by 5 different amino acids was then selected and chemically synthesized.

In order to predict the affinity of a peptide towards the hair surface, two important properties should be highlighted: the peptide size (molecular weight) and the peptide surface charge characterized (isoelectric point - pI). The smaller is the peptide, the higher the expected penetration on the scaled structure of hair. Small peptides such as KP (Mw~1600 g/mol) are expected to penetrate deep into the scaled structure of the fibre. However, the KP uptake was not as high as expected (Figure 2.3). This low affinity can be explained based on peptide isoelectric point (pI) which was found to be 5.2[2]. Thus, KP acquires negative charge at pH higher than its pI, as proved by photon-correlation spectroscopy (PCS) (-14.06 ± 2.33 mV), proving the tendency of peptide to be anionic. Such negative charges decrease the specificity towards negatively charged membranes, such as those found in hair. Human hair has an isoelectric point near 3.7 (Wilkerson 1935; Regismond et al. 1999) which explains the negative surface charges under most pH

[1] Gene information available on Pubmed at www.ncbi.nlm.nih.gov/gene/53622

[2] Calculated with "Peptide Property Calculater" available at www.innovagen.se

conditions. These electrostatic interactions are believed to play a determining role in the adsorption mechanism, which might explain the low affinity of the KP towards hair, despite its size.

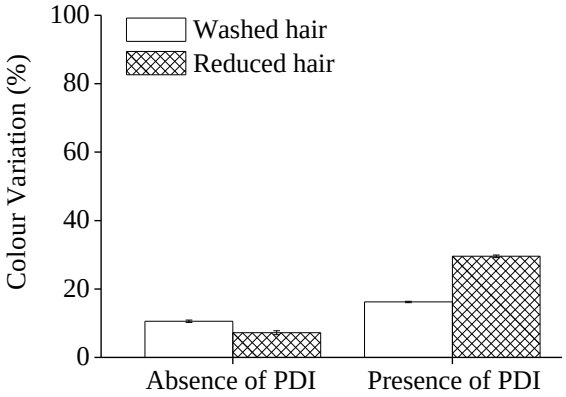


Figure 2.3 - Keratin peptide (KP) uptake on both reduced and washed hair in the absence and presence of PDI, determined by colour variation method.

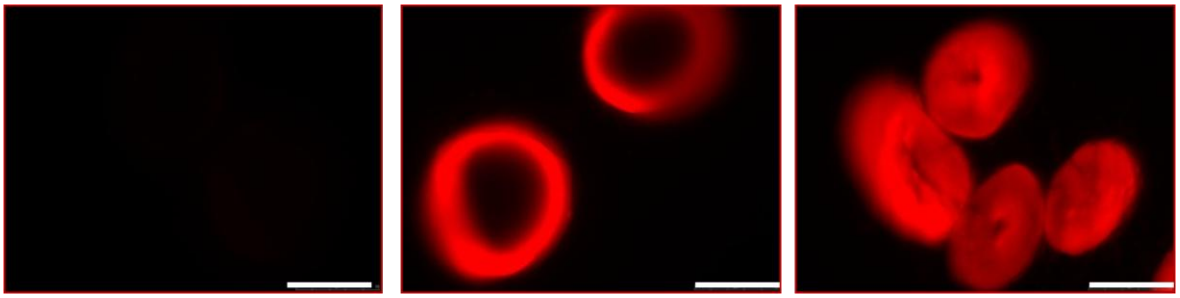
Colour variation is proportional to peptide uptake by hair. Thus, it could be observed that KP adsorption into hair fibres was favoured by the presence of PDI (Figure 2.3). This increase was more pronounced with previously reduced hair, where KP uptake was 30 % while in washed hair an uptake of 16 % was observed. The reduction treatment results in higher quantity of free thiol groups in hair and further application of PDI increased the affinity of peptide towards hair. The absence of PDI, on the other hand, resulted in poor peptide adsorption.

PDI seems to promote the covalent attachment of a cysteine-containing peptide on to reduced and washed hair. The reducing treatment, however, promoted higher yield of KP attachment probably due to the higher number of thiol groups created.

Fluorescence microscopy on hair cross sections was performed after the washing procedures in order to visualize the presence of the peptide and the effect of PDI on its penetration. The peptide is linked to a fluorescent dye (5(6)-TAMRA), which makes possible its visualization by fluorescence microscopy. The hair without peptide treatment is depicted in Figure 2.4 A. It can be seen that the hair without the peptide treatment, does not possess auto fluorescence. Comparing all the hair cross section images subjected to the same excitation conditions of brightness, time of exposure and gain, it can be seen that PDI treatment seems to favour the penetration of KP inside the fibres' cortex (Figure 2.4 C)

while the hair samples treated in the same conditions but without PDI show the presence of peptide at the cuticle (Figure 2.4 B). Spectrophotometric measurements are in agreement with these findings, since KP uptake is more efficient in the presence of PDI (~16 % for washed hair and 30% for reduced hair) than without PDI (~10 % for washed hair and 8% for reduced hair) (Figure 2.3).

These results suggest that PDI might promote the creation of new disulphide bonds between the used cysteine-containing peptide and hair. Furthermore, as KP is based on the amino acid sequence of human hair, it is thought that PDI, acting as chaperone and folding enzyme (Wang et al. 1998), may contribute to a conformational change of KP towards its minimum free energy and thus facilitating penetration inside the fibre's cortex.



A **B** **C**

Figure 2.4 - Fluorescence images of cross sections of reduced human hair fibres; (A) control (reduced hair fibres); (B) reduced hair fibres treated with KP and (C) reduced hair fibres treated with KP in the presence of PDI. Bar represent 50 μm.

Apart from being a suitable substrate for PDI because of its two cysteine residues, KP is thought to have affinity towards hair's surface. This low molecular weight peptide is a fragment of a protein encoded by the KRT85 gene, which is a keratin type II cuticular protein (Rogers et al. 1997; Koehn et al. 2010) and may be able to restore damaged cuticle parts. Also, as a type II alpha-keratin, it might possess resilient yet pliable characteristics (Coulombe et al. 2004) that could be translated to the peptide and, therefore to the hair fibre.

The attachment of a CCC onto hair in presence of PDI as presented, may constitute an alternative, milder method for hair dyeing that promotes a long lasting colouring of hair while it may simultaneously increase the fibre robustness (Cavaco-Paulo et al. 2007). Up to now, most of the many innovative treatments available are very aggressive to the hair fibre (Zviak et al. 2005a; Bhushan 2008). Therefore, a peptide that is able to form

disulphide bonds with hair and penetrate into its cortex creates new perspectives for cosmetic industry.

2.3.3.2 PDI-assisted incorporation of cysteine-containing dye on wool

Using the same approach as for studying the incorporation of KP into hair, the PDI-assisted incorporation of the cysteine-containing dye on wool was also investigated. In this case, MALDI-TOF analysis was performed after a protease digestion of wool, to evaluate the presence of disulphide bonds.

The quantification of disulphide bonds is important for gaining a comprehensive understanding of the chemical structure of a protein (Gorman et al. 1997), which in this case could be used to predict dye-fibre interactions. To confirm CCC bonding to keratinous fibres (dye-fibre interactions), a protease (subtilisin A) digestion of wool dyed in the presence and absence of PDI was performed. The hydrolysate obtained from the enzymatic digestion were then analysed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. The enzyme used in this work, subtilisin A, is a non-specific protease that preferably attacks neutral and acidic amino acids. Hence, it is expected that the enzyme will promote the rupture of most of the peptide bonds on dyed wool.

Two major fragments, with peaks around m/z 254 and m/z 397 were detected in the hydrolysate of wool dyed in absence of PDI. The peak at m/z 254 is thought to be assigned to dye A, because it fits with the corresponding theoretical molecular weight (Mw=254.166 g/mol), after losing a SH group. This adduct can be a result of the complete enzymatic hydrolysis of the peptide-like bonds between dye and wool. The other peak was attributed to the fragment composed by the dye A (that loses a NO_2 group) linked to arginine residue from wool (Table 2.1). Dye A (Figure 2.1) possess a reactive carboxyl group (-COOH) that, during the dyeing process without PDI, may react with the available amino moiety from basic amino acids such as histidine, arginin and lysine in wool, creating a new peptide-like bond. The peak at m/z 397 that was attributed to a fragment consisting of the dye attached to an arginine residue is in good agreement with this statement.

When the dyeing of wool is made in the presence of PDI, the thiol group from the dye A structure is thought to be more reactive than the carboxyl group and may react with cysteine moiety in wool, favouring the creation of disulphide bonds. In the spectra of the hydrolysate obtained after enzymatic digestion of the PDI-assisted dyed wool the single

peak correspondent to the dye disappeared and higher molecular weight peaks appeared around m/z 450 and m/z 464, suggesting the occurrence of these disulphide bonding. Disulphide bonds originated from PDI-catalyzed reactions between dye and wool are strong and stable linkages (Gilbert et al. 1995) that are not subjected to protease hydrolysis. Therefore, it may be deduced that the peaks represent fragments of the dye linked by disulphide bonds to cysteine that, in its turn, is linked to another amino acid residue, giving rise to higher molecular weight adducts. The first peak (at around m/z 450) was thought to be due to the dye A (that loses carboxyl group) bounded to a cysteine-serine fragment digested from wool, while the second peak (m/z 464) might correspond to the dye A (that also loses carboxyl group) bounded to a cysteine-threonine fragment from wool (Table 2.1).

Herein, MALDI-TOF study of the wool samples after incorporation of the dye in presence and absence of PDI suggested that disulphide bonds could be involved in the dye's attachment to the keratin substrate. It should be noted that the sequence of the possible assignments shown in Table 2.1 results from the study of the most probable combination of amino acids with the dye A, based on its molecular structure and molecular weight.

Central to the explanation of the phenomena presented here for the incorporation of both peptide and dye on keratin fibres is the oxidized form of PDI active site, which promotes incorporation of CCCs into keratin substrates (Figure 2.2). PDI-catalyzed disulphide formation occurs when oxidizing equivalents are transferred from the disulphide in the PDI active site (oxidized state) to the substrate (Ferrari et al. 1999), as depicted in Figure 2.2. Thus, in appropriate conditions, when the PDI active site is in oxidized form (as used in the study) it is possible to link CCCs onto keratinous fibres.

Table 2.1 - List of measured and theoretical (calculated) masses and corresponding structures of adduct obtained by MALDI-TOF of wool dyed with dye A, in the presence and absence of PDI, after hydrolysis by protease

	Molar mass (g/mol)		**Possible corresponding structure**
	Observed	**Theoretical**	
Absence of PDI	254. 38	254.17	Dye A(- SH)
	396.88	397.41	Dye A(- NO_2) + Arg
Presence of PDI	450.26	449.44	Dye A(- CO_2H) + Cys + Ser (- H_2O - H_2)
	464.20	463.47	Dye A (- CO_2H) + Cys + Thr (- H_2O - H_2)

2.3.3.3 PDI-assisted migration of a cysteine-containing dye on hair

As an attempt to reduce hair dyeing intervals, the potential of PDI to enhance migration of a cysteine-containing dye (dye B, Figure 2.1) was investigated. Consequently, hair samples were only partially dyed so that the central part of the hair remained un-dyed. The un-dyed width was 2.5 cm. The hair was washed, dried and then PDI in the redox buffer was applied.

A tendency of dye migration towards the middle (un-dyed) part of the hair was observed (Figure 2.5). The treatment with PDI was able to reduce the extent of the un-dyed area from 2.5 to 2.0 cm in length.

These results indicate the potential of the PDI to promote the migration of the attached CCCs over keratinous surfaces. A possible isomerisation mechanism for migration, both scission and reformation of the disulphide bonds previously created between the hair and the dye, is suggested.

In theory, the PDI isomerisation phenomenon requires one reactive thiol in the active site of PDI, thus in its reduced form (Hawkins et al. 1991a). To transform the PDI active site on the reduced form the application of redox environment with a potential of ΔE= -260 mV by the presence of equilibrium concentrations of [GSH] = 1 mM and [GSSG] = 0.2 mM, is necessary. These conditions have been reported as optimal for promoting the renaturation of RNase A by an isomerisation mechanism (Lyles et al. 1991). Nucleophilic attack of the PDI's thiol group (reduced form) on a previously formed disulphide bond between CCC and keratin is thought to result in the formation of a mixed disulphide intermediate between enzyme and substrate (keratin). If keratin has another available cysteine, that can displace PDI, an isomerisation will occur through intramolecular rearrangement (Kersteen et al. 2005) and the CCC, previously released from keratin because of the PDI modulation of the disulphide bonds, will reattach to another available cysteine in keratin. The colour migration can thus be explained as a result of the displacement of CC-dye from one disulphide bond to another allowing its interaction along the keratin substrate (Figure 2.2).

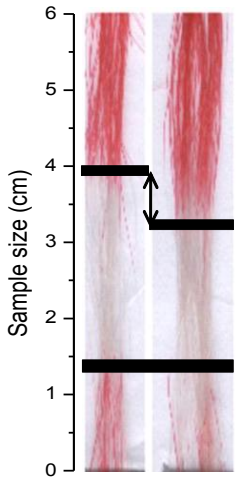


Figure 2.5 - PDI assisted migration of the dye B on hair

2.3.3.4 PDI-assisted release of a cysteine-containing protein from wool

The isomerisation mechanism that PDI catalyses is also thought to be involved in the release mechanism of a protein, namely RNase A previously attached to the wool surface. In this step, PDI was applied to manipulate the scission of the disulphide bonds created between the cysteine residues of a keratin matrix and those of a CCC, acting therefore as a switch-on agent for protein release.

Besides RNase A contains four disulphide bonds in its native state, it is one of the classic model systems used in protein science for folding studies (Chang 1999; Juminaga et al. 1998). Before the application of PDI, RNase A was attached to the wool surface via disulphide bonds. The results suggest that in an oxidizing environment, by the application of a glutathione-containing buffer (equilibrium mixture of L-glutathione reduced (GSH) and L-glutathione oxidized (GSSG)), concomitant oxidation of cysteines from reduced RNase A and wool takes place. Thus, consequent formation of new disulphide bonds between the fibre and the RNase A may occur, resulting in covalent protein fixation. A simplified cysteine oxidation in GSSG is shown in the following reaction:

2 Cysteine (-SH) + GSSG → 2GSH + Cystine (S-S)

The redox potential created ([GSH]= 2mM ; [GSSG]= 10mM) was ΔE= -228 mV, is higher than the redox potential of cystine/cysteine (E^o= -340 mV) (Åslund et al. 1997), and allows the formation of disulphide bonds between wool and RNase A. Measurements of the remaining protein content in the solution after the treatment and washing procedures revealed up to 90 % of the RNase A attached to the surface.

The incorporation of RNase A through disulphide bonds on wool fibre's surface was studied by means of infrared spectroscopy (FTIR). Although FTIR presents low sensitivity for the examination of sulphur components on textile surfaces, this technique has been thoroughly used as a standard method for the quantitative assessment of chemically modified textile surfaces. The analysis of bands at 400-500 cm^{-1} and 2600-2400 cm^{-1} give us information of S-S stretching and S-H stretching, respectively (Wojciechowska et al. 2004; Wojciechowska et al. 2002).

The tests carried out with the use of FTIR spectroscopy indicate changes within disulphide bonds on keratin, after the incorporation of RNase A. No bands are shown at the frequency of 2400 cm^{-1} on untreated samples of wool but the RNase A incorporation leads to the appearance of a band at this frequency. The bands at this wavelength are assigned to the vibration of S-H stretching of the free amino acid cysteine on protein attached to the fibre (Figure 2.6).

Another spectral region of interest is at lower frequency due to S-S stretching vibrations. Despite the fact that this band in IR spectrum possess a relatively small dipole moment changes, which are related to the symmetric nature of the bond (Urban 1993), it was possible to observe some differences. The untreated wool sample presented four different bands in the range of 400-500 cm^{-1}. After incorporation of the RNase A it can be seen an increase of the band intensity at frequency of 475 cm^{-1}, changing the shape of the spectra in these region. As the protein is attached by disulphide bonds to the wool's surface it makes sense that there is an increase on the vibration of S-S stretching.

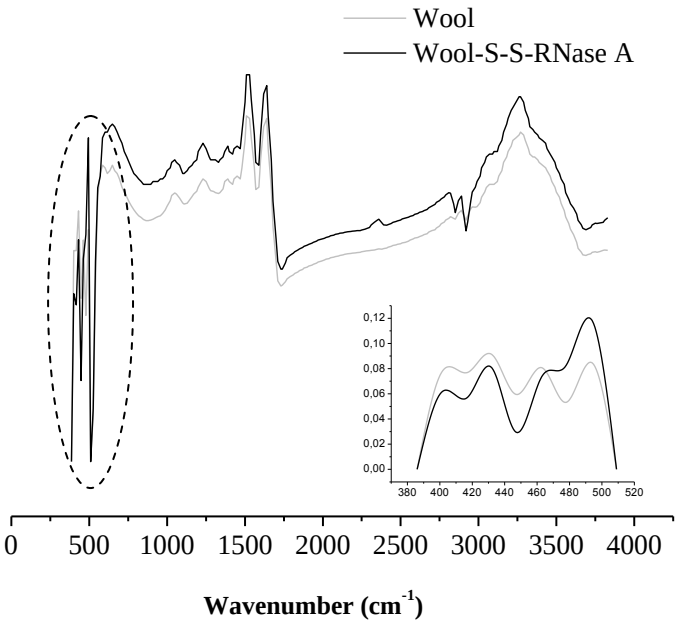


Figure 2.6 - FTIR spectra of untreated wool and wool incorporated with RNase A. Thiol groups at 2400 cm^{-1} and disulphide bonds at 400-500 cm^{-1} (zoomed area)

To corroborate these findings, incorporation of the RNase A on wool was visualized by fluorescence microscopy on wool. To do so, RNase A was conjugated with a fluorescent dye-FITC, with a ratio of fluorescence to protein (Molar F/P) of 0.79. The cross sections of the wool fabrics treated with RNase A-FITC conjugate in the presence of glutathione buffer showed a brighter layer when compared to control treated in phosphate buffer (Figure 2.7), indicating that this protein is indeed attached to wool surface in conditions above mentioned.

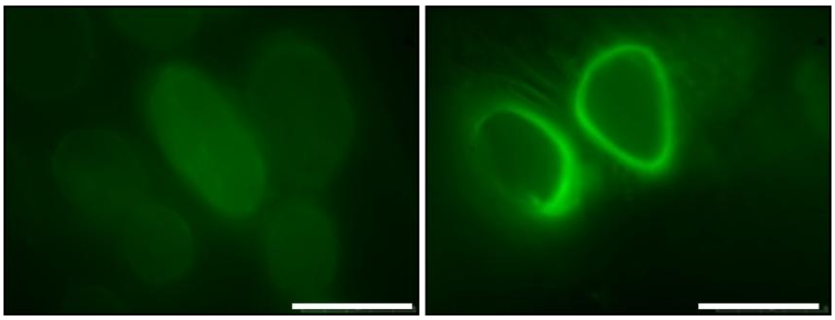


Figure 2.7 - Fluorescence microscopy images of fibre cross sections of reduced wool yarns treated with FITC-labelled RNase A in phosphate buffer (A) and in presence of glutathione-containing buffer (B). Bar represents 25 μm.

PDI was then used to release the previously attached RNase A from the wool fabrics. The measurement of catalytic activity and protein content in solution after PDI application revealed its ability to release the protein from the wool surface and to assess the percentage of protein that is in active form.

The activity of the released protein was measured based on the oxidative refolding mechanism of RNase A in the presence of its substrate, cyclic 2'3'-monophosphate (cCMP). The increase in absorbance at 296 nm was monitored for 5 minutes after incubation of the wool with RNase, treated with the PDI for 1 h. The higher activity observed after PDI application suggests that RNase A is released in its active form from the wool surface (Figure 2.8). The protein content measurements suggest about 40 % release of the protein attached to the fibre after PDI application. During the PDI treatment small amounts of keratin were also released from wool (data not shown) and these values were taken into consideration in the calculation of protein content release. The activity of the released RNase A was 46 % of that of native protein, when same protein contents compared (Table 2.2).

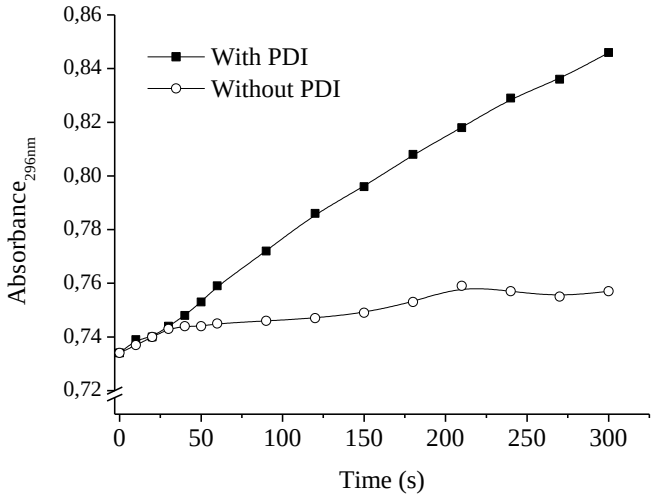


Figure 2.8 - RNase A activity in solution, after being released from the wool surface.

The results obtained indicate the possibility to link RNase A to the wool surface in oxidative conditions, while PDI is able to induce its release, thus acting as a switch-on agent to release the protein. The activity loss of the release protein was roughly half of that of native state.

Central to this explanation, and as explained for the migration experiment, is the reduced active site of PDI. It can function as a nucleophile and attack the disulphide bonds

previously formed between protein and wool. After the formation of the complex between PDI and keratin, the CCC (RNase A) is released and, in this case, the RNase A cannot be reattached to the keratin substrate probably due to its higher molecular weight (Mw), which results in release of this protein.

The first disulphide linkage-employing drug conjugate that exploits the reversible nature of this unique covalent bond was recently approved for human use (Saito et al. 2003). For that reason, an increasing number of drug formulations that incorporate disulphide bonds have been developed (Gosselin et al. 2001; Ishida et al. 2001; Kakizawa et al. 2001; King et al. 1978; McKenzie et al. 2000). The results obtained in this study show the potential of PDI to be used in enzymatic systems which act as switches for protein release. Indeed, any active substance which is incorporated to the keratinous platform using disulphide bond modulation can be released in such way. Interestingly, this system can be applied to medical textiles with previously fixed drugs in order to more efficiently deliver the necessary pharmacological compounds.

Table 2.2 - RNase A activity and protein content released from wool fabric

Conditions	Activity (U/mL)	RNase A released (mg/mL)	Percentage of recovery (%), compared to native enzyme
Without PDI	0.32 ± 0.04	0.02 ± 0.02	15.7
With PDI	1.53 ± 0.02	0.068 ± 0.007	45.4

2.4 Conclusion

The functionalization of keratinous fibres was carried out in the presence of PDI by manipulating the state of PDI active site. The results obtained led us to believe that two different reactions occurred: oxidation of disulphide bonds promoted by the oxidized active site of PDI and isomerisation of disulphide bonds promoted by the reduced active site of PDI. Both reactions occurred between cysteine-containing compounds and keratin fibres. As a result, the incorporation of peptide and dyes on hair and wool was attained as well as the migration of a dye along hair fibre and the protein release of a model protein (namely RNase A).

This work paves the way for promising biotechnological applications of keratin substrates, such as human hair and wool, whose characteristics can be modified in a

controlled manner by PDI treatments. These materials, enriched in disulphide bonds, were functionalized in order to develop novel applications for cosmetic and biomedical research.

The ability of PDI to recognize different substrates *in vitro*, such as keratin substrates and tested peptides is of major importance since most literature reports only *in vivo* recognition.

Chapter 3

Protein Disulfide Isomerase-assisted grafting of cysteine-containing peptides on over-bleached hair

3 Protein Disulphide Isomerase-assisted grafting of cysteine-containing peptides on over-bleached hair*

3.1 Abstract

The ability of Protein Disulphide Isomerase to promote the oxidation or isomerisation of disulphide bonds in proteinaceous substrates shown in previous Chapter 2 was employed for grafting two different cysteine-containing peptides on over-bleached hair. Hair tresses underwent 8 cycles of bleaching and the damage imparted by this chemical process was visualized by scanning electron microscopy (SEM). A loss of 42% of mechanical resistance and a decrease of the α-helix denaturation enthalpies were observed. PDI was further able to promote the incorporation of a keratin-based and a human lung surfactant based peptide on this damaged hair. A higher yield of peptide attachment, an increase of the quantity of disulphide bond between hair and peptide and an increase of α-helix denaturation temperature and enthalpies was observed when PDI was applied. The ability of each peptide to restore the damages imparted by overbleaching processes was also studied. Important physical and chemical properties were improved after peptides application including an increase on both mechanical and thermal properties. These achievements set these two engineered peptides as possible candidates for restorative agents for hair.

***This chapter is based on the following publication:**

Margarida M Fernandes and Artur Cavaco-Paulo, *Protein Disulphide Isomerase-assisted grafting of cysteine-containing peptides, Clinics in dermatology,* submitted.

3.2 Introduction

Keratin fibres such as wool and hair present a hierarchic structure, comprising two main morphological components: the fibrous cortex surrounded by the cuticle sheath (Wolfram 2003; Franbourg et al. 2003). Native disulphide bonds (S-S) are the main covalent crosslinks present in hair, imparting its structural integrity by forming a 3-dimensional network structure with high crosslink density (Franbourg et al. 2003; Danciulescu et al. 2004; Feughelman 1997a; Plowman 2003). Therefore, the manipulation of these reversible and stable bonds on keratin fibres can constitute a good strategy to bind or diffuse chemical compounds to the hair's cuticle or cortex. This can be enzymatically made by using Protein Disulphide Isomerase, which is able to catalyze the oxidation, isomerisation or thiol-disulphide exchange reaction in cysteine-containing proteins (Wilkinson et al. 2004). Besides, it promotes the oxidative protein folding and rearrangement of non native disulphide bonds on protein substrates leading them to the native state (Appenzeller-Herzog et al. 2008). Therefore, the PDI ability to target the disulphide bonds on protein substrates makes it an ideal enzyme to catalyze the formation of new disulphide bonds between peptides and hair.

Healthy and beautiful hair is desired and the need for products that improve the look and feel of hair surface has created a huge industry for hair care. Products such as shampoos and conditioners, along with damaging processes such as chemical bleaching treatments, alter many hair properties which results on damage to the hair fibre (Gray 2001; Bolduc et al. 2001; Dawber 1996). In recent years, many innovations have taken place and new approaches for hair treatments have been reported on literature to overcome this problem. However, these approaches are still aggressive. The PDI-assisted application of low molecular weight peptides that contain cysteine in their composition, to recover the damage imparted by the commonly used chemical treatments, may constitute a milder to treat hair fibres, which has never been reported.

Herein, two different engineered peptides derived from human lung protein surfactant B (SPB) and keratin from human hair (KP) were developed in order to study their ability to dye and restore the integrity of chemically over-bleached hair. A study involving the PDI-assisted penetration of these engineered peptides that differ in chemistry, was performed.

3.3 Materials and methods

3.3.1 Materials

3.3.1.1 Hair

Natural European blond human hair samples were provided by International Hair Importers & Products Inc. (New York) and used as supplied.

3.3.1.2 Engineered peptides

The peptides used in this study were synthesized by JPT Peptide Technologies GmbH (Berlin, Germany) and were covalently linked by their N-terminal to a fluorescent dye, (5(6)-carboxytetramethyl-rhodamine, succinimidyl ester), i.e. 5(6)-TAMRA, with spectral properties of λex=544 nm and λem=572 nm, to facilitate the analysis of peptide penetration. Peptides are fragments from keratin intermediate filament proteins (KP) or from mammal surfactant pulmonary B proteins (SPB).

The peptides were dissolved in an aqueous solution (0.05 M Phosphate buffer solution, ph 7.5). Both peptides were prepared with a concentration of 0.6 mM, corresponding to 1.0 mg/mL of KP and 0.9 mg/mL of SPB. The peptide characteristics are shown in Table 3.1. The sequence of the peptides are described by one letter code in which X represents one of known amino acid residues with the exception of cysteine residue which is represented by the letter C.

3.3.2 Methods

3.3.2.1 Hair treatments

Blond hair fibres (0.1g) were washed before treatment with a commercial shampoo. Two different types of hair were treated: virgin hair, unaltered hair without any chemicall treatment (VG); and chemically damaged hair, subjected to 8 cycles of bleaching (8xB). The bleaching procedure was applied 8 times in the same tresses of hair and consisted in the application of 10% H_2O_2 (o.w.f) in the presence of 0.1 M Na_2CO_3/$NaHCO_3$ pH 9.0 buffer at 50 ºC for 1h.

The hair samples subjected to the peptide incorporation underwent the same process as 8xB samples followed by a treatment with 600 µL of peptide solution of 0.6 mM (final concentration 120 µM; corresponding to 0.2 mg/mL of KP and 0.18 mg/mL of SPB) of the peptides diluted to 3.0 mL of 0.05M phosphate buffer pH 7.5, for 1h at 37º C and 100 rpm. Subsequently, all the samples were thoroughly rinsed with tap water and washed with a commercial shampoo.

3.3.2.2 Hair treatments with PDI

For PDI treatment, 100 µL of a 0.05 mg/mL PDI solution was added to the reaction bath, at the conditions previously described. The treatment was performed at 37 ºC, 100 rpm for 1h, and hair samples were then washed under running water and washed with a commercial shampoo.

The PDI solution was made as follow: 250 µg of PDI commercial powder (Sigma) was dissolved in 0,05M phosphate buffer pH 7.5. These environment is believed to promote a redox environment of E^o= -180 mV PDI, its intrinsic PDI redox potential, that stabilizes the active site oxidised state.

3.3.2.3 Colour variation

The colour variation was measured directly from the hair-peptide solution before and after the treatment, at 555 nm. Since the colour of the solution comes from the peptide, from the differences in absorbance, the quantity of peptide that penetrates/attach into the hair was determined and expressed as colour variation. The % of colour variation was calculated based on the following Equation.

$$\text{Colour Variation} \quad (\%) = \quad \frac{\text{Abs}_{\text{initial}} - \text{Abs}_{\text{final}}}{\text{Abs}_{\text{initial}}}$$

3.3.2.4 Free thiol and disulphide bond determination

The amount of free thiol groups in hair was determined spectrophotometrically using 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent). Initially, 10 mg of hair samples were added to 5 mL of 0.5 M phosphate buffer, pH 8.0 and 100 µL of Ellman's reagent solution (4 mg/mL). The samples were mixed and incubated for 1 h at room temperature in the dark and the absorbance was measured at a wavelength of 412 nm (Ellman 1959).

To determine the total amount of sulfhydryl groups, the reaction with Ellman's reagent was performed after complete reduction of hair samples disulphide bonds with sodium borohydride ($NaBH_4$): also 10 mg of hair samples were hydrated with 350 µL of water for 10 min. Then, 150 µL of 0.05 M Tris buffer pH 6.8 and to 1.0 ml of a freshly prepared 4% (w/v in 0.2 M NaOH) $NaBH_4$ solution was added. The samples were incubated for 1 h in an oscillating waterbath at 37 ± 0.5 ºC. Thereafter, the remaining $NaBH_4$ was inactivated by addition of 200 µL 5 M HCl and agitating for 10 min. The pH of the reaction mixture was adjusted to 8.0 with 2 mL of 1 M Phosphate buffer pH 8.0. The thiol groups were then measured by adding 100 µL of Ellman's reagent (4 mg/mL) on these samples and incubating 15 min at room temperature. L-cysteine reagent standards were used in both methods to calculate the amount of thiol groups.

The disulphide bonds present on the different hair samples were calculated by subtracting the thiol groups present after complete reduction with $NaBH_4$ to the initial free thiol groups. The thiol determination in both cases, before and after complete reduction with $NaBH_4$, was repeated 3 times in order to validate the results.

3.3.2.5 Fluorescence microscopy

Hair fibres transversal cuts were analysed by fluorescence microscopy. Hair fibres were embedded into an epoxy resin and transversal cuts of the fibers with 15 µm were prepared using a microtome (Microtome Leitz). Fibers cross sections were analyzed by a Fluorescent microscope LEICA DM 5000B with a magnifications of 40x and 100x. The fluorescence microscopy images were taken using the same conditions of filter, time of exposure, brightness and gain. The most representative images among all were chosen.

3.3.2.6 Mechanical properties

The method used broadly follows the guidelines laid down in ASTM D1445-95 for the tensile testing of fibres. The measurements were performed with an Instron 4505 tensile tester with a maximum load cell capacity of 2.5 N. For each measurement, 10 single hair fibres were taken randomly from the hair tress. Each hair was individually mounted in the tensile jig by means of a paper device with a fixed gauge length of 20 mm. Before the test begins the paper device was slashed. The measurements were performed under controlled conditions (20 ± 0.5 ºC; 55 ± 4 % humidity), at a rate of 1.5 mm/min, until breakage occurred. For each hair, records of applied load against extension were taken and

using as average mean diameter of 70 μm, the data were converted to stress (load/unit area) against strain (% extension).

3.3.2.7 Scanning Electron Microscopy (SEM)

Some hair fibres were taken randomly from the hair tress and mounted onto aluminum stubs using conductive carbon adhesive tape and sputter coated from a gold/palladium leaf source to impart conductivity to the surface of the sample. The thickness of the coating was approximately 10 nm. Samples were studied using a NOVA Nano SEM 200 FEI. The microscope was operated at 10 kV and samples viewed at a working distance of 8 mm and 10 000 x magnifications.

3.3.2.8 Differential Scanning Calorimetry (DSC)

All investigations were conducted on a power-compensated DSC instrument (DSC-7, Perkin Elmer) using pressure-resistant (25 bar), stainless steel, large-volume capsules in the temperature range of 50-250 ºC (heating rate: 5 ºC/min, sample weight: 7-8 mg). The DSC device was calibrated using indium and palmitic acid, both of high purity. The samples were stored at selected levels of humidity (RH of 45%) and temperature (20–22 °C) for 24 h prior to the analyses and each hair sample was measured at least three times, in order to validate the results.

3.3.2.9 Data analysis

Results were analyzed using Graph Pad Prism version 5.04 for Windows (Graph Pad Software, San Diego, CA). Statistical significances were determined using a one-way ANOVA followed by the Dunnett post-hoc test or by the unpaired two tailed Student's t test method. P values ≤ 0.05 were considered statistically significant.

3.4 Results and Discussion

3.4.1 Engineered peptides

The peptides used in this work were developed to be a PDI substrate, due to their variable content in cysteine residues (Table 3.1) and with the objective of recovering

damaged hair properties. They are fragments of the keratin intermediate filament proteins (KP) or fragments of the mammal surfactant pulmonary B proteins (SPB).

The amino acid sequence of the keratin type II cuticular Hb5, a protein that in humans is encoded by the KRT85 gene (Rogers et al. 1997; Koehn et al. 2010) was found and a fragment of 13 amino acids comprising two cysteines, separated by 4 different amino acids, was then selected and chemically synthesized. Due to its origin, this peptide is thought to comprise several properties: affinity towards hair's surface and ability to restore damaged cuticle parts. Also, as a type II alpha-keratin, it might possess a resilient and pliable characteristic (Coulombe et al. 2004) that can be translated to the peptide and consequently to the hair fibre.

The amino acid sequence of a pulmonary-associated surfactant protein B (SPB) from mammals' lungs was also searched (Moore et al. 1992; Pilot-Matias et al. 1989) and a fragment of the amino acid sequence was chosen.

Table 3.1 - Peptides' nomenclature and characteristics: sequence, number of amino acids, molecular weight, isoelectric point, and zeta potential. All peptides are attached to a fluorescent dye TAMRA at C terminus and to $CONH_2$ at N terminus

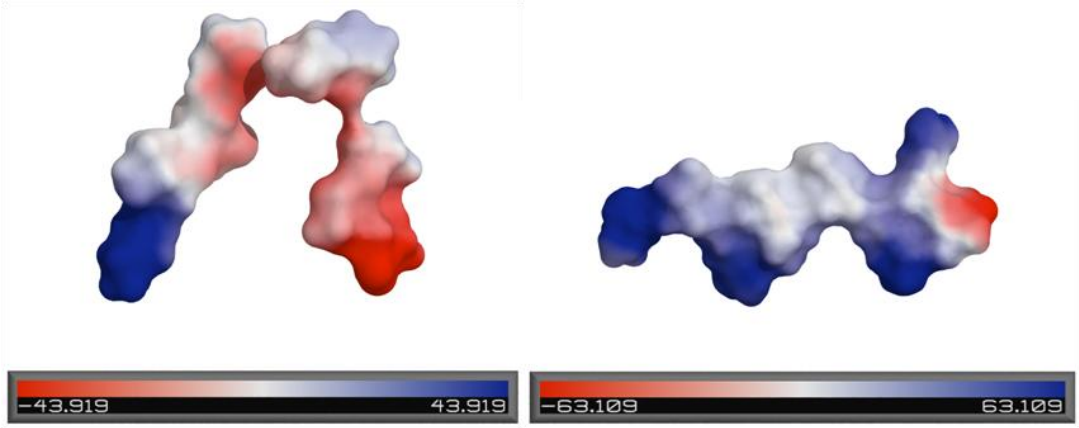
Peptide name	Peptide Sequence C-N	Nº aa	Mw (Da)	Isoelectric point (pI)*	Zeta potential at pH 7.5 (mV)
KP	X_3**C**X_5**C**X_3	13	1599.84	5.2	4.28 ± 1.21
SPB	X**C**X_7	9	1496.87	11.3	-24.93 ± 0.71

*Calculated with "Peptide Property Calculater" available at www.innovagen.se

The diffusion of compounds into hair can be hindered by the presence of the hydrophobic lipid layer. This thin layer covers the hair surface, bonded by thio-ester linkages to hair proteins and is thought to act as a barrier to the penetration of compounds into its interior (Breakspear et al. 2005). The development of SPB peptide was thought to overcome this problem. Surfactant proteins from mammal's lungs are able to recognize and interact with lipids and fragments or models representing those proteins could recognize and interact with hair lipids, increasing therefore the penetration of several compounds inside hair (Cavaco-Paulo et al. 2007). Such properties make these peptides as a new interesting compound for hair care application.

The peptides were visualized by a molecular visualization program (PyMol v1.4) that creates the structure based on their amino acids sequence (Can et al. 2006). This program allows the visualization of peptide's structure in vacuum, the identification of

their surface charge and the major differences in their structure (Figure 3.1). Besides illustrating their amphipathic nature, property that increases their affinity for biological membranes (Bessalle et al. 1993; Wimmer et al. 2005), it also shows that KP is mainly anionic (negative surface charges (red) predominate) while in SPB is mainly cationic (predominance of positive surface charges (blue)).



A B

Figure 3.1 - Peptides surface charge analysis from PyMol v1.4. Representation of the sequence and structure of A) KP and B) SPB, from C to N terminal. Red denotes the negatively charged and blue denotes the positively charged.

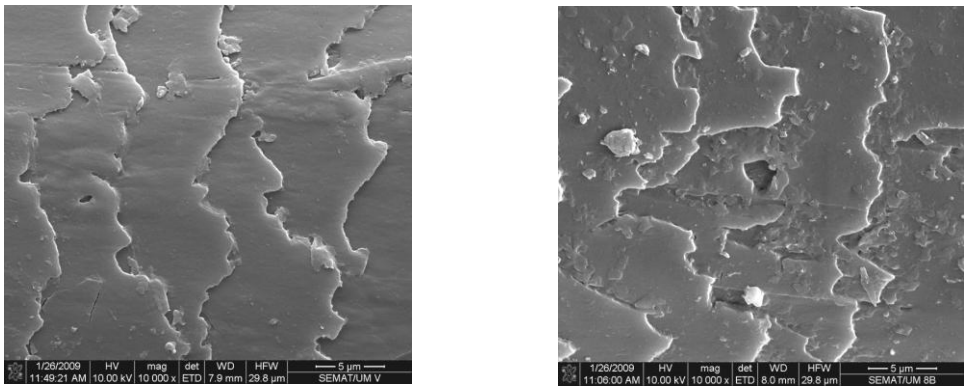
This tendency was also observed in terms of isoelectric point (pI), a characteristic that predict the peptides' affinity towards hair. The pI is the pH at which the peptide carries no electrical charge and the net charge on the surface is zero (i.e. number density of positive and negative sites is equal) (Franks et al. 2003). Therefore, at pH 7.5 (pH of the treatment) peptides will possess different net charges. At pH lower than pI, the peptide acquires positive charges (SPB) and at higher pH than pI the peptide acquires negative charges (KP), confirmed by their zeta potential values (Table 3.1).

Human hair isoelectric point has been reported by others to be near 3.7 (Wilkerson 1935; Regismond et al. 1999) thus possessing a negative surface charge under most pH conditions. The electrostatic interactions are believed to play a determining role in the adsorption mechanism. Therefore, it is expected that the positive charges found on SPB will increase its specificity towards the negatively charged hair surface while the negative charges found on KP will decrease it.

3.4.2 Over-bleached hair

Chemical bleaching is a technique commonly used in current cosmetic applications aiming two main objectives: to give hair a lighter look or, more often, to prepare it for the application of a dye. These lightning treatments have the advantage of modifying the characteristics of melanin pigments generally yielding a lighter shade than the natural one (Zviak et al. 2005a), having also the disadvantage of modifying hair properties along the cuticle after repetitive applications, resulting on damage of the hair fibre (Gray 2001; Bolduc et al. 2001; Dawber 1996).

To mimic several bleaching applications in a blond virgin hair, 8 cycles of bleaching were performed. Figure 3.2 shows the SEM images of a normal untreated hair (a) and an over-bleached hair (b). Over-bleached hair experienced more chemical damaged when compared to virgin hair that maintains complete cuticles and no holes are observed. Damaged, over-bleached hair presents less visible cuticles layers (5 layers in virgin hair and 3 layers in over-bleached hair in the same area shown in Figure 3.2), pronounced holes and debris fragment along the damaged cuticle layers. Chemical bleaching destroys the edges of the cuticle releasing small cuticle fragments on the hair shaft.



A B

Figure 3.2 - SEM images of normal virgin hair (A) and of over-bleached hair (B). Bar represents 5 μm.

3.4.3 PDI-assisted incorporation of peptide on over-bleached hair

The colour variation method measures the uptake, and therefore the affinity, of peptide on hair while it is being applied. According to expectations, a high affinity towards the negatively charged hair was observed for the positively charged SPB while a low

affinity was observed for the negatively charged KP (Figure 3.3). Therefore, the initial peptide uptake seems to be dependent on peptides' isoelectric point, highlighting the importance of electrostatic interactions on the peptides initial uptake. Nevertheless, the presence of PDI on peptide solution bath promoted a higher colour variation ratio when treating hair with KP, indicating that PDI might promote the covalent linkage between this peptide and hair. In the contrary, the application of SPB along with PDI decreased the peptide uptake, indicating that PDI decreased the peptide affinity towards hair (Figure 3.3). The presence of PDI is somehow inhibiting the initial contact of SPB towards hair. This decrease on the affinity is not well understood but we believe that some changing in conformation of peptide might occur upon PDI application, which does not hinder the peptide attachment through disulfide bonds on hair (Figure 3.4 & Table 3.2).

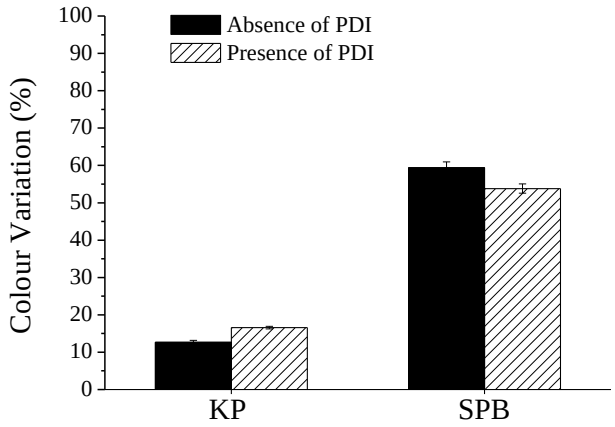


Figure 3.3 - Uptake of peptides on over-bleached hair treated with or without PDI, measured by colour variation method in bath treatment solution at 555nm.

After the treatment, the hair was submitted to a washing procedure to remove the loosely bond peptide. To visualize the presence of the remaining peptide on hair, fibre transversal cuts were made and fluorescence microscopy images ware taken. Although this method does not give information about the amount of peptide or type of bonding present after hair treatment, it demonstrates without doubt the presence of peptide.

Both peptides are covalently bonded to a fluorescent dye (5(6)-TAMRA) and similarly to what Regismon and co-workers reported (1999), it was possible to qualitatively visualise the peptide on hair by fluorescence microscopy. The samples were subjected to the same conditions of brightness, time of exposure and gain. Figure 3.4 shows the fibres transversal cut images of unaltered human hair (VG) treated with peptide,

over-bleached hair treated with peptide (8xB-KP; 8xB-SPB) and over-bleached hair treated with peptide in the presence of PDI (8xB-KP-PDI; 8xB-SPB-PDI).

The intensity of the layer around the hair cuticle of VG hair treated with SPB is more intense than when VG treated with KP. This result demonstrates the high affinity of SPB towards human hair (Figure 3.4), as expected from the properties shown previously (Figure 3.3, Table 3.1). On the other hand, when peptides are applied to chemically damage (over-bleached) hair, fluorescence microscopy images shows that both SPB and KP are highly attached to hair's surface (Figure 3.4).

These results suggest that more important than the peptide's isoelectric point are the hair conditions, i.e., if it is damaged or not. Over-bleached hair is believed to possess different physic-chemical properties than those reported for unaltered hair (VG). The surface charge of over-bleached fibres might be altered and the interaction with the peptides different. Indeed, the surface charge of the fibres depends on several factors such as microstructure, porosity, specific surface, fibre swelling capacity or interaction energy of the fibre and solution (Barba et al. 2009) and the chemical process of bleaching certainly change them. As the SEM images depict in Figure 3.2, bleaching is a chemical process that results in hair's cuticle damage, like cracks, holes and cuticle lifting. This damage may constitute a pathway for the penetration of small peptides, despite their chemical properties. In fact, previous studies indicate that, when mainly electrostatic interactions occur, the amount of adsorption on hair is increased in bleached (damaged) hair rather than virgin hair and with decreasing molecular weight compounds (Faucher et al. 1976).

Further PDI application along with peptides was also visualize by fluorescence microscopy. The presence of PDI seems to favour the penetration of the KP and SPB inside the fibres' cortex, as reported before in chapter 2 for KP, (Fernandes et al. 2011) while the control (KP and SPB without PDI) possess the peptide at the cuticle (Figure 3.4).

We believe that these results are indicative that PDI promote the creation of stronger linkages between the cysteine-containing peptides and hair, which results in peptide attachment to hair. Besides penetrating into the cortex, these strong linkages are believed to resist the washing procedures.

To prove that disulphide bonding occurs, a quantification of disulphide bonds on hair treated with peptides was performed. Using the approach of Nagy and co-workers (2007) we were able to quantitatively measure the disulphide bonds present in hair treated with peptides in the presence and absence of PDI. Disulphide bonds were calculated using Ellman's reagent (Ellman 1959) from the difference between the free thiol groups present

on hair and the total thiol groups present on hair after complete reduction with $NaBH_4$ (Hansen et al. 2007).

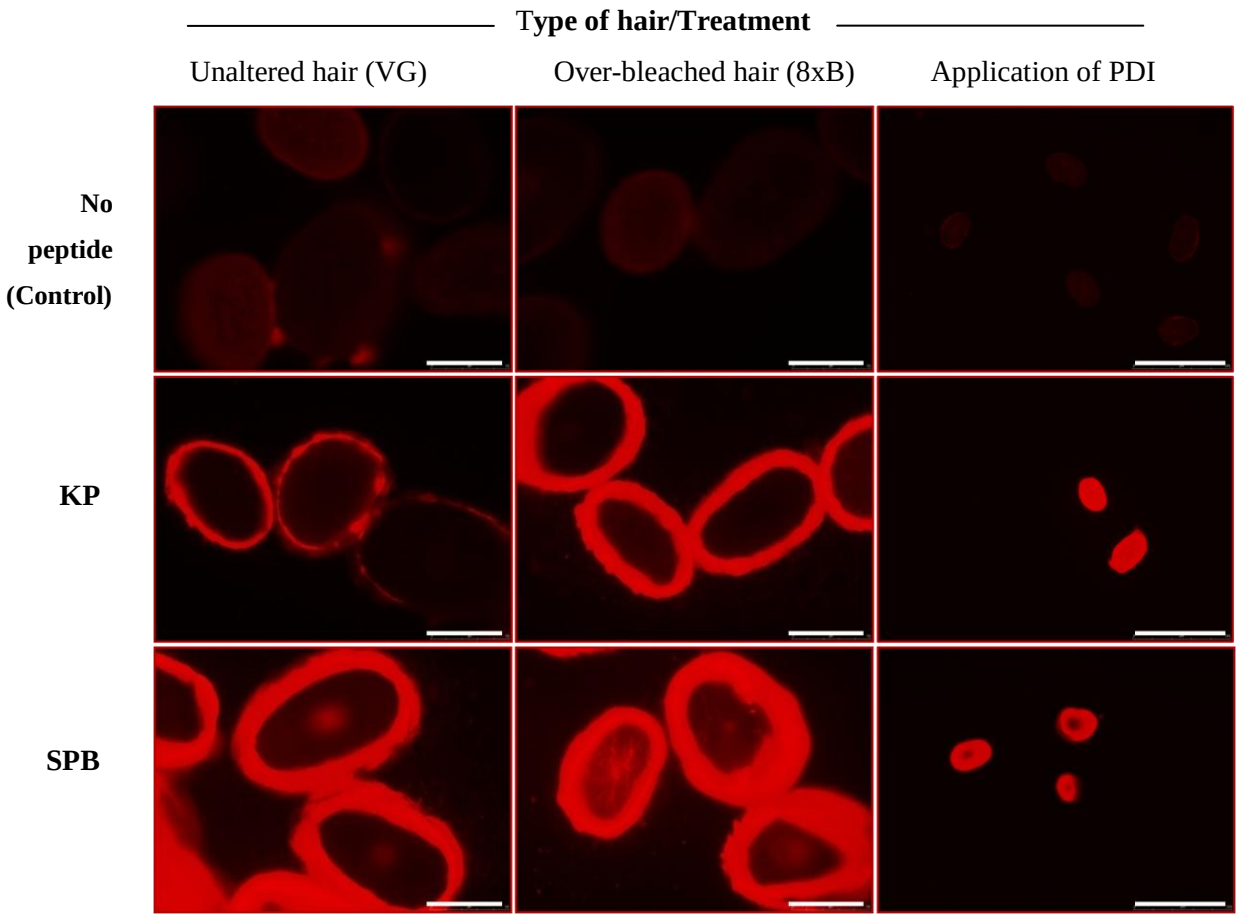


Figure 3.4 - Fluorescence images of human hair cross sections. Unaltered human hair and over-bleached hair treated with KP and SPB peptide in the presence and absence of PDI. Smaller bars indicate 50 μm and bigger bars indicate 250 μm.

Table 3.2 shows the free thiol and disulphide bonds on hair treated with both peptides in the presence and absence of PDI. Starting with the analysis of free thiol groups, before complete reduction, a higher content was found to be present on hair treated with KP when compared with hair treated with SPB. KP possesses two cysteine residues separated by five amino acid residues and only one cysteine is thought to be involved in disulphide bond formation, when PDI is present. We believe that the other peptide's cysteine might be free and then sensitive to Ellman's reagent.

In the absence of PDI, more thiol groups are observed in hair treated with KP than with SPB (Table 3.2). Due to its positives charges, the C-terminal of SPB has high affinity to hair (Figure 3.1) and when applied on hair without PDI, electrostatic interactions are believed to play a major role. The cysteine residue is the second amino acid present in the sequence of this peptide (Table 3.1), so if peptide binds to hair through the C-terminal, no free thiol group is detected by Elman's reagents even though PDI is not applied.

With regard to disulphide bonds, a higher quantity was calculated when 8xB hair was treated with both peptides along with PDI (Table 3.2), corroborating the assumptions raised from the free thiol group results. By the light of these results, we believe that PDI is able to catalyze the covalent linkage through disulphide bonds of these cysteine-containing peptides on hair.

Table 3.2 - Quantification of thiols (-SH) in the form of free thiol groups and in the form of disulphide bonds on over-bleached hair treated with peptides in the absence and presence of protein disulphide isomerase (PDI)

	Free thiol -SH (µM/mg of hair)	**S-S bonds (µM/mg of hair)**
8xB	1.6 ± 0.3	1.3 ± 0.1
8xB-KP	3.4 ± 0.6	1.0 ± 0.1
8xB-KP PDI	**2.6 ± 0.6**	**2.7 ± 0.1**
8xB-SPB	0.040 ± 0.008	2.26 ± 0.08
8xB-SPB PDI	**0.04 ± 0.01**	**3.27 ± 0.01**

3.4.4 Recovery of hair mechanical and thermal properties

The way that hair care products interact and change chemical and physical properties of hair are of main interest in hair-care science, since these properties are closely tied to product performance.

It was previously shown that KP and SPB have affinity towards over-bleached hair and are covalently bonded in the presence of PDI. However, are these peptides able to recover the mechanical strength and thermal properties of damaged hair?

To answer this question, a study of the physical and chemical properties of damaged hair fibres after peptides' treatment, was performed. The Young's modulus and

tensile strength of hair treated with peptides are presented in Figure 3.5. It is common knowledge that the determination of these parameters is quite prone to variation, depending on the method chosen, the part and type of hair measured, among other factors. However, a set of 10 different hair samples were used in order to validate the results.

The damage imparted by the consecutive chemical bleaching treatments was translated in resistance's loss. It was observed that over-bleached hair lost around 42% of mechanical resistance, measured in terms of Young's modulus and 37% of tensile strength (Figure 3.5). Young's modulus, also known as the tensile modulus, measures the stiffness of an elastic material, such as hair. It is a measure of the ratio of stress to strain therefore reflecting the resistance of hair to elastic deformation (Kitano et al. 2009). The other parameter analysed is the ultimate tensile strength, which is the maximum stress that a material can withstand when subjected to tension. This may be considered an indirect measure of the important mechanical property of hair: its elasticity. In fact, bleaching treatments are reported as processes that alter the elasticity of hair, by decreasing it by up to 25% (Erik et al. 2008).

However, the lost resistance was regained with after SPB and KP treatment. As an exciting result, KP was able to recover the hair's resistance towards higher levels than those of unaltered hair with an increment on hair's resistance of 112% on Young's modulus parameter and 102% of tensile strength. SPB was also able to recover the hair physical properties: an increment of ~94% of Young's modulus parameter and ~43% of tensile strength was observed (Figure 3.5).

As mentioned above, we believe that the high cuticular damage observed after several bleaching treatments induce loss of resistance. The peptides application seems to protect the cuticle layers thus improving the mechanical resistance.

SPB high affinity may be in the origin of these improvements. It might be able to cover the surface of the damaged hair, therefore increasing their stiffness.

On the other hand, the origin of KP may be the key factor for the explanation of the high resistance recovery. As a fragment of a cuticular keratin protein it is thought to be able to recover the cuticle structure. Filling the holes of damaged hair cuticle and penetrating inside the fibre's cortex is a possible pathway for the resistance recovery observed with KP.

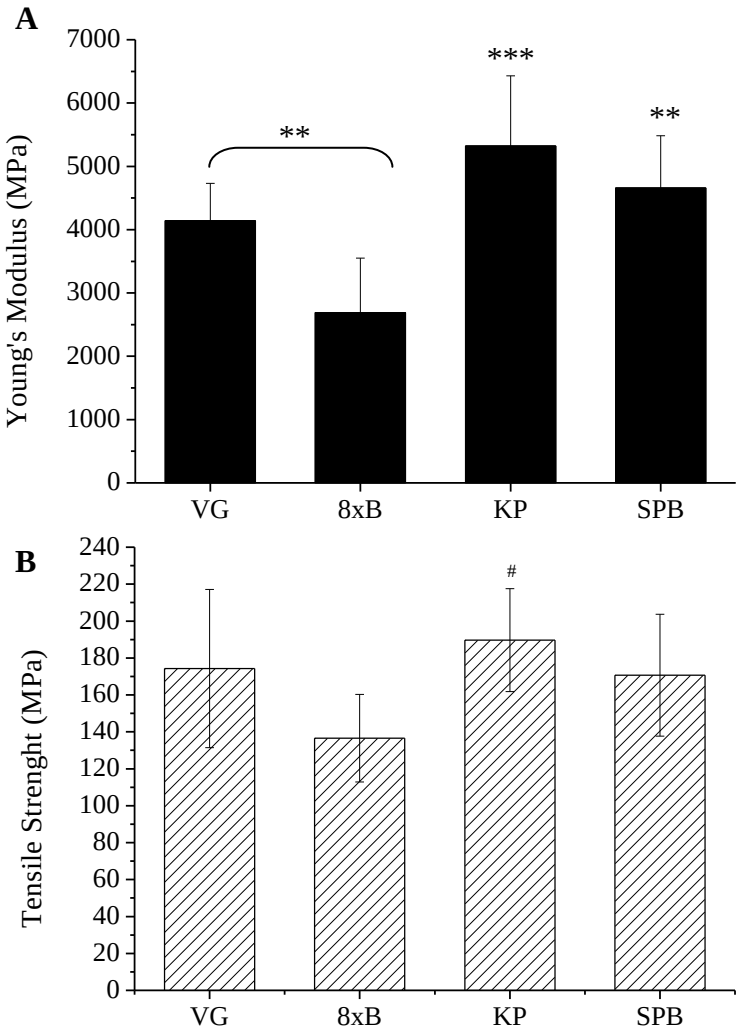


Figure 3.5 - Mechanical resistance parameters: A) Young's modulus and B) tensile strength of a virgin (VG) and over-bleached (8xB) hair, and over-bleached hair treated with peptides (KP and SPB). Data presents mean ± SD of ten independent experiments. ** P≤ 0.01, when compared with each other,*** P≤ 0.001, ** P≤ 0.01 when compared to 8xB,$^{\#\#\#}$ P≤ 0.05 when compared with the 8xB. No significant differences were observed between each concentration of peptide and the respective control.

It is widely accepted that mechanical properties of hair, which are important in its acceptable cosmetic performance, are strongly influenced by their immediate chemical environment. Therefore, to corroborate the previous findings and to prove that the chemical environment on hair after treatment with peptides in the presence and absence of PDI is changed, a differential scanning calorimetry (DSC) was performed. This technique has been widely used to assess the denaturation performance of human hair keratin

submitted to different chemical effects (Wortmann et al. 2008; Wortmann et al. 2006; Wortmann et al. 2002).

Although human hair exhibits a complex morphology as an α-keratin fibre, for the context of thermal analysis it is considered as a filament/matrix-composite, as originally proposed by Feughelman (Feughelman 1959, 1997a). In this model, the α-helical fraction of the intermediate filaments (IF) comprises the crystalline filament phase, that is embedded in an amorphous matrix. The matrix is represented by the intermediate filament associated proteins (IFAPs), i.e., the matrix, and the rest of the amorphous morphological components.

DSC experiments were therefore conducted at dry state under the same conditions of temperature and humidity. The effects of bleaching treatment, subsequent peptide application and presence of PDI on the main morphological components of human hair were analyzed. Table 3.3 shows the temperatures and enthalpy related to the removal of loosely bound water as well as the temperatures and enthalpy values for the α-helix keratin denaturation.

The thermal transitions in the keratin are strongly affected by the amount of water and a broad endothermic signal, related to the removal of loosely bond water at temperatures, appears at 100°C (Humphries et al. 1972). A decrease on the temperature and enthalpy at which the removal of water occurs was observed in chemically damaged hair (over-bleached). The damage imparted by the overbleaching processes on the surface of the hair may be the reason for this decrease. The holes that were observed in SEM (Figure 3.2) on damaged hair fibres may constitute a faster pathway for the water removal, thus lowering the temperature and enthalpy values when compared to the virgin hair, in which no holes or damaged surface was observed. Further incorporation of both peptides on this chemically-damaged hair increased the temperature and enthalpy of water removal, probably due to the ability of this peptide to cover the surface of the hair, partially filling the holes observed previously, as above mentioned (Table 3.3). Less water adsorption on hair is thought to occur and therefore the water removal when peptide is present is delayed to higher temperatures. PDI is able to highly increase the temperature at which the water is removed, corroborating one more time the results that shows the PDI-induced attachments of peptides on damaged hair. The enthalpy, however, is highly decreased, indicating that a lower energy needed to remove the water. This result is not well understood but we believe that if PDI promotes the incorporation on cortex of this cysteine-containing peptides, less peptide will cover the hair surface. Therefore, the disulphide bonds promoted by PDI at

hair's surface increase the water removal temperature but the lower quantity of peptide on surface decrease the enthalpy.

The other important parameter analyzed was the denaturation of the helical keratin fraction, characterized by a signal with a maximum temperature at the 210–250°C interval (Monteiro et al. 2005).

Table 3.3 - Temperatures of water removal (Tdw) and α-helix denaturation (Tdα); and enthalpies of water removal (ΔHw) and α-helix denaturation (ΔHα) on over-bleached hair 8xB, over-bleached treated hair (8xB-SPB and 8xB-KP) and virgin hair

	Tdw (ºC)*	**ΔHw (J/g)***	**Tdα(ºC)***	**ΔHα (J/g)***
Virgin hair (VG)	92.8 ± 0.8	244.1 ± 4.4	229.3 ± 0.7	6.8 ± 0.2
8xB	86.5 ± 1.9	178.4 ± 18.5	230.6 ± 4.5	4.2 ± 1.1
8xB-KP	93.7 ± 2.4	262.9 ± 11.8	229.0 ± 1.7	5.1 ± 0.5
8xB-KP-PDI	116.2 ± 1.7	95.5 ± 9.8	241.0 ± 2.6	9.9 ± 1.5
8xB-SPB3	89.4 ± 0.3	226.6 ± 9.5	235.7 ± 5.3	6.6 ± 2.4
8xB-SPB3-PDI	128.0 ± 4.1	54.4 ± 8.2	241.0± 7.8	10.3 ± 3.1

*Data presents mean ± SD (standard deviation) from a minimum of three experiments

The α-helix denaturation temperature (Tdα) of hair fibres chemically bleached did not change significantly when compared to the virgin, non-damaged, hair (Table 3.3). However, after application of peptides on over-bleached hair in the presence or absence of PDI, the α-helix denaturation temperatures shifted towards higher values, with the exception of hair treated with KP in the absence of PDI.

This signals are thought to be strongly affected by chemical treatments providing information about the α-helix content (Wortmann 1993). It is therefore interesting to study the changes that results from the different treatments applied on hair. A prove of this concept is that α-helix denaturation enthalpy decreased on over-bleached hair, and increased after peptides treatment, indicating the negative effect of bleaching treatments and the positive effects of subsequent peptide application. It is evident that more energy is needed to disorganize the α-keratin structure of healthy, virgin hair or the hair treated with peptides then to disorganize the over-bleached hair.

3.4.5 Conclusion

Surfactant and keratin-based peptides were applied on chemically over-bleached hair and their ability to restore its mechanical and thermal properties was studied. The overbleaching process highly decreased the mechanical resistance of the hair fibres. The results suggested that integrity of human hair was improved after both peptides treatment. Interestingly, KP highly increased the mechanical properties of damaged hair towards non-damaged (VG) hair values. Further application of PDI promoted a higher yield of attachment of peptides on hair via disulphide bonds, proving the ability of PDI to catalyze the formation of disulphide bonds between a cysteine-containing compound and keratin substrate. Fluorescence microscopy images have indicated that over-bleaching process was determinant in the peptide uptake on hair. The higher the fibre damage, the higher the peptide attachment. This behaviour discloses an advantageous system since damaged hair will need more peptide to treat it. Colour variation measurements have shown that SPB is highly substantive to human hair and PDI application did not improve the initial affinity of peptide towards hair. The KP, however, showed less affinity towards hair but PDI application increased it.

Chapter 4

Keratin-based peptide: a new restorative agent for hair

4 Keratin-based peptide: a new restorative agent for relaxed hair

4.1 Abstract

In the previous Chapter, a peptide based on a fragment of keratin type II cuticular protein, keratin peptide (KP), was able to restore the mechanical and thermal properties of over-bleached damaged hair, in the absence of PDI. In the quest to accomplish the same goal, this peptide was studied as a new restorative agent for weakened relaxed hair, only by surface modification. The peptide was prepared in both aqueous (WF) and organic solvent formulations (OF), in order to determine if organic solvents facilitate the interaction of peptide with hair and to assess the differences on hair recovery. Both peptide formulations were shown to induce an improvement on weakened hair being that OF seems to improve this characteristics at higher yield. As a possible new hair-care product, its cytotoxicity and genotoxicity was also studied. These tests are indicators of the potential of peptide to cause irritation on skin or to be carcinogenic, respectively. To assess the cytotoxicity effects, cell viability was measured by Alamar Blue assay. To evaluate the peptide carcinogenic potential, an evaluation of cell death and the increase of DNA mutation rate were performed. The peptide in WF did not show cytotoxicity or genotoxicity at all tested concentrations. The presence of OF, however, induced cytotoxicity on cells by decreasing cell viability in 20% in all range of concentrations after 72h. Moreover, OF inhibited cell growth and was considered genotoxic.

This chapter is based on the following publication:

Margarida M Fernandes, Cristovão F Lima, Ana Loureiro, Andreia Gomes and Artur Cavaco-Paulo, *Keratin-based peptide: a new restorative agent for relaxed hair*, Journal of the American Academy of Dermatology, submitted.

4.2 Introduction

Black African hair is characterized by its extremely curly shape (Khumalo et al. 2000). Compared to straight hair, excessively curly hair is likely a twisted ribbon in terms of its physical configuration. It is difficult to comb both in its wet and dry states, is hard to style and is highly unmanageable (Syed et al. 1998). As a consequence, requires more drastic approaches to styling than any other type of hair. Whether it is alkaline relaxing or repeated hot combing, it leaves the hair more fragile and requires of the consumer the outmost care to maintain its healthy appearance. Even when intact, the African hair demonstrates its potential for breakage (Berardesca et al. 2007). More fragile than straight hair, excessively curly hair breaks more readily upon stretching, excessive combing and brushing. Thus, this type of hair requires special handling and specially formulates products that are different from Caucasian hair care formulas (Syed et al. 1998).

Herein, a 13-aminoacid keratin-based peptide (KP) was applied in relaxed damaged hair due to its characteristics of recovering the mechanical properties of over-leached damaged hair, shown in the Chapter 4. Due to its properties, this peptide may constitute a potential candidate for a new product development. As a consequence, further investigation on the peptide risk assessment, including the study of cell viability, cell morphology and genotoxicity in the presence of peptide was carried out. The first two tests evaluated the skin irritation potential while the third test evaluated the induction of cell death and/or the increase of DNA mutation rate and carcinogenesis.

The cosmetic products safety assessment is one of the most important steps when a new product is created. Cosmetics and their ingredients must not be harmful to human health under normal or foreseeable conditions of use (Nohynek et al. 2004). Prior to human testing, an initial product safety assessment is usually performed *in vivo* on animals. However, ethical and financial objections to these methods have been raised as well as scientific drawbacks such as intra- and interlaboratory variability, good predictability only for strong irritants, and the fundamental difference between animal and human in morphological aspects of skin (Lee et al. 2000). As a consequence, several *in vitro* alternatives to animal testing have recently been proposed to predict the *in vivo* skin irritancy potential of a variety of test agents (Osborne et al. 1994; Ponec 1992). The Alamar blue (AB) assay has been introduced as an alternative cell viability indicator and used to estimate the cytotoxicity of chemicals on cultured cells (Nakayama et al. 1997; Page et al. 1993).

The genetic toxicity tests are important analysis because they not only predictive the potential carcinogenic, but also mutagenic hazards. They are principally regarded as short-term screening studies for the prediction of the carcinogenic potential of substances. The strategies to evaluate the genotoxic hazard of a substance have been proposed by several authors and organisations, and are based on a combination of *in vitro* and *in vivo* tests (Ashby et al. 1996; Nohynek et al. 2004).

In this study, KP was studied as a new restorative agent for damaged hair. The restoration of damaged hair was studied by the improvement of the physical and chemical properties. Several concentrations of peptide in the presence of both aqueous and organic solvent formulations were tested and the cytotoxicity and carcinogenic potential was studied, as well as the increase of the mechanical properties. Alamar Blue assay was performed to evaluate the cytotoxicity of the peptide. To assess its genotoxicity, an alkaline version of the comet assay in immortalized human foreskin fibroblasts was performed.

4.3 Materials and Methods

4.3.1 Materials

4.3.1.1 Hair

Natural African black curly human hair samples were provided by International Hair Importers & Products Inc. (New York) and previous to use, a washing procedure with a commercial shampoo was performed.

4.3.1.2 Peptide

Keratin peptide (KP) used in this work was synthesized by JPT Peptide Technologies GmbH (Germany). The peptide was developed based on the amino-acid sequence of cuticular keratin type II from human hair (Naeem et al. 2006) and possess a sequence with thirteen (13) amino-acids comprising two (2) cysteines residues (TAMRA-X_3**C**X_5**C**X_3-$CONH_2$) with a molecular weight of 1599.8 g/mol. The characteristics of this peptide were presented in the previous chapters.

The peptide was either dissolved in an aqueous solution (0.05 M Phosphate buffer solution, ph 7.5) (WF) or in an organic solvent formulation (OF). In this case, a 10% ethanol, 1.5% propylene glycol, and 0.5% benzyl alcohol mixture in 0.05 M Phosphate buffer solution, pH 7.5 was added to the peptide. Concentrations from 0.025 g/L to 0.5 g/L of peptide were prepared (Table 4.1). The sequence of the peptides is described by one letter code in which X represents one of known amino acid residues, with the exception of cysteine residue which is represented by the letter C.

Table 4.1 - Peptide concentration (KP) and conditions used in the cytotoxicity (Alamar Blue) and genotoxicity assay

	Concentration KP
WF Phosphate buffer	0 (negative control) 0.025 g/L 0.05 g/L 0.1 g/L 0.2 g/L 0.5 g/L
OF 10 % ethanol 1,5% propilenglicol 0,5% benzylalcohol	0 (negative control) 0.025 g/L 0.05 g/L 0.1 g/L 0.2 g/L 0.5 g/L

4.3.2 Methods

4.3.2.1 Hair relaxing treatment

The relaxing treatment in black African hair was carried out by breaking and reforming the disulphide bonds through the rearrangement of internal protein structure of curly hair. Hair tresses of 0.2 g were placed in contact with a 0.75 M sodium hydroxide (NaOH) solution for 30 min. The high alkalinity (pH=12-14) swells the hair and opens the cuticle, allowing the alkaline agent to penetrate the hair fibre and diffuse into the cortex. The NaOH reacts with the keratin protein, breaking the structural disulphide bonds in the hair. The hair was then extended to a straight configuration by the use of a comb. The alkaline agent was then washed out from hair, and a solution of 0.1M acetic acid was used as a neutralizing agent to initiate the reformation of new disulphide crosslinks and to close

down the cuticle. These new bonds lock and the hair new shape is created. The neutralizing agent was then washed out using a commercial shampoo.

4.3.2.2 Application of peptide on relaxed hair

Hair relaxed samples (0.2 g) were treated with 3 mL of water formulation (WF) 0.05 M Phosphate buffer solution pH 7.5; or with 3mL of organic solvent formulation (OF) in the presence of KP at final concentration of 0.2 mg/mL. The treatment was carried out at 37 ºC in a bath with orbital agitation, 100 rpm for 1 h. After treatment, the hair samples were washed with commercial shampoo and under running water. As control, the hair was treated in the same way, omitting the peptide in the solution.

4.3.2.3 Mechanical properties

The effect of peptide application on both WF and OF buffers was assessed by the differences on the mechanical properties. The method used broadly follows the guidelines laid down in ASTM D1445-95 for the tensile testing of fibres. The measurements were performed with an Instron 4505 tensile tester with a maximum load cell capacity of 2.5N. For each measurement, 10 single hair fibres were taken randomly from the tress. Each hair was individually mounted in the tensile jig by means of a paper device with a fixed gauge length of 20mm. Before the test begins the paper device was slashed. The measurements were performed under controlled conditions (20±0.5ºC; 55±4% humidity), at a rate of 1.5 mm/min, until breakage occurred. For each hair, records of applied load against extension were taken and using as average mean diameter of 70 µm, the data were converted to stress (load/unit area) against strain (% extension).

4.3.2.4 Differential Scanning Calorimetry (DSC)

To study the thermal and chemical characterisitics of hair fibres, a DSC study was performed. All investigations were conducted on a power-compensated DSC instrument (DSC-7, Perkin Elmer) using pressure-resistant (25 bar), stainless steel, large-volume capsules in the temperature range of 50-250ºC (heating rate: 5 C/min, sample weight: 7-8 mg). The DSC device was calibrated using indium and palmitic acid, both of high purity.

The samples were stored at selected levels of humidity (RH of 45%) and temperature (20-22°C) for 24 h prior to the analyses and each hair sample was measured at least three times, in order to validate the results.

4.3.3 Peptide safety assessment

Different peptide concentrations in WF or OF buffers (Table 4.1) were filtered in a sterile environment and, subsequently, diluted (100%, 10% (v/v)) in complete culture medium. The culture medium itself was used as a negative control, whereas a 200 µM solution of the oxidant *tert*-butyl hydroperoxide (*t*-BOOH) (Sigma) prepared in fresh culture medium was used as a toxicity positive control.

4.3.3.1 Culture of human skin fibroblasts cell line (BJ5TA)

The BJ5TA cell line (normal human skin fibroblasts) was maintained according to ATCC recommendations (4 parts Dulbecco's BJ5TA Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 1 part of Medium 199, supplemented with 10% (v/v) of fetal bovine serum (FBS), 1% (v/v) of Penicillin/Streptomycin solution and 10 µg/ml hygromycin B). The cells were maintained at 37 C in a humidified atmosphere of 5% CO_2. Culture medium was refreshed every 2 days.

4.3.3.2 Cytotoxicity evaluation: Alamar blue assay

Cells were seeded at a density of $20x10^3$ cells/100 µl/well on 96-well tissue culture polystyrene (TCPS) plates (TPP, Switzerland) the day before experiments and then exposed to the peptide and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cells were examined at 24, 48 and 72 hours for signs of toxicity, using AlamarBlue assay (alamarBlue® Cell Viability Reagent, Invitrogen). Resazurin, the active ingredient of alamarBlue® reagent, is a non-toxic, cell permeable compound that is blue in colour and reduced to resorufin, red colour compound, by viable cells. 10 µL of alamarBlue® reagent were added to each well containing 100 µL of culture medium. After 4 h of incubation at 37 °C the absorbance at 570 nm was measured, using 600 nm as a reference wavelength, in a microplate reader (Spectramax 340PC). The quantity of resorufin formed is directly proportional to the number of viable cells. The procedure was performed at least 3 times and the average values are presented.

4.3.3.3 Cell morphology

Morphological changes in cells were also followed by phase contrast microscopy (IX71, Olympus), after 1h and 72h of contact with peptide.

4.3.3.4 Genotoxicity evaluation: Comet assay

One day before use, fibroblasts were seeded at 40,000 cells/ml for the 72 h genotoxicity assays, and at 100,000 cells/ml for the 1h genotoxicity assays. After treatment, cells were harvested by trypsinization and used for the comet assay. The alkaline version of the comet assay was performed as previously described (Lima et al., 2006). In brief, 40,000 cells were embbebed in 1% w/v low melting point agarose and spread onto agarose-coated slides. Slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 with NaOH, Triton X- 100 1% v/v added fresh) at 4ºC for 2 h. Then slides were immersed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) at 4ºC and incubated for 40 min for alkaline unwinding of DNA. Afterwards, electrophoresis was performed for 20 min, 300 mA, at 0.8 V/cm in a cold room (4ºC). Finally, the slides were neutralized by washing them 3 times for 5 min each with 0.4 M Tris, pH 7.5, at 4ºC. For the analysis of the comet images, slides were stained with SYBR Gold (Invitrogen) and images taken under a fluorescent microscope (IX71, Olympus). Images were then analyzed using a free comet scoring software Comet Score (TriTek Corp.).

4.3.4 Data analysis

Several samples were used for each measurement and all the values are presented as averages ± the respective standard deviations (SD) or standard error of mean (SEM). Results were analyzed using Graph Pad Prism version 5.04 for Windows (Graph Pad Software, San Diego, CA). Statistical significances were determined using a one-way ANOVA followed by the Dunnett post-hoc test or by the unpaired two tailed Student's t test method. P values ≤ 0.05 were considered statistically significant.

4.4 Results and discussion

4.4.1 Recovery of mechanical and thermal properties

KP, a peptide derived from keratin intermediate filaments, was previously shown to recover the mechanical and thermal properties of over-bleached blond hair (Chapter 4). For that reason, we have used the same peptide to study its ability to recover the mechanical resistance of weakened relaxed African hair. Young's modulus and Tensile Strength were the parameters analysed to determine the hair's resistance. The Young's modulus, also known as the tensile modulus, is a measure of stiffness of an isotropic elastic material, applied only in the elastic (Hookean) region of a stress/strain curve (Kitano et al. 2009; Druhala et al. 1971; Erik et al. 2008; Nikiforidis et al. 1992). It is a measure of elasticity and refers to a fibre's capacity to recover its original configuration after being stretched (Druhala et al. 1971). The tensile strength is the measure of the maximum point of strength required to stretch the fibre (Druhala et al. 1971; Erik et al. 2008). It is of great importance to evaluate these parameters on hair subjected to a chemical relaxing treatment, due to the harsh conditions employed and consequent damage imparted on hair. This technique is often used in cosmetic to permanently straight curly hair and use strong bases, such as sodium hydroxide, to disrupt the disulphide bonds that are responsible for the hair shape and rearranging them in order to make it straight. It is an effective treatment but very aggressive to the hair fibre. During the process of relaxing, one third of the disulphide bonds are modified permanently to lanthionine bonds, which decrease the elasticity and tensile strength along with cuticular damage at the surface of the hair (Syed et al. 1998). Indeed, the measurements of relaxed hair fibres resistance lead us to the conclusion that relaxing treatment induces 35% of resistance loss when compared to virgin, unaltered hair, measured in terms of Young's modulus. The maximum strength that hair holds also decreased around 36% (Figure 4.1).

Knowing that KP was able to increase the mechanical resistance and thermal properties of over-bleached hair to similar values of untreated virgin hair (Chapter 4), the ability of this peptide to improve the relaxed hair properties was evaluated.

The application of KP on hair was tested in two different environments, WF and OF. The reason for using this organic solvent formulation is that, despite this peptide is soluble in aqueous solutions, it possess a certain hydrophobicity

(ratio of hydrophilicity = -0,4)[3]. Hydrophobic peptides are usually applied together with a formulation comprising a surfactant such as DPPC or an organic solvent such as ethanol. This formulations are added to attain a peptide formulation compatible with a water environment (Silva et al. 2010), thus improving the interaction with biological membranes. Using the same approach, we have used a mixture of 10% ethanol 1.5% propylene glycol and 0.5% benzyl alcohol mixture in 0.05 M Phosphate buffer solution, pH 7.5. It is thought that peptide might possess different conformation when in presence of OF, facilitating its penetration.

KP treatment imparted an increase on mechanical hair properties when it was applied either in WF or OF environment. The resistance that was lost by the relaxing treatment was partially regained after KP treatment (Figure 4.1). When peptide was applied in the presence of OF the hair regained ~28% of Young's modulus and ~13% of maximum strength. No significant differences were observed on the mechanical properties of hair when peptide was applied in WF when compared to OF: an increase of ~31% of Young's modulus and 13% of maximum strength. An increase on the elongation values were also observed (data not shown), however, the statistic program did not validate these results, showing no significant differences.

An interesting result was that application of OF alone, i.e., in the absence of peptide, was able to increase the Young's modulus in ~28%, despite the maximum strength and elongation parameter still low. The Young's modulus parameter is strongly affected by the water content that hair holds. Since the hydrophobic lipid protection layer of damaged hair is depleted, the hydrophilic molecules of the inner cellular structure of hair are exposed to water. Water can adsorb and diffuse easily into hair via the defects on the surface, therefore softening the hair. The hair softening can be translated in an increase of Young's modulus (Bhushan 2008).

In Chapter 4 it has been shown that KP was able to change the chemical environment on over-bleached hair. KP imparted an increase on the temperature and enthalpy of denaturation of α-helix keratin on damaged hair, indicating a higher energy needed to disrupt the keratin α-helix structure. To understand if this peptide is also able to improve the thermal properties of relaxed hair. Similarly to what has been done before, a differential scanning calorimetry (DSC) was also performed on relaxed hair treated with KP in the presence of both formulations.

[3] Calculated with "Peptide Property Calculater" available at www.innovagen.se

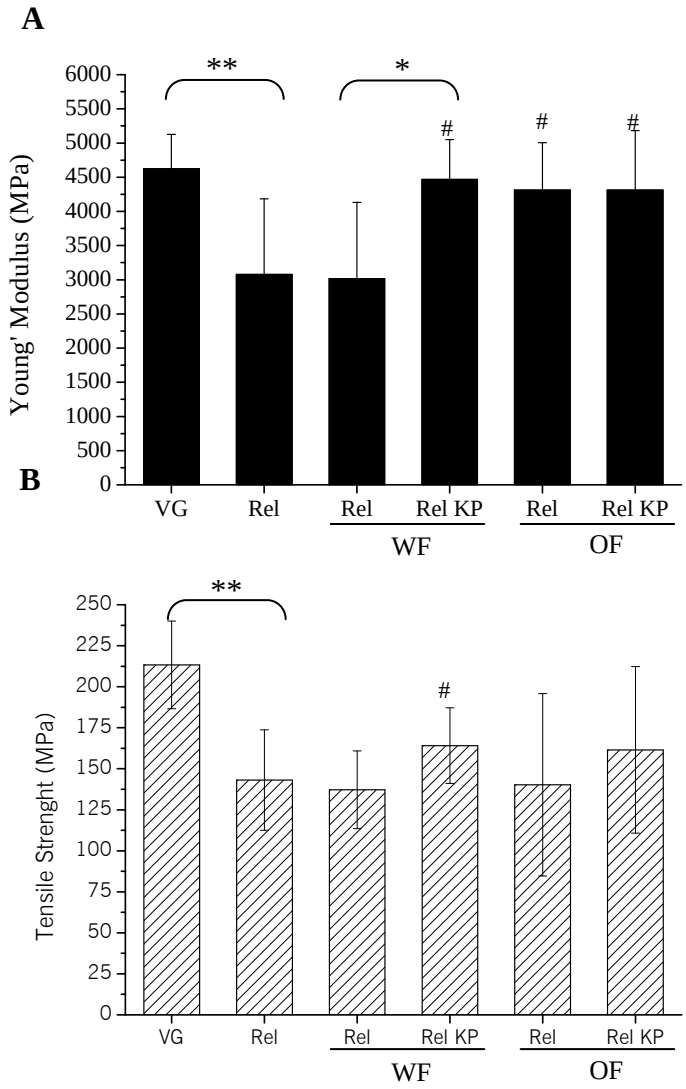


Figure 4.1 - Mechanical resistance parameters: A) Young's modulus, B) Tensile Strength and C) Elongation of unaltered virgin hair (VG) and relaxed black hair (Rel) treated with 0.2 mg/mL keratin peptide (KP) in the presence of aqueous formulation (WF) and organic solvent formulation (OF). Values are mean ± SD (standard deviation) of eight independent experiments. * P≤ 0.05, ** P≤ 0.01 when compared with each other, # P≤ 0.05 when compared with relaxed hair. No significant differences were observed between the other conditions and the relaxed hair. Elongation results do not present statistic significance.

Four main parameters were analyzed by DSC: the denaturation temperature and enthalpy of water removal; and the denaturation temperature and enthalpy of α-helix keratin. The last two parameters are strongly affected by chemical treatments (Wortmann

et al. 2002). All these parameters decreased when hair was subjected to a relaxing treatment indicating a degree of chemically damage on the fibre (Table 4.2). The removal of water temperature dropped around 6ºC, meaning that water is more easily desorbed due to the fibre damage, decreasing also the energy needed for that to happen (Table 4.2). The α-helix denaturation temperature decreased 2ºC and the α-helix denaturation enthalpy dropped from 6,76 J/g to 3,33 J/g. Relaxing treatment break the disulphide bonds and chemically reform them to change the shape of the hair. The environment is therefore more fragile due to the strong chemicals applied, explaining the decrease of thermal properties.

Interestingly, the hair samples treated with OF without peptide, showed an increase in these parameters, corroborating the increment of Young's modulus shown for this sample in Table 4.2. The application of KP improved the thermal characteristics at the same level as without peptide (Table 4.2). As a consequence, one question is raised: are these increments due to the peptide or to the OF formulation?

By the light of previous results, there are no doubts that OF makes a positive influence on the recovery of hair properties, but still lack in recovering the tensile strength and elongation (Figure 4.1), despite the increment on thermal properties. We believe that OF plays an important role on swelling the hair fibre allowing the peptide to penetrate inside the cortex, due to the presence of organic solvents. Inside the fibre cortex it would change the chemical environment reflecting therefore an increase on thermal characteristics results. When KP is present, we believe that the real benefit to relaxed hair emerges. lf KP is able to interact with α-helix in the hair's cortex, as a type II intermediate filament, it would be able to improve the α-helix chemical environment and thus increase the α-helix denaturation enthalpy.

The thermal properties are increased when hair is treated with KP in both WF and OF but higher yield was observed when OF was used. The characteristics of both KP and OF seem to disclose a good approach for recover hair damage. While OF opens the structure of the hair fibre, KP penetrate inside the fibre cortex therefore increasing its mechanical properties (Figure 4.1). As the water removal (ΔHd_{H2O}) and α-helix denaturation enthalpies ($\Delta Hd_{\alpha\text{-helix}}$) are higher than the unaltered virgin hair, we believe that KP may interact on the hair's cortex at the same time that covers the hair's surface, increasing the energy that is needed for the water removal and the degradation of α-helices, respectively.

Table 4.2 - Thermal characteristics of relaxed black hair treated with 0.2 mg/mL keratin peptide (KP) in the presence of aqueous formulation (WF) and organic solvent formulation (OF)

	Td_{H2O} (ºC)	ΔHd_{H2O} (J/g)*	$Td_{\alpha\text{-helix}}$ (ºC)*	$\Delta Hd_{\alpha\text{-helix}}$ (J/g)*
Unaltered hair	92.8 ± 0.8	244 ± 4	229.7 ± 0.1	6.8 ± 0.2
Relaxed hair	87 ± 9	206 ± 49	227.9 ±0.5	3.33 ± 0.05
Relaxed hair (WF)	88 ± 3	207 ± 23	227.2 ± 0.9	3.41 ± 0.8
Relaxed hair (OF)	97 ± 1	267 ± 1	227.9 ± 0.2	4.9 ± 0.1
Relaxed hair-KP (WF)	91 ± 1	262 ± 2	228.9 ± 0.1	4.66 ± 0.03
Relaxed hair-KP (OF)	93 ± 1	270 ± 16	228.2 ± 0.4	4.8 ± 0.3

Data presents mean ± SD (standard deviation) from a minimum of three experiments

4.4.2 Cytotoxicity evaluation

The cytotoxicity of KP in the presence of WF or OF was assessed through the use of BJ5TA cell line cultures (normal human skin fibroblasts). A wide range of peptide concentrations were tested to perform the cytotoxicity evaluation, according to Table 4.1. The concentrations were chose according to the conditions applied on treatment of hair.

Using the same approach of Lee and co workers (Lee et al. 2000) a possible skin irritation potential of peptide in both WF and OSF environments by using Alamar blue assay in cells exposed to peptide was evaluated. Figure 4.2 shows the cell viability of human skin fibroblasts after 72h of incubation with KP in WF and OF. The cell viability at 24h and 48h time points was also evaluated (data not shown), and the same behaviour of 72h was observed.

When peptide is applied in the presence of WF, cell viability was close to 100% after 72h indicating that this formulation is not toxic and can be in contact with hair scalp (Figure 4.2). No significant changes on cell viability were observed between the different concentrations of peptide tested, which means that at these concentrations the peptide is not toxic and may not cause skin irritation.

However, when the peptide is in presence of OF, less cell viability is observed. We believe that this toxicity is due to the OF environment and not due to the peptide since there is no significant differences between the peptide concentrations in OF and the negative control (OF alone) (Figure 4.2).

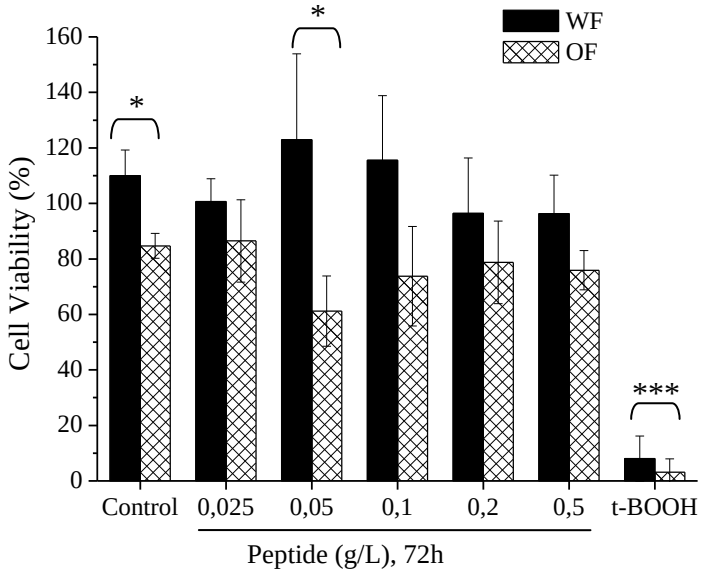


Figure 4.2 - Viability of human normal skin fibroblasts cells after 72h of contact with conditioned peptide (KP). Values are mean ± SD (standard deviation) of four independent experiments. * P≤ 0.05, when compared with each other, *** P≤ 0.001 when compared with respective control. No significant differences were observed between each concentration of peptide and the respective control.

4.4.3 Cells morphology evaluation

In order to visualize the impact of the peptide formulations on cells, the morphological changes in cells was followed by phase contrast microscopy after 1h and 72h of contact with peptide. When fibroblasts were incubated with peptide dissolved in WF for 1 h, no cytotoxicity was observed (Figure 4.3). However, OF induced significant cell morphological damages, i.e. cells detach and round up, with or without peptide (Figure 4.3). The positive control, the oxidant tert-butyl hydroperoxide (t-BOOH), induced cell morphological damages both in the presence of phosphate buffer or alcohol formulation (data not shown).

To observe if these damages to cells have long lasting effects, an experiment with 72 h of incubation was performed. As observed in Figure 4.3, when cells were incubated in the presence of WF, with or without peptide, cells presented normal morphology and grew continuously along the time, as observed by the increased cell density along the time. Peptide did not perturb cell growth.

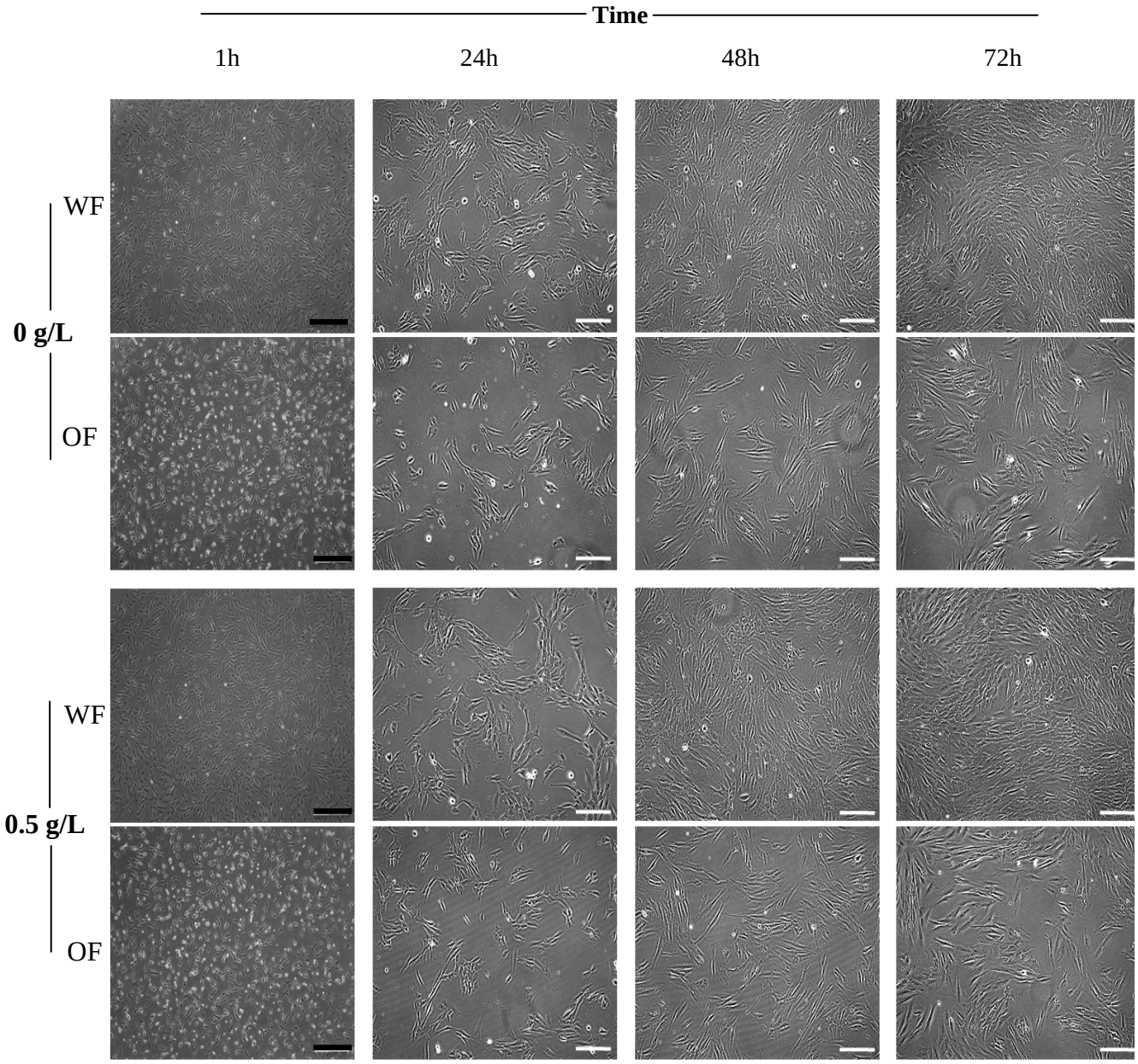


Figure 4.3 - Effect of peptide on aqueous (WF) and organic solvent (OF) formulation on human fibroblasts after 1 h and during 72h of incubation. Photos are of a representative experiment from a total of four independent experiments. Black bar indicates 500 μm while white bar indicates 200 μm.

However, when cells were incubated with OF, cell density was smaller than the one observed with WF after 24 h of incubation. In addition, some cells were floating and others morphologically affected (stressed), indicating induction of cell damage and death by OF. Nevertheless, some cells appear to resist better to OF after 24 h of incubation. Along the time of exposure with OF, although cells growth slightly and with improved morphology, probably derived from resistant cells, cell densities are still much lesser than the ones incubated with WF (Figure 4.3, 48 h & 72 h of incubation). Peptide did not improve or worsen the damaging effects of OF in cells until the highest concentration tested.

The decrease on cell viability observed when KP is in OF formulation (Figure 4.2) can thus be explained by the OF capacity of inhibiting cell growth.

4.4.4 Genotoxicity evaluation

Damage to DNA have strong cellular implications and is involved in the stop of cell cycle, in the induction of cell death and/or in the increase of DNA mutation rate and carcinogenesis. The single cell gel electrophoresis, or comet assay, is a sensitive, quick and simple method for evaluating DNA damage in single cells, and commonly used nowadays in genotoxic studies. After embedding cells in agarose and quick and simple method for evaluating DNA damage in single cells and commonly used nowadays in genotoxic studies.

After embedding cells in agarose and following cell lysis, and alkaline unwinding of the DNA, the negatively charged DNA is pulled towards the anode. However, only loops of DNA that contain breaks are able to migrate in the gel from the supercoiled DNA (head) forming the comet tail (Collins et al. 2008). The percentage of DNA that is in tail, evaluated under fluorescence microscopy and using image analysis programs, is a reliable and one of the most used parameters to express DNA damage (Collins et al. 2008).

The potential genotoxicity of peptide was evaluated by the comet assay exposing human fibroblasts to the peptide for a short (1 h) and prolonged (72 h) period of time. This methodology allows discriminating among short and direct damage to DNA that can be or not then repaired, and prolonged DNA damage including crosslinking of DNA with DNA and protein.

Cells were used for the comet assay to evaluate DNA damage. As shown in Figure 4.4, peptide did not induce DNA damage until the highest concentration tested (0.5 g/L).

However, OF induced significant DNA damage and this was not dependent on peptide. Damage induced by OF was not, however, significant to almost every cell contrarily to what happen to the positive control. In this case, there was heterogeneity on cell damage between cell populations. That can also be observed in a representative image taken from the comet assay (Figure 4.5, 1h). Taken together, we observed that some cells are more prone to DNA damage than others in the presence of OF for 1 h.

After 72 h of incubation, cells were used for the comet assay to evaluate DNA damage. As shown in Figure 4.4, peptide in WF did not induce DNA damage until the highest concentration tested (0.5 g/L). OF still induced significant DNA damage and does not seem dependent on peptide. However, the extent of damage was remarkably smaller than the one observed after 1 h of incubation (Figure 4.4), indicating that most of the cells after 72 h of incubation repaired DNA damage or are resistant cells. The carcinogenic compound N-methy-N-nitrosourea (MNU) used as positive control induced significant DNA damage (Figure 4.4) after 72 h of incubation. The overall DNA damage after 72 h of incubation can also be observed in a representative image taken from the comet assay (Figure 4.5).

Comet images after 72 h of incubation with peptide and/or alcohol formulation did not show DNA condensation when compared with controls (Figure 4.5), which indicates no significant DNA-DNA and/or DNA-protein crosslinking induced both by peptide and OF. In addition, after 72 h of incubation of cells with peptide and/or OF did not prevent extensive DNA damage induced by the oxidant t-BOOH when compared with controls (data not shown), confirming therefore that DNA crosslinking are not at place.

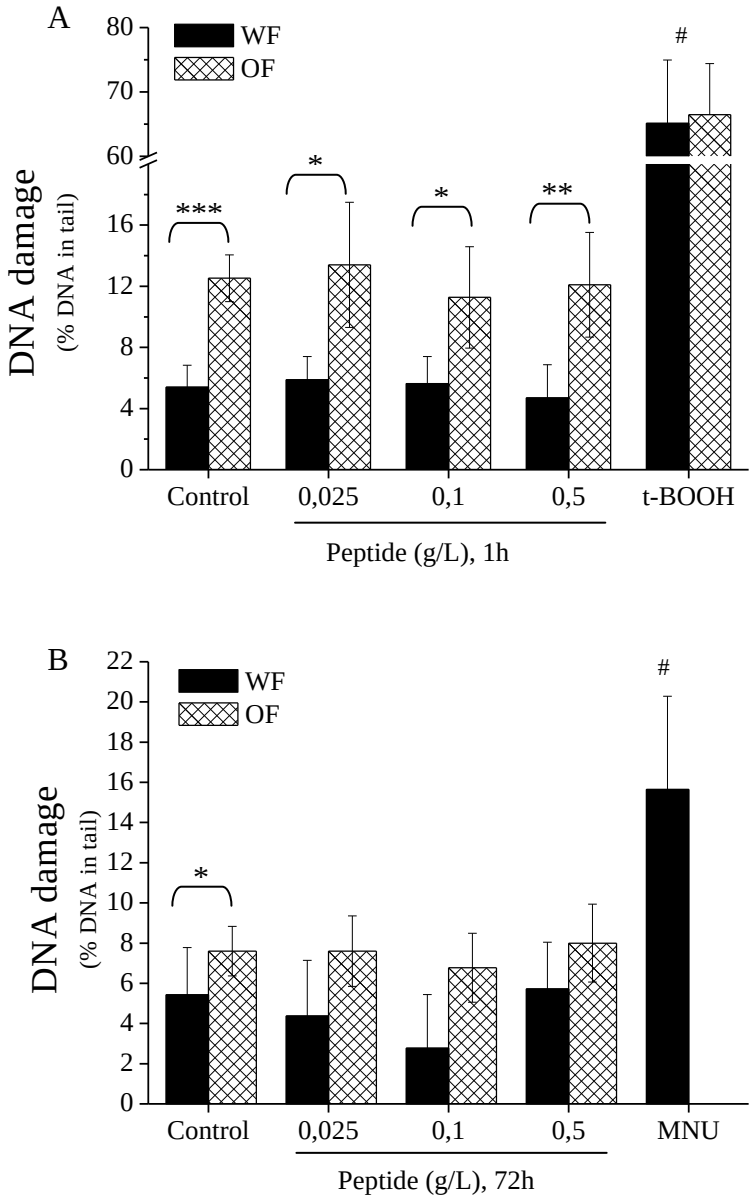


Figure 4.4 - Effect of peptide and solvent formulation on DNA damage, expressed by the % of DNA in the tail of the comet (values are the average of 100 cells counted per condition), of human fibroblasts after A) 1h and B) 72h of incubation, measured by comet assay. *tert*-Butyl hydroperoxyde (*t*-BOOH) was used as positive control for 1h evaluation and N-methy-N-nitrosourea (MNU) for 72h evaluation. Values are mean ± SD of four independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ when compared with each other. $^{\#}$ $P \leq 0.05$ when compared with the respective control. No significant differences were observed between each concentration of peptide and the respective control.

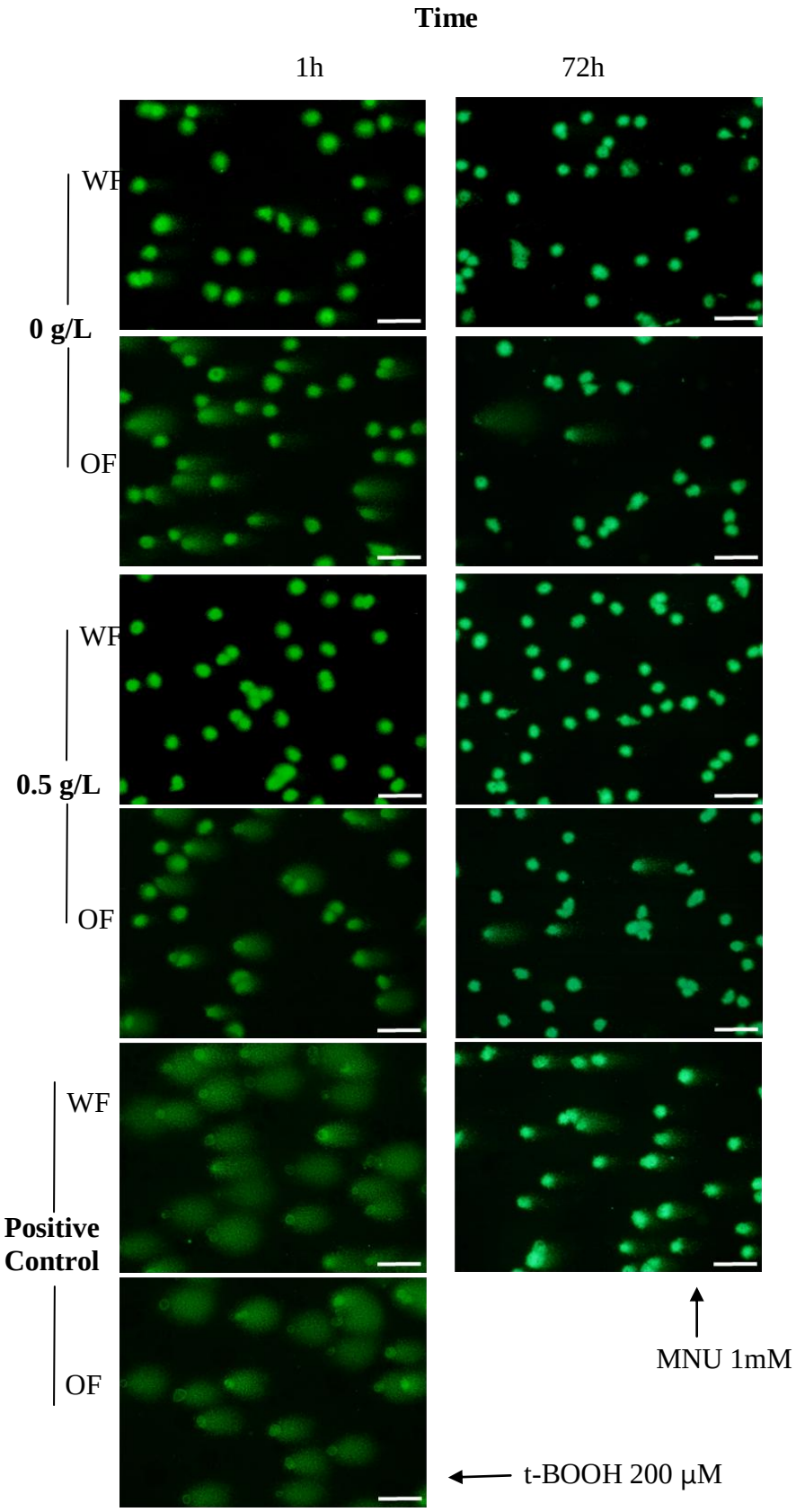


Figure 4.5 - Effect of peptide in aqueous (WF) and organic solvent (OF) formulation on DNA damage of human fibroblasts after 1h and 72h of incubation. Photos are representative of a comet assay experiment from a total of four independent experiments. Bar indicates 100 µm.

4.5 Conclusions

KP was able to improve the physical (mechanical) and chemical (thermal) properties of relaxed hair. The peptide in the presence of organic solvent formulation (OF) was shown to possess higher ability to recover these properties, indicating that it would constitute a good approach for the application of peptide. Since this formulation is to be applied on human hair and therefore is in contact with human scalp, a study of its cytotoxicity and genotoxicity was performed. The risk assessment was then valuated and peptide was found to be non-cytotoxic. In the presence of OF less cell viability and a inhibition of cell growth was observed while in the presence of aqueous formulation (WF) around 100% of cell viability and normal cell growth was observed. These results indicated that OF may induce skin irritation. The peptide in WF, on the other hand, did not show toxicity, meaning that this peptide can be safely applied with all the concentrations tested and will not induce skin irritation. The observations made by comet assay corroborate the previous results.

The peptide was found to be non-cytotoxic, it does not inhibit cell growth nor induce DNA damage or DNA crosslinking in human foreskin fibroblasts in culture up to a concentration of 0.5 g/L and 72 h of exposure. The OF formulation, used to dissolve peptide inhibits cell growth, is cytotoxic and genotoxic, but does not induce DNA crosslinking in human foreskin fibroblasts.

Despite the fact that KP together with OF promotes the highest recover of weakened hair properties, it seems to induce certain toxicity on cells. Conversely, the KP in WF does not induce toxicity or genotoxicity and is also able to recover the properties of damaged hair. This formulation is therefore an excellent candidate for a new hair-care product development.

Chapter 5

Protein Disulphide Isomerase-induced refolding of RNase A microspheres

5 Protein Disulphide Isomerase-induced refolding of RNase A microspheres

5.1 Abstract

The results presented in Chapter 2 and 3, in which PDI was able to functionalize cysteine-containing substrates led to the belief that it would be able to functionalize proteinaceous particles such as Ribonuclease A microspheres. Therefore, the application of PDI as a new methodology to recover the biological function of protein-based microspheres was studied. The microspheres were developed by application of high-intensity ultrasound on a two-phase RNase A aqueous-dodecane system. Particles with sizes with less than 1 µm and negative charges (-20 ± 3 mV) were found. The sonopreparation induced no changes on the secondary structure of the RNase A microspheres, despite the fact that partial reduction of disulphide bonds seems to have occurred upon ultrasound application. This suggested, however, that protein tertiary structure might be destroyed. During microspheres formation, RNase A lost 35 % of enzymatic activity. Application of PDI in a redox environment that promotes the oxidation of PDI active site (GSSG/GSH; ΔE= -180mV) was then able to fully recover the lost activity. The use of another redox buffer with stronger reductive power (GSSH/GSH; ΔE= -260mV) or DTT_{ox}/DTT_{red}, along with PDI, promoted the release of the active RNase A protein into solution in its native state.

This chapter is based on the following publication:

Margarida M Fernandes, Raquel Silva, Helena Ferreira, Ilaria Donelli, Giuliano Freddi, Artur Cavaco-Paulo, *Protein Disulphide Isomerase-induced refolding of RNase A microspheres*, Biotechnology Journal, 2011, submitted.

5.2 Introduction

Protein disulphide isomerase (PDI) is an enzyme involved in the correct folding of many proteins *in vivo*. It is expressed on endoplasmic reticulum on cell and possesses three major activities: reduction, oxidation, and isomerisation of disulphide bonds in protein substrates. The PDI's active site contain two cysteines that can either form an intramolecular disulphide (oxidized PDI) which mediate the oxidase activity, or exist in the dithiol form (reduced PDI) which mediate the isomerase or reductase activity. To become functionally active, a protein chain must fold into a unique three-dimensional structure, the native fold, and PDI is thought to be able to induce these changes upon unfolded proteins. This enzyme recognizes an unknown but large number of cysteine-containing substrates including a multitude of native like, partially unfolded and non native states (Wilkinson et al. 2004; Lyles et al. 1991; Gilbert et al. 1991; Freedman 1995). The first studies involving PDI was based on the study of its ability to catalyze the oxidation and folding of reduced Ribonuclease A (RNase A) to its native conformation (Goldberger et al. 1963; Venetianer 1963). Since then, the oxidative refolding of RNase A has been thoroughly investigated and had led to the postulation of the thermodynamic hypothesis of protein folding, which states that the folded form of a protein represents the minimum of its free energy (Anfinsen 1973). In fact, PDI is thought to induce the state of lower energy on reduced RNase A promoting therefore the proper refolding that leads to the native state.

RNase A is a well-characterized protein of 124 amino acids with a molecular weight of 13.7 KDa (Berisio et al. 2002). With several high-affinity binding sites, RNase A is a possible target for many organic and inorganic molecules (Neira et al. 1999; Leonidas et al. 2003). As an interesting approach, various forms of this protein, including its oligomers, have been demonstrated for antitumor and other biological activities (Matousek et al. 2003; Fu et al. 2004; Soucek et al. 1999).

In this study, RNase A microspheres was developed in order to study the ability of PDI to refold proteins stabilized in the form of microspheres. Several techniques have been reported for protein microspheres generation, among which the ultrasound technique presents a low-cost with high efficiency method for microspheres production. Early in 1990, Suslick and co-workers (1990) have used this method for the synthesis of proteinaceous microspheres filled with air or water insoluble liquids.

This one-step procedure usually yields microspheres at high concentrations with high stability and long shelf life (Gedanken 2008). To prove this stability concept, enzyme-

based microspheres developed by ultrasound technique have been reported. These studies have shown that the particles prepared by ultrasonication are still catalytically active and the spherization process, unlike denaturation in which the protein biological activity is destroyed, reduces its biological activity but does not destroy the enzyme active sites (Avivi et al. 2007, 2005). Additionally, the lower surface densities reached after microspheres preparation are thought to lead to a decrease, but not complete loss of protein biological activity (Fei et al. 2009). For example, studies indicated that the environment surrounding the active heme site in hemoglobin is not altered significantly during the microsphere formation process (Gedanken 2008; W. Wong 1995), proving that sonication do not lead to denaturation. However, the attempt to restore the lost biological activity of enzyme based microspheres through conformational changes has not been reported so far.

Herein, RNase A microspheres were prepared and their biological activity was assessed and compared to the native enzyme activity. The PDI ability to refold the protein microspheres was then studied. This possibility raised from the results shown in Chapter 2, where PDI was able to catalyze the functionalization of keratin-based matrices with cysteine-containing compounds (CCC) by modulation of PDI active site (Fernandes et al. 2011). Likewise, in this work, the conditions that lead to different states of PDI active site were tested in order to induce the activity of PDI upon spherical non native protein states, which has not been studied so far. Also, to date, RNase A microspheres were only prepared by miniemulsion polymerization method (Tan et al. 2006) and their development by ultrasound application have never been reported in literature. Therefore, the ultrasound prepared RNase A microspheres, together with the ability of PDI to recover their biological function are novel approaches.

The microspheres formed were investigated for the thiol group content using Ellman's reagent method. The size and polydispersity index of the microspheres were evaluated using the photon-correlation spectroscopy (PCS) and the zeta-potential were measured using Laser Doppler Anemometry (LDA). The morphology was studied using scanning electron microscopy (SEM) and the study of the possible conformational changes induced by ultrasound was made by FTIR spectra analysis.

5.3 Materials and methods

5.3.1 Materials

Ribonuclease A from bovine pancreas (RNase A), protein disulphide isomerise from bovine liver (PDI), L-glutathione reduced (GSH), L-glutathione oxidized (GSSG), dithiothreitol (DTT) as well as all other reagents were purchased from Sigma Aldrich. Stock solutions of native RNase A (1.0 mg/mL), hydrated freeze-dried RNase A microspheres (0.1 mg/mL) and PDI (2.5 μg/mL) were prepared in 0.1 M phosphate buffer, pH 7.5.

An equilibrium mixture of GSH and GSSG was made and two different redox buffers were obtained: 1 mM GSH and 0.2 mM GSSG to promote a potential redox of ΔE= -260 mV, and 0.1 mM GSH and 1 mM GSSG to promote a potential redox of ΔE= -180 mV. The redox buffer DTT, in its turn, was composed by DTT at final concentration of 100 mM in 0.1 M phosphate buffer, pH 7.5.

5.3.2 Methods

5.3.2.1 Microspheres preparation

The microspheres were prepared by an adaptation of Suslick method (Suslick et al. 1990). Briefly, a two phase solution containing 60 % of 0.1 g/L RNase A aqueous solution and 40 % of *n*-dodecane (organic phase) was prepared. The bottom of the high-intensity ultrasonic horn was positioned at the aqueous-organic interface and amplitude of 40 % with a temperature of 10 ºC (±1 ºC) and with a total treatment of 3 min, was applied. To obtain a more complete separation of the microspheres from the mother solution, the flasks were placed in refrigerator (4 ºC) for 24 h.

Microspheres emulsion were collected by centrifugation (3000 g, 30 min) using the centricon tubes (Amicon Ultra-15), a centrifugal filter unit with a cellulose membrane with a molecular weight cut-off of 100 kDa, that allows the free protein to pass through the pores and separate from the protein microspheres. Dry microspheres, in their turn, were obtained by a freeze drying process. First, the microspheres were frozen at -20 ºC for 48 h and the freeze drying procedure was made using a LabConco FreeZone 2.5 Liter Benchtop

Freeze Dry System at a pressure of approximately 0.03 mbar and a condenser temperature of -50 ºC for 48 h.

5.3.2.2 Microspheres formation efficiency

The efficiency of the microspheres formation was assessed by the quantification of the free protein in the aqueous medium after microspheres separation. The protein quantification was made via Lowry method (Lowry et al. 1951), using BSA as standard and using Sigma test kit nº P 5656. The efficiency of microspheres formation was calculated as follows:

$$\text{Microspheres formation } (\%) = \frac{[C]_i - [C]_f}{[C]_i} \times 100$$

where $[C]_i$ and $[C]_f$ are the initial and final concentration of protein in the aqueous solution, respectively.

5.3.2.3 Size, polydispersity and zeta potential

Malvern zetasizer NS (Malvern Instruments) equipment was used to determine the microspheres' size distribution and polydispersity index, using photon-correlation spectroscopy and zeta-potential, using laser doppler anemometry technique. In order to verify the reproducibility of the proteinaceous microspheres preparation assembled under the typical process described above, the procedure was carried out at least three times for each of the individual microspheres. The instrument was routinely calibrated with a ± 66 mV latex standard.

5.3.2.4 Light microscopy

The morphology of both emulsion and hydrated freeze dried microspheres was analysed on a Leica Microsystems DM-5000B light microscope using a 100× oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

5.3.2.5 FT-IR Spectroscopy

Infrared spectra were recorded at room temperature using a Perkin Elmer Spectrum 100 infrared spectrophotometer from 4000 cm^{-1} to 500 cm^{-1}. To eliminate spectral contributions due to atmospheric water vapor, the instrument was continuously purged with dry air. After mixing, the RNase A microspheres emulsion were quickly transferred to a potassium bromide (KBr) Liquid Omni Windows support cell in a Omni Cell Assembly accessory, specially designed for liquid solutions. In the case of native RNase A protein, approximately 2 mg of protein was mixed with 200 mg of KBr and a pellet was formed by pressing with a Specac KBr 13 mm Die Kit and a Specac 10-ton hydraulic press. All samples were measured at least three times.

Deconvolution of amide I band region was performed using OriginPro 8.5 software. The number of components and their peak position were determined from the second derivative spectrum of the same region. 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. For all data, a linear baseline was fitted and a smoothing of 15 pt with Savitzky-Golay method was applied.

5.3.2.6 Determination of thiol (-SH) group content

The amount of free thiol groups in RNase A (native and microspheres) was determined spectrophotometrically using 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent). Initially, 250 µL of native RNase A (5 mg/mL) or RNase A microspheres solution (5 mg/mL) were added to 2.5 mL of 0.5 M phosphate buffer (pH 8.0) and 50 µL of Ellman's reagent solution (4 mg/mL). The samples were vortexed and incubated for 1 h at room temperature in the dark. The absorbance was measured at a wavelength of 412 nm (Ellman 1959).

To determine the total amount of thiol groups, the reaction with Ellman's reagent was performed after complete reduction of disulphide bonds with sodium borohydride ($NaBH_4$): 350 µL of native or microspheres sample solutions (5 mg/mL) was added to 150 µL of 0.05 M Tris buffer (pH 6.8) and to 1.0 mL of a freshly prepared 4 % (w/v in 0.2 M NaOH) $NaBH_4$ solution. The samples were incubated for 1 h in an oscillating waterbath at 37 ± 0.5 ºC. Thereafter, the remaining $NaBH_4$ was inactivated by addition of 200 µL of 5 M HCl and agitating for 10 min. The pH of the reaction mixture was adjusted to 8.0 with 2 mL of 1 M phosphate buffer (pH 8.0). The thiol groups were then measured by adding 100

µL of Ellman's reagent (4 mg/mL) on these samples and incubating 15 min at room temperature. L-cysteine reagent standards were used in both methods to calculate the amount of thiol groups.

The disulphide bond present on protein and microspheres were calculated by subtracting the free thiol groups present after complete reduction with $NaBH_4$ from the initial free thiol groups. The protein of each RNase A protein solution (native and microspheres) was quantified by Lowry method (Lowry et al. 1951), using BSA as standard and using Sigma test kit nº P 5656.

5.3.2.7 Activity assay on native RNase A and RNase A microspheres

The activity of hydrated freeze dried RNase A microspheres and native RNase A was measured spectrophotometrically in a procedure based on the PDI oxidative folding of RNase A (Lyles et al. 1991). Activities were determined at 25 ºC using cytidine 2':3'-cyclic monophosphate (cCMP) as a substrate. Assay mixtures were composed of 0.1 M phosphate buffer (pH 7.5), 4.5 mM of cCMP (final concentration) and 250 µL of RNase A solution, native or microspheres. The reaction was followed at 296 nm in a quartz cuvette of 0.1 cm path length for 10 min. The slope that results from the linear increase on absorbance was calculated and activity was determined using Beer Law, where ε is the cCMP molar extinction coefficient (190 $M^{-1}cm^{-1}$):

$$\text{Activity} \,.\, L^{-1} = \text{Slope} \times V_{\text{Total}} \times \frac{1}{\varepsilon} \times \frac{1}{V_{\text{Sample}}} \times 10^{3}$$

5.3.2.8 PDI induced refolding of RNase A microspheres:

The activity was also determined in presence of 8 µM PDI with a redox buffer of GSSG/GSH (ΔE= -180 mV) buffer at pH 7.5, 0.05 M phosphate buffer, and together with the two redox buffers: GSSG/GSH (ΔE= -260 mV) and DTT_{ox}/DTT_{red} buffer. The last two buffers are able to promote the reduction of the active centre of PDI, which is believed to promote the rapid oxidative folding *in vitro* (Lyles et al. 1991).

The dry microspheres (10 mg) were diluted in a solution containing 500 µL of PDI (8 µM PDI final concentration) and 9.5 mL of 0.05 M phosphate buffer pH 7.5. Then the solutions were vigorously mixed and incubated at room temperature. Samples of 250 µL were taken from this solution at 0, 5, 20, 40, 60 and 80 min and the RNase A activity by the procedure above mentioned, and protein content was then measured.

On the other hand, the refolding process on microspheres emulsion was initiated by adding 500 µL of PDI (8 µM PDI final concentration) and 9.5 mL of both GSSH/GSH and DTT redox buffers to 1 mL of microspheres emulsion. The microspheres were vigorously mixed and then centrifuged at 3000 g for 10 min in order to separate microspheres phase. Samples of 250 µL were taken from the aqueous phase at 0, 20, 40 and 60 min and the protein content on the aqueous separated phase was then measured.

Figure 5.1 schematically shows the experimental set up performed in the quest to accomplish the goal of this study.

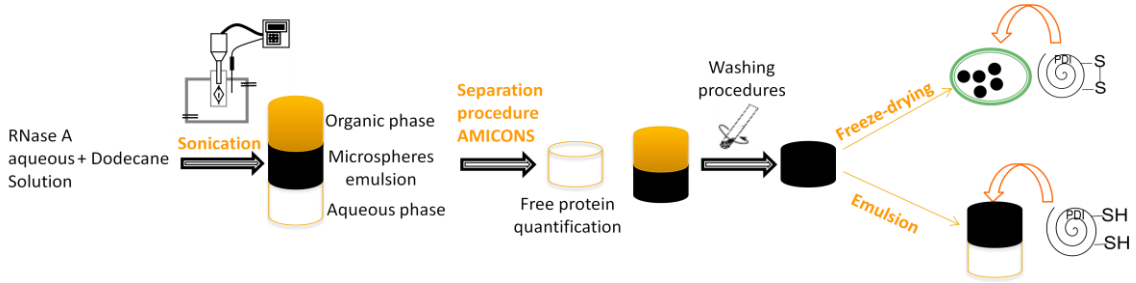


Figure 5.1 - Experimental set up used to measure the PDI-assisted refolding of RNase microspheres.

5.3.3 Results and discussion

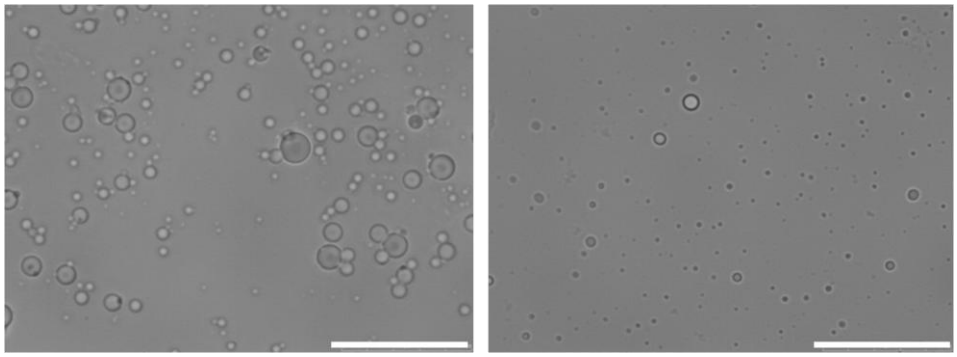
5.3.3.1 RNase A microspheres characterization

A group of parameters were determined to characterize the sonochemically prepared RNase A microspheres samples: morphology, particle size, polidispersity index, zeta-potential, efficiency of microspheres formation and thiol group content.

The size distribution of RNase A microspheres ranged from 400 nm to 1000 nm presenting a high polidispersity index (near 1). Bigger sizes of these particles were clearly observed while studying its morphology by light microscopy (Figure 5.2). The images demonstrate the spherical shape of the particles and that freeze drying process does not compromise that morphology.

The surface charge of the microspheres was also analyzed in terms of zeta-potential values. The zeta-potential represents the overall surface charge that is present at the surface of microspheres. This parameter can also be related with the microspheres stability because a more pronounced zeta potential value, either positive or negative, favours the particle-suspension stability (Riddick 1968). Therefore, the RNase A microspheres were found to be reasonably stable due to the significant negative zeta potential value presented (-20 ± 3

mV). This value also indicates that microspheres have affinity to positively charged particles/surfaces.



A B

Figure 5.2 - Light microscope images of proteinaceous RNase A microspheres: A) particles emulsion and B) hydrated freeze dried particles. Bar represents 25 µm.

The microspheres development method yielded a high degree of microspheres formation. More than 84 % of the protein was calculated to be transformed in microspheres, measured by the free protein in aqueous soltution (Figure 5.1).

The free thiol groups content were also analysed. A comparison was made between native protein and microspheres. There are evidences that in microspheres more thiol groups are present (Table 5.1). The particles development seems to partially open the protein structure by disrupting some of the disulphide bonds. Therefore, there are more thiol groups bonds when RNase A is in the form of microspheres (2.3 µM/mg of protein) rather in its native form(0.14 µM/mg of protein).

Table 5.1 - Quantification of thiols (-SH) in the form of free thiol groups and sulphur in the form of disulphide bonds on native and RNase A microspheres

	Free thiol -SH groups µM/mg of protein	**Disulphide S-S groups µM/mg of protein**
RNase A microspheres	2.3 ± 0.2	21.6 ± 2.4
Native RNase A	0.14 ± 0.06	23.5 ± 1.1

5.3.3.2 Conformational assessment analysis of proteinaceous microspheres

To characterize the microspheres in terms of structural conformation, Fourier Transform Infrared (FTIR) spectroscopy measurements were made on RNase A microspheres. FTIR is one of the techniques which have recently become very popular for structural characterization of proteins. This is due to the presence of characteristic bands such as amide I, amide II and amide III in the infrared spectra of proteins and polypeptides. These bands arise from the peptide bonds that link the amino acids. The absorption associated with the amide I band leads to stretching vibrations of the C=O bond of the amide, while absorption associated with the amide II and III bands leads primarily to bending vibrations of the N-H bond (Haris et al. 1999).

Depicted in Figure 5.3 is the infrared spectrum of amide I and II region of native protein and RNase A microspheres. It can be seen that native RNase A infrared spectra shows two peaks corresponding to amide I and amide II bands, at 1600-1700 cm^{-1} and 1510-1580 cm^{-1}, respectively, while in the form of microspheres the amide II band disappears. This might indicate that a conformational change have occurred upon ultrasound application on the occasion of microspheres formation.

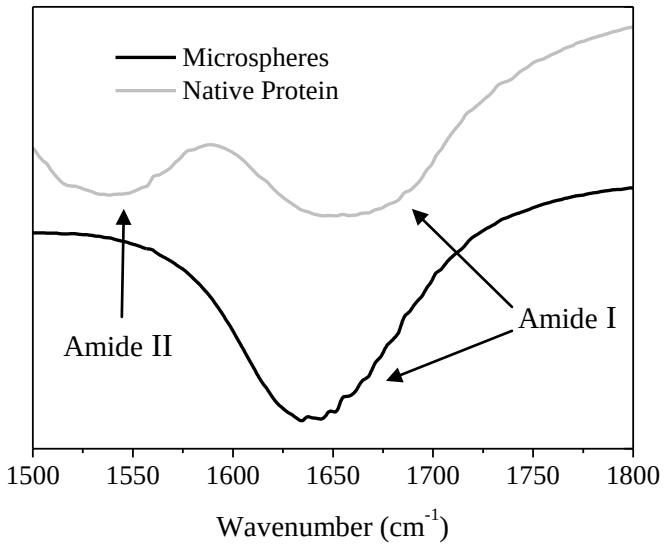


Figure 5.3 - Infrared spectra of amide I and amide II region of RNase A microspheres and native RNase A.

A deeper study on amide I band was then performed to characterize microspheres' secondary structure. Studies with proteins of known structure have been used to correlate

systematically the shape of the amide I band to secondary structure providing a powerful tool in the analysis of the secondary structure of proteins in aqueous solutions (Byler et al. 1986). The amide I band of proteins typically consists of many overlapping component bands that represent different structural elements such as α-helices, β-sheets, turns and non-ordered or irregular structures. Therefore, the individual component bands cannot be resolved in the experimental spectra. The Fourier deconvolution procedure, sometimes referred to as 'resolution enhancement' involves narrowing the widths of infrared bands, allowing increased separation of the overlapping components present within the broad band envelope (Byler et al. 1986; Kong et al. 2005).

A quantitative analysis of the secondary structure of native and RNase A microspheres in water is given in Table 5.2. The native protein contained major β-structure 38.9 %, followed by turn structure 34.8 % and α-helix 26.3 %. These results are consistent with other FTIR spectroscopic studies made on native RNase A protein that indicates 38 % of β-sheet and 28 % α-helix (Goormaghtigh et al. 1990) or 38% of β-sheet and 20% of α-helix (Seshadri et al. 1994).

After microspheres development, some differences were observed; the β-sheet structure was increased from 38.9 % to 42.3 %, whereas the α-helix decreased from 26.3 % to 20.2 %. It should be noted, however, that the molecular conformation is essentially maintained. The observed spectral changes are consistent with the perturbations of the RNase A secondary structure by the microspheres formation, also observed by the difference in the number of thiol groups in the microspheres.

Table 5.2 - Percentage of α-helix, β-sheets and other structures by deconvolution of amide I bands RNase A native and RNAse A microspheres

	RNase A native	**RNase A microspheres**
β-sheets	38.9 %	42.3 %
α-helix	26.3 %	20.2 %
Others (unordered)	34.8 %	37.5 %

5.3.3.3 RNase A microspheres enzymatic activity

The enzymatic activity of RNase A microspheres was carried out in the presence of its substrate, cCMP. Previously they were subjected to a freeze-drying process in order to

assist the accurate conditions for the biological activity measurement. For the determination of protein activity it is important to determine the catalytic ratio between the enzyme and substrate (Biochemistry-USPark et al. 2003). It is, therefore, more accurate to predict the protein content on RNase A microspheres if they are in the dry state rather than as emulsion. Moreover, the freeze-drying technique is a dehydration process that avoids loss of activity and other damages (Matejtschuk 2007). It was however, important to prove that lyophilisation process did not compromise the microspheres' morphology. For that, light microscope images were taken on both suspension and freeze dried RNase A microspheres. The images reveal that microspheres maintain their sphere-like morphology after the freeze-drying process (Figure 5.2).

By carrying out the RNase A's substrate hydrolysis the biological activity and the concentration of catalytically active RNase A that is present was determined. Hence, by measuring the catalytic activity on native RNase A and on freeze dried RNase A microspheres, it was found that the activity of microspheres is ~ 65 % when compared to the native RNase A, losing nearly 35 % of its activity (Table 5.1).

The lower density reached after microspheres formation can be a reason for these activity loss. The active sites of the enzymes' microspheres might be buried and, in this case, the interaction with the reactants is more difficult and slows down the reaction (Avivi et al. 2007), inhibiting therefore the enzymatic process.

The ultrasound application induced a loss in protein activity but it didn't destroy the secondary structure of the protein. We believe that this indicate that protein conformation might be changed due to the presence of mixed non-active and native disulphide bonds, together with disrupted disulphide bonds (free thiol groups). For a protein that contain eight cysteine residues such as RNase A, scission or intra and inter molecular arrangements of disulphides bonds could occur upon ultrasound application (Lyles et al. 1991). This is in agreement with the presence of higher quantity of free thiol groups on microspheres when compared with the native protein (Table 5.1). On the other hand, we also believe that disruption of disulphide bonds may also affect the protein tertiary structure, which is stabilized by disulphide bonds, among others (Wedemeyer et al. 2000; Welker et al. 2001; Rothwarf et al. 1998). These conformational changes could explain the disappearance of the amide II band on FTIR spectra (Figure 5.3).

Considering that ultrasound only disrupt some of the disulphide bonds in the RNase A protein, it is expected that PDI will be able to recover the activity lost during sonication.

This is due to its ability to act upon disulphide bonds in a wide range of substrates, as previously proved by our group (Fernandes et al. 2011).

Table 5.3 - Activity and protein quantification of freeze-dried RNase A microspheres and native RNase A

RNase A	Activity U/mg
Freeze-dried microspheres	3.56 ± 0.02
Native	5.49 ± 0.04

5.3.3.4 Refolding of RNase A microspheres

The PDI ability to catalyze the oxidation of thiol groups and the isomerisation of non-native disulphide bonds, in appropriate redox conditions, was used to refold RNase A microspheres towards the native state. The presence of an equilibrium concentrations of oxidized and reduced glutathione, [GSH] = 0.1 mM and [GSSG] = 1.0 mM, was applied together with PDI. These conditions promoted a redox environment with a potential of ΔE = -180 mV, which is reported as the optimum condition for PDI oxidase activity (Chivers et al. 1997). Redox buffers typically consist of a small molecule thiol and a small molecule disulphide that besides facilitating the formation of the correct disulphide bonds on proteins incorrectly folded, when added to PDI are crucial to obtain the active protein (Gough et al. 2005b).

Figure 5.4 shows the biological activity of RNase microspheres (freeze-dried) along time in the presence of three different environments: PDI-GSSG/GSH (ΔE = -180 mV), the control redox buffer GSSG/GSH (ΔE = -180 mV), and the control in phosphate buffer. The microspheres activity was recovered at the same yield in both catalyzed and non-catalyzed refolding. The presence of PDI increased the activity of microspheres to 5.18 U/mg, in approximately the same extent of the redox buffer (5.22 U/mg; Table 5.3). When compared with the native protein activity (5.49 U/mg; Table 5.1), it can be stated that both conditions are able to fully recover the microspheres activity. However, the initial velocity of RNase A microspheres refolding is higher when PDI is present. We believe that PDI provides a faster and alternative pathway for microspheres refolding, thus indicating catalytic ability of PDI to act on RNase A microspheres (Table 5.4).

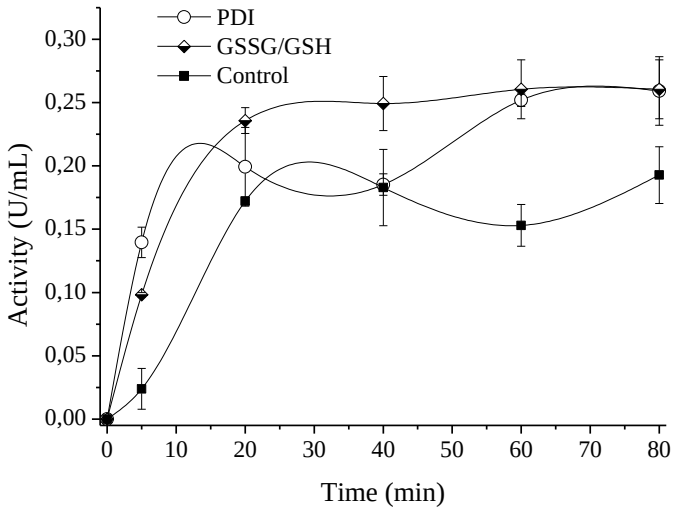


Figure 5.4 - Time-course reaction of the oxidative folding of RNase A lyophilized microspheres in the presence of: 1) PDI with GSSG/GSH buffer, 2) GSSG/GSH buffer and the 3) control with 0,1 M Phosphate buffer pH 7.5.

In fact, the role of the redox buffers in protein folding was reported as essential in the formation of folding intermediates by increasing the rate of thiol-disulphide interchange reactions involved in protein folding (Ono et al. 2005; Gough et al. 2005b). Alone, the redox buffer (GSSG/GSH) was able to alter the distribution of the non-native disulphide bonds and promote the oxidation of thiol groups found in RNase A microspheres, inducing the most stable protein configuration, and leading to the maximum of biological activity similarly to what happen in the presence of PDI. On the other hand, these results corroborate the findings that the structural conformation of microspheres does not undergo major changes otherwise the same behaviour on catalyzed and non-catalyzed reaction should not be observed.

Table 5.4 - Initial velocities of RNase A microspheres folding and biological activity after 80 minutes, in the presence and absence of protein disulphide isomerase (PDI)

Conditions	**V_0 (µM/min)**	**Final activity (U/mg)**
PDI with GSSG/GSH	0.197	5.18 ± 0.03
GSSG/GSH	0.157	5.22 ± 0.02
Control	0.129	3.86 ± 0.02

The microspheres in the presence of water (control) are also able to refold in certain extent (3.86 U/mg), which is in good agreement with results shown in Table 5.3

where microspheres are still catalytic active after sonication process, although loosing around 35 % of activity (3.56 U/mg).

The novelty appeared when the PDI-assisted refolding of microspheres was tested in microspheres emulsion. The refolding tests were performed in the presence of two redox buffers: GSSG/GSH (ΔE= -260 mV) and DTT_{ox} /DTT_{red}. These redox buffers were applied in microspheres emulsion to create the appropriate redox environment for the reduction of PDI active site accordingly to equations (1) and (2).

$$PDI_{ox} + GSH \rightleftharpoons PDI_{red} + GSSG \quad (1)$$

$$PDI_{ox} + DTT_{red} \rightleftharpoons PDI_{red} + DTT_{ox} \quad (2)$$

The conditions applied in equation (1) have been reported as optimum for promoting the renaturation of RNase A through the isomerisation of non-native disulphide bonds, by the application of a redox potential of ΔE= -260 mV (Lyles et al. 1991). The refolding activity on microspheres was controlled by the quantification of active RNase A that was released to the aqueous medium after the addition of the PDI in the presence of both redox buffers, as Figure 5.1 depicts. For that, the protein release over time and the final RNase A activity was quantified and compared with the controls without PDI.

A tendency of increasing the protein content on aqueous phase is shown when PDI is applied in the presence of both redox buffers (Figure 5.5). The absence of PDI however, did not promote an increase on protein content, expressing the failure of redox buffers alone in promoting refolding in this case. The PDI-refolding mechanism in the presence of DTT redox buffer seems to induce more protein release, probably due to the most reducing environment promoted by this buffer. It is thought, however, that PDI and its reducing environment might promote the degradation of microspheres, rather than the refolding of the protein, also explaining the protein release. The final activity measurements show that the released protein was in its active form when PDI was applied with GSSG/GSH buffer and in non native form when PDI is applied with DTT (Figure 5.5). These results indicate that PDI promote the protein release into aqueous medium through two different mechanisms: by refolding when GSSG/GSH redox buffer is used; or by degradation when DTT is present.

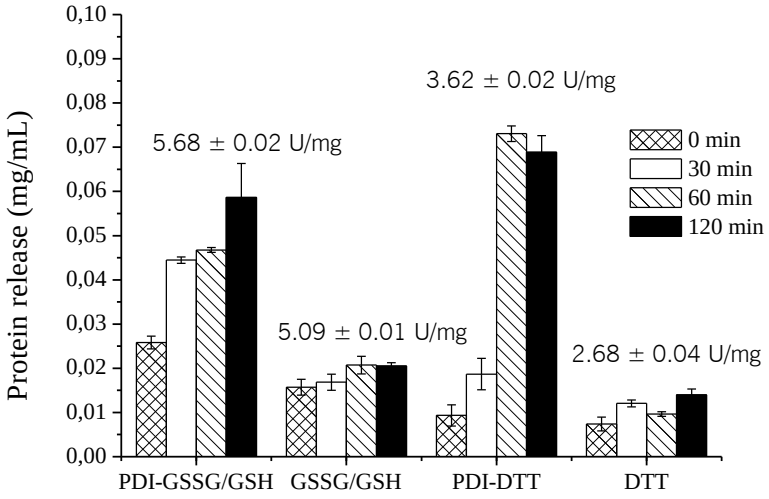


Figure 5.5 - RNase A release from microspheres emulsion on aqueous phase after treatment with: 1) PDI with GSSG/GSH buffer, 2) GSSG/GSH buffer, 3) PDI with DTT and 4) DTT, and final protein activity.

In the case of refolding, the microspheres are probably opening and acquiring the native state conformation by a PDI-induced structural change. These achievements can be further studied for the possibility of microspheres to open by the mentioned refolding mechanism, in the presence of an appropriate buffer and PDI, for protein delivery purposes.

Involved in the correct folding of proteins are oxidation and isomerisation reactions (Xiao et al. 2005). Through the application of an appropriate redox environment, PDI is thought to catalyze the oxidative renaturation of reduced/denatured RNase A, by controlling the redox state of the PDI active site (Darby et al. 1995).

Figure 5.6 synthesizes the reactions that are the base for PDI-assisted refolding of microspheres. The active site should be on the oxidized form in order to assist the oxidation of thiol groups, and in the reduced form to assist the isomerisation of non-active microspheres' disulphide bonds. These reactions resulted in the recovery of microspheres' biological function of and on protein release, respectively.

The oxidation pathway of thiol groups in microspheres catalyzed by PDI, leading them to an oxidized state, is depicted in Figure 5.6 A. The application of a redox potential of -180 mV promotes oxidized state of PDI active site, which then reacts with the

microspheres' thiols groups, explaining the increase in activity and the faster initial reaction of RNase A microspheres when PDI is applied.

On the other hand, an isomerisation is thought to be the reaction involved on the release of proteins from microspheres in the aqueous medium (Figure 5.6 B). When PDI active site is reduced, due to a redox environment of -260 mV or lower, the low pK_a property of the N-terminus cysteine, allows it to act as nucleophile. As a consequence, the isomerisation initiates by attacking a substrate disulphide followed by several intramolecular rearrangements within microspheres itself. The mechanism that regulates the PDI microspheres' refolding is probably dependent on the disulphide bonds' ability to open and close in an equilibrium medium. We believe that manipulation of PDI active site is essential to define the reactions that occur on protein substrates.

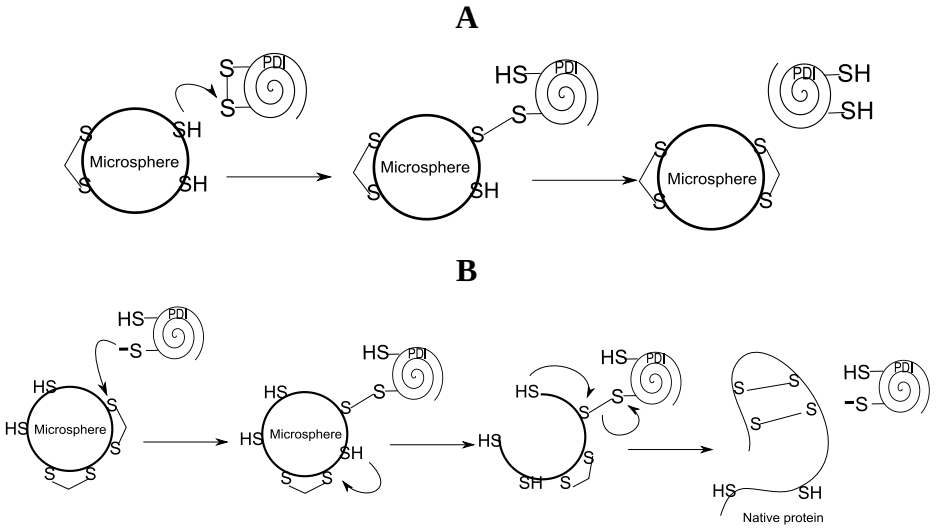


Figure 5.6 - Schematic representations of the PDI-assisted formation/scission of the disulphides in RNase microspheres. Nucleophilic attack is indicated with a continuous curved arrow. (A) Representation of PDI-assisted oxidation of a thiol groups in microspheres. (B) Representation of PDI-assisted oxidative refolding of RNase A microspheres.

The protein oxidative folding is a complex process that is not yet fully understood, however, it is known that it is intimately associated to the stability of protein disulphide bonds. *In vitro* refolding of inactive proteins is one of the major challenges in genetic and protein engineering and the protein folding pathway is still case of many hypotheses (Freedman 1995). While debating its driven force, numerous studies agree in the fact that disulphide bonds play one of the most important roles on folding process (Witt 2008).

Disulphide bonds are thought to control the conformational flexibility on unfolded proteins, decreasing its conformational entropy and then favouring the protein folded state (Bulaj 2005; Jungbauer et al. 2007). Indeed, both mechanism shown for PDI-assisted refolding on RNase A microspheres is thought to involve proper disulphide bonds formation by creating a highly optimized environment that lead microspheres to a more stable state.

5.3.4 Conclusion

This work demonstrates that ultrasound application is a useful method for the preparation of RNase A microspheres without destroying the enzymatic activity, only reducing it in 35 %. The loss of the enzymatic activity is probably due to alteration in protein tertiary structure by ultrasound. Nevertheless, the secondary structure was conserved and not involved in microspheres development.

The application of PDI in presence of GSSG/GSH (ΔE = -180 mV) restored the lost activity. The ability of PDI to act upon protein substrates was demonstrated for recovering the biological function of proteinaceous microspheres.

On the other hand, the study performed on RNase A microspheres emulsion suggested that PDI in presence of GSSG/GSH (ΔE = -260 mV) or DTT_{ox}/DTT_{red} was able to induce the release of the active protein into aqueous medium by refolding or degradation mechanism, respectively.

In conclusion, the PDI application on proteinaceous microspheres formed by ultrasound showed potential in recovery of biological function of proteins or can serve as a system for controlled release.

Chapter 6

General discussion and future perspectives

6 General discussions and future perspectives

The catalytic role of protein disulphide isomerase (PDI) in the oxidation or isomerisation of protein disulphide bonds *in vivo* has been and continues to be the subject of many studies. However, this enzyme has been sparsely reported as a tool for modification of proteinaceous substrates. The functionalization of keratinous fibres, namely hair and wool, by PDI-assisted grafting of specially designed cysteine-containing molecules through disulphide bond formation, was successfully performed. Moreover, the PDI-assisted rearrangement of the disulphides in the proteinaceous (RNase A) microspheres was also presented, with the possibility of using such system in protein delivery applications.

This work can thus be divided into two main parts: 1) PDI functionalization of keratinous fibres and 2) PDI functionalization of proteinaceous microspheres. The summary of the main achievements and general conclusions are presented bellow:

1) PDI functionalization of keratinous fibres

The main characteristic feature of keratin fibres is the high content of cysteine residues, which impart them great mechanical resistance. Taking advantage of these disulphide bonds was the strategy used to functionalize keratin fibres, for several purposes, from chemical compound delivery to fibres dyeing or reinforcement.

In a first approach, the PDI-assisted functionalization of **wool fabric** was made in order to incorporate a CC-dye or to assist the release of a model protein from the wool fabric's surface.

The incorporation of the CC-dye containing cysteine residue was proved by MALDI-TOF analysis, performed after digestion of wool by protease. Higher molecular weight fragments observed in the samples of the wool dyed in the presence of PDI suggested the disulphide bonding might have occurred, since proteases do not cleave disulphide bonds. Therefore, the cysteine residues of wool hypothetically linked to the cysteine group from the dye would remain intact and not hydrolysed by proteases. The reason for the disulphide bond formation by PDI lies in the intrinsic redox potential (E^o = -180 mV) used in this study, that stabilises the oxidised form of the enzyme active site, promoting catalysis of the oxidation reaction. The redox potential of PDI is higher

than the redox potential of cystine/cysteine (E^o = -340 mV) which, in this study, favoured the oxidation of cysteine residues between dye and wool.

The reversible and at the same time stable nature of disulphide bonds together with properties such as high mechanical strength of wool were advantageously used to develop a system that uses PDI as an agent for the release of a protein model (namely RNase A). Regenerated keratin has been used as biomaterial for controlled release purposes, however, applied in this form loses its characteristic high mechanical strength. The approach of using native keratin materials as biomaterial is desirable not only due to its good mechanical properties, but also due to its biocompatibility and biodegradability.

The reduced form of RNase A was successfully attached on the wool fabric by disulphide bonds formation (confirmed by FTIR analysis), using a redox buffer (GSSG/GSH) with the oxidizing power of ΔE= -228 mV. PDI was then able to promote the release of around 40 % of the attached protein from the wool, and more important the released enzyme conserved its activity. The release was due to the PDI reduced active site, achieved by the application of the environment with the redox potential of ΔE= -260 mV. The reduction and/or the isomerisation of disulphide bonds (thiol/disulphide interchange reactions) are thought to be the reason for the protein release, as explained in Chapter 2.

In a second approach, PDI was used to functionalize **human hair**. The linkages between the hair and the engineered cysteine-containing peptides were created by the application of PDI in the redox environment that promotes the PDI oxidative activity (E^o = -180 mV). The peptides used were synthesised based on the amino-acid sequences of a cuticular keratin (KP) and a human surfactant protein (SP), and were meant for the restoration of the hair damaged parts and for the recognition and interaction with hair lipids for increased penetration inside hair, respectively.

The first studies conducted with KP were performed in reduced hair. The reducing treatment was performed to set hair's thiol groups available for further PDI application. PDI promoted the covalent attachment of a CC-peptide onto the cortex of reduced hair, demonstrated by fluorescence microscopy on hair transversal cuts. The thiol groups formed by the reduction treatment performed on hair played a key role on the efficiency of PDI-assisted incorporation. Further application of KP and SPB was carried out on over-bleached hair. PDI-assisted formation of new disulphide bonds between hair and peptides was proved by determination of total thiol groups on hair treated with both peptides in the presence and absence of PDI. A loss of 42% of mechanical resistance (Young's modulus) was observed when hair was subjected to the bleaching process. Both peptides were shown

to recover both mechanical and thermal properties of this damaged hair. KP induced an increment of 112% of Young's modulus parameter and 102% of tensile strength. SPB also increased these parameters in 93% and 43%.

The successful attachment of the peptides onto hair, assisted by PDI, may constitute a promising approach for the treatment of damaged hair. Products that use milder methods of attaching compounds on its surface or penetrating into its cortex, with simultaneously increasing the fibre robustness creates new perspectives for cosmetic industry.

1.1) Application of KP on relaxed hair (absence of PDI)

Due to the great properties imparted by one of the peptides applied on hair, the keratin peptide (KP), a different part of the thesis was created (Chapter 4). This part was devoted to the study of KP application on damaged relaxed hair as well as the evaluation of its cytotoxic and genotoxic potential. Two different peptide formulations were applied: KP in water solution formulation (WF) and KP in organic solvent formulation (OF). Similarly to what happened in Chapter 3, KP was able promote the mechanical and thermal properties of relaxed damaged hair in the presence of both formulations. Interestingly, KP in OF induced a higher recovery of the hair's mechanical and thermal properties. However, this formulation was shown to be cytotoxic and genotoxic in all range of peptide's concentrations, inhibiting cell growth. The peptide in WF, however, was not cytotoxic and did not inhibit cell growth nor induce DNA damage or DNA crosslinking in human skin fibroblasts in culture up to the concentration of 0.5 g/L and 72 h of continuous exposure.

2) PDI functionalization of protein microspheres

Ribonuclease A is a small and well characterized protein that contains 8 cysteine residues connected to form 4 disulphide bonds. Being the classic substrate of PDI, RNase A was involved on the first studies of PDI ability to refold non-native proteins towards the native configuration (Goldberger et al. 1963; Venetianer 1963). In this thesis RNase A was used for the formation of enzymatic microspheres, based on Suslick method for microspheres generation through application of ultrasound (Suslick et al. 1990)

A full characterization of RNase A microspheres indicated that they possess spherical shape, particle sizes of less than 1 μm and negative particle surface charge. Surprisingly, the application of the ultrasound was found to induce the cleavage of disulphide bonds in protein, affecting the protein tertiary structure, while no changes in the secondary structure of the protein during microspheres formation was observed.

The partially loss in the enzymatic activity after ultrasound application was recovered by the application of PDI in the oxidative environment (GSSG/GSH; ΔE = -180 mV), using the PDI intrinsic ability to promote the oxidation of substrates with lower redox potential. On the other hand, the application of the appropriate reductive environment (e.g. -260 mV, GSSH/GSH buffer or DTT_{ox}/DTT_{red} solution) promoted the release of the active RNase A protein from the microspheres.

It is thought that the reduction of PDI active site is the key factor for these results, since in that state it would be able to promote the cleavage of disulphide bonds and more probably the thiol/disulphide interchange reactions within the microspheres, leading to the release of the protein. It is worthy to mention that with the application of PDI in both oxidation and reduction environment the recovery of the loss in the enzymatic activity was achieved.

6.1 Future perspectives

The results presented in this thesis highlighted the PDI application as a promising strategy for functionalization of proteinaceous substrates. The successful grafting of the cysteine-containing functional molecules on keratinous substrates broadens the perspectives in the treatment of damaged hair, and in the general cosmetic purposes. However, further risk assessment should be performed before the development of a new hair-care product. Important parameters such as inflammation, induction of oxidative stress on cultured cells, as well as topical application *in vivo* should be performed.

Another perspective is that the presence of PDI through its diverse chemistry could trigger the release of active compounds in the desired site. Recently, PDI was reported to act as an injury response signal that enhances fibrin generation in the wound site via tissue factor activation (Reinhardt et al. 2008). These results, together with the results shown in Chapter 2, shows that PDI not only could be used as a trigger for the active agent release, but also be an active participant in the tissue regeneration processes.

Chapter 7

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