Cosmetic Science

Keratins and lipids in ethnic hair

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Synopsis

Human hair has an important and undeniable relevance in society due to its important role in visual appearance and social communication. Hair is mainly composed of structural proteins, mainly keratin and keratin associated proteins and lipids. Herein, we report a comprehensive study of the content and distribution of the lipids among ethnic hair, African, Asian and Caucasian hair. More interestingly, we also report the study of the interaction between those two main components of hair, specifically, the influence of the hair internal lipids in the structure of the hair keratin. This was achieved by the use of a complete set of analytical tools, such as thin layer chromatography-flame ionization detector, X-ray analysis, molecular dynamics simulation and confocal microscopy. The experimental results indicated different amounts of lipids on ethnic hair compositions and higher percentage of hair internal lipids in African hair. In this type of hair, the axial diffraction of keratin was not observed in X-ray analysis, but after hair lipids removal, the keratin returned to its typical packing arrangement. In molecular dynamic simulation, lipids were shown to intercalate dimers of keratin, changing its structure. From those results, we assume that keratin structure may be influenced by higher concentration of lipids in African hair.

Résumé

Les cheveux humains ont une pertinence importante et indéniable dans la société en raison de leur importantrôle dans l'aspect visuel et la communication sociale. Les cheveux sont principalement composés de protéines de structure, surtoutdes kératines et des protéines kératiniques et des lipides. Nous rapportons une étude approfondie du contenu et de la distribution des lipides dans les cheveux ethniques, Africains, Asiatiques et Caucasiens. Plus intéressant, nous présentons également l'étude de l'interaction entre ces deux composantes principales des cheveux, en particulier l'influence des lipides internes du cheveusur la structure de la kératine des cheveux. Ceci a été réalisé par l'utilisation d'unensemble complet d'outils d'analyse tels que la chromatographie sur couche mince-au détecteur d'ionisation de flamme (FID), l'analyse aux rayons X, la simulation de dynamique moléculaire et de la microscopie confocale. Les résultats expérimentaux indiquent des quantités différentes de lipides dans des compositions capillaires ethniques et des pourcentages plus élevés de lipides internes dans les cheveux africains. Dans ce type de cheveux, la diffraction axiale de la kératine n'a pas été observée dans l'analyse aux rayons X, mais après l'enlèvement des lipides, la kératine revient à sa disposition de compactage typique. La simulation dynamiquemoléculaire montre que les lipides s'intercalent entre les dimères de kératine, changeant ainsi sa structure. A partir de ces résultats, noussupposons que la structure de la kératine peut être influencée par l'augmentation de la concentration de lipides dans les cheveux de type africain.

Introduction

Human hair has an important and undeniable relevance in society due to its value as a communication or signal device, and also protective, sensory and sexual attractiveness functions. Human hair is composed by approximately 90% proteins and 1–9% lipids (dry weight). Hair is constituted by follicle and shaft [1]. The hair shaft, the part of the hair seen above the skin, has a thickness of 50–100 μm. It is composed of three main parts: medulla, cortex and cuticle. The medulla, with a thickness of 5–10 μm, is made from loosely packed cells and is not always present in the hair. The cortex, which is 45–90 μm thick, corresponds to 90% of the total hair mass. It is composed of macrofibrils, constituted by seven to ten tetramers spiral units - the intermediate filaments. The intermediate filaments are surrounded by a matrix, constituted by high sulphur proteins, ultra-high sulphur proteins and high glycine-tyrosine proteins. Those proteins interact with the intermediate filaments through intermolecular disulphide bonds, which provide the high mechanical strength, inertness and rigidity to the keratin fibres [1–3]. The tetramers of intermediate filaments are comprised by anti-parallel coiled-coil dimers of individual filament keratin chains, with low sulphur content [4]. The cuticle, at the surface of the hair, corresponds to a 5 μm thick protective covering consisting of seven to ten superimposed layers of flat overlapping structures [4–7].

Hair lipids are scattered all over the hair fibre and mainly consist of cholesterol esters (ChE), free fatty acids (FFA), cholesterol (Ch), ceramides (Cer) and cholesterol sulphate (ChS). Those can be separated in exogeneous and endogeneous lipids according to their origin, sebaceous glands or hair matrix cells respectively [6].

Considering the virgin human hair, which corresponds to a completely intact and chemically unprocessed hair, human hair

accounts for a large part of the phenotypic variation between different human races. Therefore, it is usually categorized into three major distinct ethnic groups: Asian, Caucasian and African. Looking from the perspective of biological variability, environmental effects and diversity of fibre texture, the amino acid makeup of protein components across ethnic groups is remarkably uniform [2, 8, 9]. Although a considerable amount of data on human hair has been reported, very few data on the influence of lipids on ethnic hair is available. Some studies regarding the lipid composition on human hair have been published [6, 7, 10–14]. However, they do not correlate the ethnicity of the hair and their lipid content and, in those, results were not always consensual, or the differences between ethnicities completely explained [7]. Still, there is one indication that the African hair has more hair lipids compared to other ethnic hairs [12].

Herein, we report for the first time a comprehensive study of lipids content among ethnic hair, their distribution along the different parts of the hair and more interestingly, the influence of the hair internal lipids with the structure of the keratin in the hair, achieved by the use of a complete set of analytical tools, such as thin layer chromatography-flame ionization detector, X-ray analysis and confocal microscopy.

Materials and methods

Materials

Natural European, African and Asiatic virgin human hair samples were provided by International Hair Importers & Products Inc. (New York) and prior to analysis the hair tresses were previously subjected to an extensive rinsing and washing procedure with a commercial shampoo. All chemical were supplied by Sigma Aldrich (Germany), unless otherwise stated.

Methods

Extraction of lipids from ethnic hair: Hair fibres from different hair tresses were randomly collected in the three ethnicities of hair. The external surface lipids were initially removed from the virgin hair surface using a soxhlet extraction either with t-butanol or n-hexane for 4 h [15]. The internal lipids were then removed by extraction with different mixtures of chloroform/methanol (2:1 v/v, 1:1 v/v and 1:2 v/v) for 2 h and 100% methanol overnight, at room temperature and in a stirring system, using the same hair samples. The different extracts of each hair were then evaporated to a volume of approximately 15 mL and dissolved in methanol to a final volume of 50 mL prior to analysis.

Thin Layer Chromatography- Flame Ionization Detector (TLC-FID): The quantification of lipids on ethnic hair was performed using thin layer chromatography (TLC) coupled to automated ionization detection (FID) system (Iatroscan MK-5, Iatron Lab. Inc., Tokyo, Japan). Samples were applied on Silica gel S-III Chromarods using a SES (Nieder-Olm, Germany) 3202/15-01 sample spotter. The determination of lipid content was performed using an optimized TLC/FID protocol [16] using a methodology where rods (in set of 10) were developed using the following mobile phases:
(1) n-Hexane/diethyl ether/formic acid (53:17:0.3, v/v/v) for a distance of 10 cm and analysed by a partial scan of 72% to quantify the non-polar lipids of the samples.
(2) Chloroform/n-hexane/acetone/methanol (55:5:7:3, v/v/v) to 10 cm and another partial scan of 85% to quantify ceramides.

(3) Chloroform/methanol/formic acid (57:12:0.3, v/v/v) to 10 cm and a total scan (100%) to quantify the most polar lipids.

X-ray diffraction of ethnic hair: X-ray diffraction experiments were performed in virgin Asian, Caucasian and African hair samples with lipids and after its extraction from ethnic hair. Diffraction data was collected using a Gemini PX Ultra (Oxford Diffraction) equipped with CuKα radiation (λ=1.54 184Å), a four-circle kappa goniometer and a CCD Detector. Data collection and processing was carried out using CrysAlisPro Software System (Oxford Diffraction). X-ray exposition time was 3000 s for all the experiments.

Molecular Dynamics Simulation: The simulations were performed using GROMACS 4.0.7 [17] package using the Martini force field [18]. The system size was chosen according to the minimum image convention taking into account a cut-off of 1.2 nm. The bonds lengths were constrained with LINCS [19]. Non-bonded interactions were calculated using a twin-range method, with short and long range cut-offs of 0.9 and 1.2 nm respectively. Neighbour searching was carried out up to 1.2 nm and updated every ten steps. A time step of integration of 30 fs was used. A reaction field correction for the electrostatic interactions was applied using a dielectric constant of 15. Pressure control was implemented using the Berendsen barostat [20], with a reference pressure of 1 bar, 3.0 ps of relaxation time and isothermal compressibility of 3.0×10^{-5} bar^{-1}. Temperature control was set using the Berendsen thermostat [20] at 300 K. The keratin, free fatty acids and the water were included in separated heat bath, with temperatures coupling constants of 0.30 ps. Two replica simulations of 480 ns in length were carried out using different initial velocities taken from a Maxwell–Boltzman distribution at 300 K, leading to a total simulation time of 960 ns for each system. The initial structures for the simulations without the lipids were the final structures from the simulations with lipids.

Staining hair lipids with Nile Red: Two different Nile Red formulations were used to incorporate in ethnic hair samples: Nile Red dissolved in t-butanol and in a mixture of chloroform/methanol (1:1 v/v) both in a concentration of 0.1 mg/mL. Ethnic hair tresses were treated with both formulations for 1 h. The hair fibres were embedded into an epoxy resin. Transversal cuts with 15 μm were prepared using a microtome (Microtome Leitz).

Confocal microscopy on hair transversal cuts and 3D reconstruction: Hair cross-sections were analysed on a confocal laser scanning microscopy (CLSM; Olympus IX81) using × 60 amplification. The images were processed using Olympus Fluoview FV1000 software. The images were collected with the same conditions of brightness, time of exposure and gain. The Nile Red fluorescence was examined at the spectral setting of the green fluorescent protein (GFP) (wavelength absorption at 488 nm and emission at 520 nm) and a rhodamine filter (wavelength absorption at 559 nm and emission at 618 nm) for all the images collected. The confocal reconstructions were made using "IMARIS" image analysis software.

Results and discussion

Despite the recent advances in the characterization of hair lipids, little progress has been made in extending our understanding on lipids composition and their distribution among ethnic hair. Herein, a complete set of analytical tools were used to characterize it with the objective to broaden the knowledge related to the lipids role on the different types of hair.

Thin layer chromatography – Flame ionization detector (TLC-FID)

To determine the difference on internal lipids between ethnicities, their extraction from hair was performed and the resulting adducts were analysed using TLC/FID. The external lipids were not analysed due to a possible interference of hair daily care. Three independent experiments were performed for quantitative lipids analysis. Internal hair lipids are mainly constitutive lipids biosynthesized in the hair matrix cells, which includes polar-like components, such as ceramides and cholesterol sulphate and non-polar components [6, 10, 11].

Analysing the overall content of extracted hair lipids among ethnic hair, it could be observed that Asiatic and Caucasian hair possess identical quantity of internal lipids (~ 2.0%), whereas African hair lipids are slightly increased (3.5%) (Table I).

The major lipid classes analysed in ethnic hair were cholesterol ester (ChE), free fatty acid (FFA), fatty alcohol (R-OH), cholesterol (Ch), ceramide (Cer), glucosyl ceramide (GC) and cholesterol sulphate (ChS), as shown in Table I. Analysing the percentage of each lipid component over the Total Lipid analysed it is possible to observe that the three hair types have similar lipid distribution. The major differences are in the cholesterol ester, ceramides and cholesterol sulphate content. African hair is richer in ChE and ChS, but on the other hand, it presents a smaller amount of ceramides. The level on FFA is slightly higher for the Caucasian hair and for cholesterol, a slightly lower amount was found for African hair. Also, regarding the percentage over weight of hair fibre, the content of FFA is higher in African hair. However, the main difference in terms of lipid content is the total amount of lipids extracted: African hair has 1.7 times more internal lipids than the other two ethnicities, which corresponds to around 70% higher internal lipid content.

The most abundant lipids in all the hair types are the free fatty acids and the cholesterol ester. The high percentage of fatty acid in the hair was already expected, as reported in other studies [11, 13], which did not include the analysis of cholesterol esters. Previous studies indicate lower hydration properties in the African hair [9]. The higher percentage of lipids is putatively related to this property, as hydrophobic domains avoid the entrance of water into the hair bulk thus preventing its swelling.

Table I Quantification of internal hair lipids in ethnic hair (percentage over weight of hair fibre,% o.w.f., and percentage over the total lipid analysed): cholesterol ester, free fatty acids, fatty alcohol, cholesterol, ceramide, glucosyl ceramide and cholesterol sulphate, using TLC-FID

Lipids	Asiatic		African		Caucasian	
	% Hair	% Lipids	%Hair	% Lipids	%Hair	% Lipids
ChE	0.20	16.39	0.32	19.05	0.21	14.29
FFA	0.66	54.10	0.92	54.76	0.85	57.82
R-OH	0.04	3.28	0.05	2.98	0.05	3.40
Ch	0.06	4.92	0.06	3.57	0.07	4.76
Cer	0.16	13.11	0.15	8.93	0.15	10.21
GC	0.03	2.46	0.04	2.38	0.03	2.04
ChS	0.07	5.74	0.14	8.33	0.11	7.48
Total Lipid Analysed	1.22		1.68		1.47	
Total Lipid Extracted	2.07		3.48		2.05	

X-ray determination

X-ray diffraction was performed to verify differences in the architecture of the hair filaments. These measurements were performed in both natural and delipided hair samples to determine the lipidic influence on hair structure and to verify which diffractions were exclusively from the lipids. The results are represented in Table II.

The structure formed by the alpha-keratin chains corresponds to a supercoiled coil structure and provides some typical reflections. The reflections 12.3 Å and 24.6 Å are characteristics of the unit length of alpha-keratin, which corresponds to 450Å. Previous studies refer that those molecules are assembled into dimers with a rod-like central part comprised of alpha-helical coils [21–23]. Due to the degree of packing of those helices, authors indicate an axial reflection located at 5.15 Å [22, 24]. In this study, these reflections do not change between samples, with exception of the African hair with lipids, which does not possess any of these reflections or other low order reflections.

The reflections 9.5 Å and 27 Å are related with the lateral packing of the helices, where 9.5 Å was reported to be due to interferences between coiled-coil chains and 27 Å due to a dense lateral of intermediate filaments [22, 25, 26].

The results reveals that there are reflections, which exist in the samples with lipids and do not exist in the samples without lipids (3.7 Å, 4.1 Å, 5.0 Å and 15.0 Å), which indicates that those reflections are characteristics of hair lipids. Besides, in the lipid-rich African hair, no meridian reflections correspondent to keratin were found, indicating that the degree of organization of the alpha-helices is not high enough to generate usable data in the diffraction patterns. Considering the fact that this behaviour is only observed in African hair and that only free lipids were removed in this procedure, we could assume that the excess of internal free hair lipids causes a great interference to the keratin structure, providing some disturbance to the spatial organization of the hair regarding the unit length of alpha-keratin and to the degree of packing of the alpha-helix coils.

Molecular dynamics simulation

It was possible to observe in the X-ray diffraction experiments that in African hair, the keratin structure is somehow different from the keratin found in Asiatic or Caucasian hair, and this difference was assumed to be caused by the higher amount of lipids present in African hair. To better understand the lipidic influence and interaction with the keratin structure, some molecular dynamics simulations were performed.

For these simulations, we used Martini force field [18]. A simplified model of the hair structure was constructed, based on the sequence of natural hair keratins. For the representation of hair lipids, a model of a free fatty acid was used, because this is the major lipids present in all kinds of hair.

The presence of lipids seems to disorganize the ordered structure of keratin, as the lipids have the ability to intercalate a dimer of keratin, as can be visualized in Fig. 1a and Fig. 2a. The disorganization is able to change the X-ray diffraction. Thus, the presence of higher concentration of lipids in the African hair may influence a higher disorganization of the keratin structure. This is a confirmation of the results of X-ray analysis, as there is an absence of those reflections related to the keratin structure, such as the packing of keratin.

Table II Scattering of the ethnic hair fibres before and after lipids' extraction, in Å. (M – Meridian, E – Equator)

| Before lipids' extraction | | | | | | After lipids' extraction | | | | | |
| African | | Asiatic | | Caucasian | | African | | Asiatic | | Caucasian | |
M	E	M	E	M	E	M	E	M	E	M	E
4.1	9.5	5.0	9.5	3.7	9.5	5.15	9.6	5.15	9.5	5.15	9.5
5.0	27	5.15	28	5.0	28	12.3	28	12.3	28	12.3	28
		12.3		5.15		24.6		24.5		24.5	
		15.0		12.3							
		24.6		15.0							
				24.5							
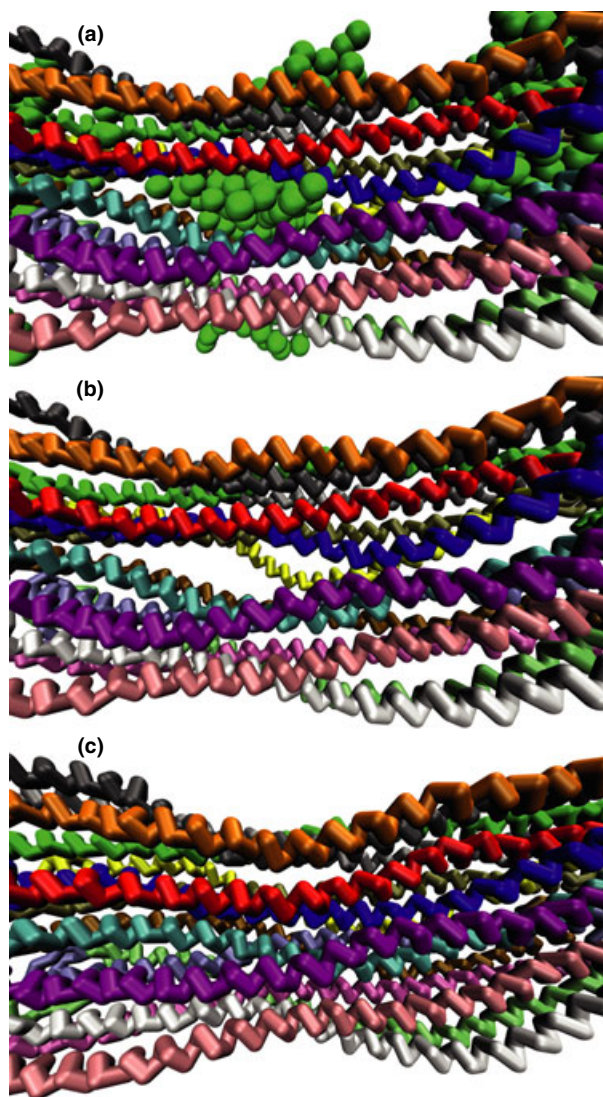
Figure 1 Model of hair keratin using Martini force field: a) and b) with the presence of hair lipids (free fatty acids), represented in a) with the spheres, c) after the removal of the lipids.
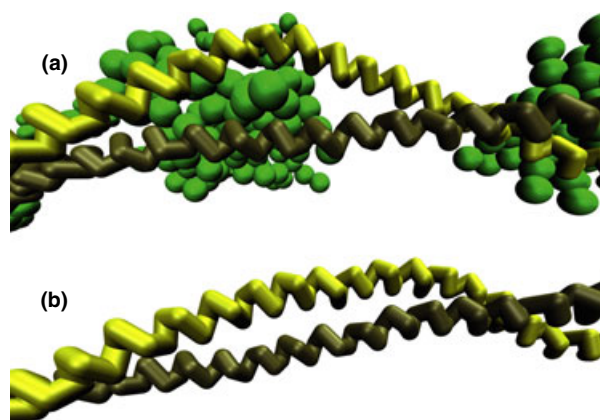
Figure 2 Detail of a dimer of keratin model of keratin dimers using Martini force field a) with the presence of lipids and b) after the lipids removal.

Just like what happens in the X-ray, the removal of the lipids from the simulated keratin/lipids mixture allows the keratin to organize itself, returning to its typical packing structure, Fig.1c and Fig. 2b. Together with the results from X-ray diffraction, these results from the simulations demonstrate the importance of the hair lipids in the structure of hair keratin. This effect should be dependent on the quantity of lipids, explaining why it is only possible to observe this behaviour for African hair.

Confocal microscopy on hair transversal cuts

The lipidic effect demonstrated by molecular dynamics simulations should be dependent on the distributions of the lipids at the cortex. The ability of the lipids to intercalate the keratin dimers should be more significant in the lipids, which do not form aggregates in the cortex. Hence, it is important to verify the distributions of the lipids in the hair. To achieve this, the hair stained with a fluorescence dye was observed under confocal microscopy. These experiments were also conducted using fluorescence microscopy, which provided similar results.

Nile red is a phenoxazone dye that fluoresces intensely, and in varying colour, in organic solvents and hydrophobic lipids, being able to detect some lipid deposits not usually seen in traditional fat stains [27]. Therefore, it is an excellent probe for the detection of lipids using confocal microscopy because its spectra of excitation
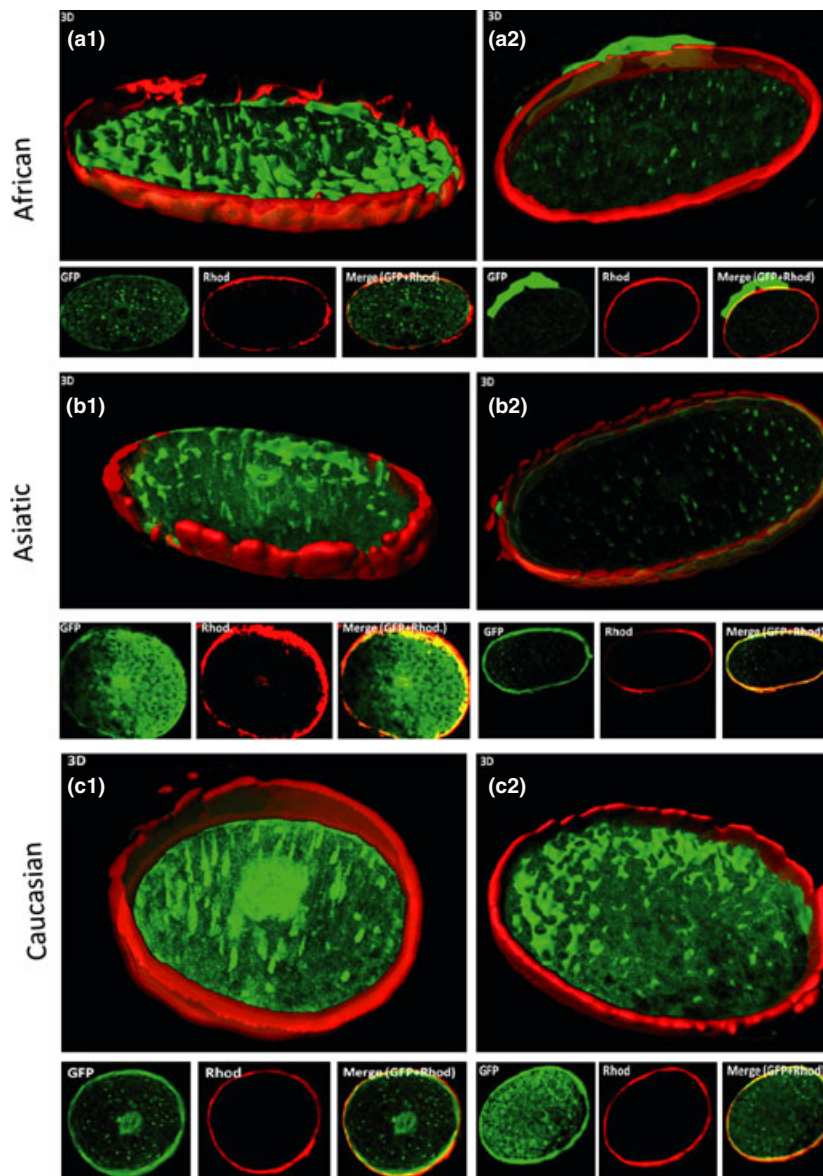
Figure 3 Confocal microscopy of ethnic hair (a) African hair, b) Asiatic hair and c) Caucasian hair) treated with Nile Red solved in: 1) chloroform/methanol and 2) t-butanol. All the images present the result with the filter with GFP filter, rhodamine filter ("Rhod.") and the combination of both filters ("Merge (GFP+Rhod)"), as well as 3D reconstruction from "IMARIS" software.

and emission vary according to the surrounding environment, being shifted to shorter wavelengths with decreasing solvent polarity. The fluorescence wavelength of Nile Red is, therefore, due to the chemical environment where it is inserted [28]. The solvents where Nile Red was solved in are used to have information about the chemical environment at different depths in the hair fibre.

From Fig. 3 it is clear that there is a polar region located at the cuticle, as indicated by the red fluorescence of Nile Red. The exogenous hair lipids are mainly constituted by free fatty acids and cholesterol esters [6], and these lipids are essentially non-polar.

However, it is known that the major component of exogenous lipids, 18-Methyleicosanoic Acid, is covalently attached to the cuticle surface, with the tails align to each other [29]. This particular arrangement leads to the formation of a very polar region at the lipid/protein interface. The polarity of this region is responsible for the red fluorescence observed in Fig. 3.

In the remaining hair fibre, it is not possible to observe polar regions. Regarding the similarities in the fluorescence levels in Nile Red solved in chloroform/methanol, they do not indicate similar levels of lipids, but a similarity in the chemical environment. As it can be observed at Table I, there are some polar lipids at the hair's

interior, but as they are scattered all over the cortex, apparently they donot have the ability to form polar aggregates with the ability to shift the Nile Red fluorescence to red. This result is in accordance with the results from X-ray analysis and from Molecular Dynamics simulations. If the lipids do not form aggregates within the cortex they will have the ability to interact strongly with keratin fibres, interfering with its structural arrangement. However, only in the African hair, which has a higher internal lipid concentration, this phenomenon was observed, indicating that, in the hair, a higher concentration of hair lipids influences the keratin structure. The X-ray results showing meridian reflections characteristics of hair lipids can be in accordance with the lack of lipid polar aggregates. The lipids can be organized in such a way that they give usable data in the X-ray experiments and at the same time presenting a fully hydrophobic character.

Conclusions

In summary, ethnic hair presents different amounts of lipids on its composition, both in total amount and types of lipids present, where African hair has, in general, higher lipid content and specifically cholesterol ester, free fatty acids and cholesterol sulphate.

In the X-ray analysis of the African hair, the presence of lipids was assumed to interfere with the keratin structure, as no axial diffraction correspondent to keratin was found. After the removal of the lipids, the structure of keratin returned to its typical arrangement. To better understand this influence, molecular dynamics simulation of keratin and lipids were performed, where lipids were shown to intercalate dimers of keratin, changing its structure. Therefore, we can assume that higher lipid content, mainly from lipids that do not aggregate within the cortex, will have the ability to interact and interfere with the structure of keratin fibres, which might have influences in the texture of the hair.

Acknowledgement

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