	Experiment title: **Evaluation of breast cancer and auto-immune diseases diagnosis from hair microstructure**	**LS1735**
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15/15/run	**Local contact(s)**: ID13 : M. Burghammer/D. Flot ID21: J.Susini,/M. Salomé	*Received at ESRF:*

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Scientific background

Hair abnormalities are associated with certain diseases. For instance, vitiligo, canities, alopecia, wooly hair or hirsutism are often observed during dysthyroidies, autoimmune diseases, vitamin deficiency, various syndromes (Vogt-Koyanagi-Harada) and even heart diseases. In addition to the breast cancer debate, an abnormality was reported on X-ray diffraction patterns of hair from insulin-dependent diabetes.
Such abnormalities could be used as markers for diagnosis or supervision of diseases. However, X-ray diffraction for hair had not yet been used for such purposes because of the lack of complete and convincing analysis based on medical criteria, in particular carried out on a sufficiently large sampling. It appeared therefore highly necessary to undertake extensive studies of the physiological and pathological variations of the molecular and supramolecular organisations in hair in order to evaluate the diagnostic and prognostic interest of the observed abnormalities.

Objectives

The objectives of the present proposal were threefold:
1. to study the physiological variations of the molecular and supramolecular organisations in scalp hair from healthy subjects.
2. to determine possible changes in the molecular and supramolecular organisations in hair of patients developing diseases like cancer, diabetes and other autoimmune diseases.

3. to establish correlation between abnormalities and clinical characteristics in order to evaluate the diagnostic and prognostic interest.

Techniques

Several techniques were used. Classical diffraction and Infrared microscopy experiments were carried out at LURE, we have benefited of the microdiffraction and microfluorescence techniques at the ESRF.

Microdiffraction experiments

Samples

About 300 hair samples were analysed, 200 from healthy people and 100 from people developing diseases. The collection from patients was carried ot in the CHU-Grenoble, it was accompanied by medical documents. In addition, all donors had to fill a form giving basic information as age, sex, race, size, weight as well as hair status. All the samples were analysed, many of them have been examined various time, systematic analysis were also carried out on a same hair as a function of the position along the hair, or for a given person as a function of location on scalp. Data collections were also obtained on hair sections.

Setup

Experiments were carried out on the microfocus beamline ID13 with a 13 keV energy beam size-limited down to a 5 μm diameter section by a collimator. A pinhole was added close to the sample to remove scattering signal from air. Samples were mounted on a computer-controlled Physik Instrument X/Y stage coupled to a microscope which permitted to position them in the beam with 0.1 μm spatial resolution. For each sample a series of scattering patterns along a line perpendicular to their axis were collected with a 10 μm step size between data points. Patterns were recorded on CCD MARRESEARCH camera located at 150 mm from the sample using 10 s exposure time. Using a small diameter beamstop (0.3 mm) located about 40 mm from the sample allowed us to collect both SAXS and WAXS data in the 150Å–2Å region.
We have designed a sample holder containing about 50 hairs that are located at regular 1mm intervals. The automatic data collection using such a sample holder required about 1 shift including positioning.

Results

The main scattering features are visible on a single pattern of an hair perpendicular to the beam. They consist in a broad equatorial spot centered at 9.7 Å, corresponding to the mean distance between α-helical axes, a fine meridian arc at 5.15 Å, related to the projection of the α-helix pitch along the coiled coil axis and broad equatorial peaks at 91Å, 45Å and 29.5Å due to the dense lateral packing of microfibrils. On the meridian, the most intense reflection is seen at 67Å, it arises from the axial stagger between molecules along the microfibril. The ring arising from lipid granules is located at 45Å.

Statistical study: the position and intensities of the reflections display some variability, high for the 45Å ring due to crystallised lipids, or low for 67 and 91Å reflections that are due to keratin. The statistical analysis is not yet completed, however several significant differences have already been detected, they arise either from physiological parameters or from medical history (auto-immune diseases). Let us give a few examples of striking differences:

- Physiological variability
    - The average distance between microfibrils varies significantly according to the ethnic groups: it is about 3% higher in African and Chinese hair than in Caucasian hair. This could reveal a different interfilaments matrix quantity since the microfibril diameter is the same, and therefore explain different macroscopic properties.
    - Also quite significant are the differences in lipid content: fair hair always contain crystallised lipids whereas they are missing in about 30% in other hairs. Similarly, they are nearly always present in hair belonging to skinny people ans missing in about 30% of other groups.
- Disease-induced modifications
    - Diabetic patient hair display diffraction rings from lipids which position correspond to a 10% swelling compared to non diabetic people. Insulin seems to modify the axial architecture of the microfibrils (shift of the 67 Å reflection).

- Cancer-induced modifications are oberved for the 91Å, but it is not yet possible to ascertain their origin, chemotherapy or radiotherapy.

Hair cuticle: During our measurement campaigns we have observed that the diffraction patterns from hair cuticle and cortex were different. We have successfully interpreted the diffraction signal from cuticle, the results are now published:
L. Kreplak, C. Mérigoux, F. Briki, D. Flot, J. Doucet, Biochim. et Biophys. Acta157(2), 268-274 (2001) "Investigation of human hair cuticle structure by microdiffraction. Direct observation of cell membrane complex swelling"
We reproduce here the abstract:
"*The cuticle of mammalian hair fibres protects the core of the fibre against physical and chemical stresses. The structure and some of the properties of the cuticle have been extensively studied by electron microscopy. However there is still a need for a less-invasive structural probe. For this purpose, microdiffraction experiments have been carried out on human hair samples showing a characteristic small-angle X-ray scattering pattern for the cuticle. This pattern has been assigned to the cell membrane complex (CMC) between each cuticle scale. Using a simple model of the electron density within the CMC, values have been derived for the average thickness of the β-layer and δ-layers which are close to those obtained by electron microscopy. In order to illustrate the potentialities of microdiffraction in studying the properties of the cuticle, the effect of water-sorption has been monitored. Using the intensity modelling described above, a 10% swelling of the δ-layer's thickness has been observed. This study shows that structural modifications of the CMC by physical or chemical stresses can be followed directly on the cuticle of human hair fibres by microdiffraction analysis.*"

Micro-fluorescence experiments

Samples

Due to the duration of the X-ray fluorescence data collections in mapping only about 30 hair, selected from X-ray diffraction observations, have been examined: low and high lipid content and some a few ones from various patients. Thin slices (about 50 μm thick) of human scalp hair shaft cut perpendicular to the hair axis were prepared. They were inserted into a plastic pipe and embedded in a resin. One to five cross-sections were analyzed for each hair. The slices were mounted between two 10 μm thick ultralene foils a plastic foil devoid of contaminants, and then fixed on the sample holder.

Setup

Samples were examined using the X-ray microscopy beamline ID21. The Scanning X-ray Microscope used Fresnel zone plates as focusing optics, which demagnify the synchrotron X-ray source to generate a sub-micron probe .The microprobe sizes were $0.3x0.3\mu m^2$ at the Calcium K-edge. Fluorescence and transmission signals can be used simultaneously to investigate the sample, which is mounted on a piezo-electric stage and raster scanned in the beam to acquire a two-dimensional image point-by-point. In order to minimise the contribution of the unwanted elastic scattering, the sample stage is tilted about 75° with respect to the beam direction, and the energy-dispersive high-purity Germanium single element detector mounted in the horizontal plane perpendicular to the beam is used to collect the fluorescence signal. The fluorescence signal was processed via a multiple Channel analyser allowing fluorescence emitted by various elements to be recorded simultaneously. The energy tuning was obtained using by a fixed-exit silicon monochromator. Finally a fixed-exit double mirror system, located upstream from the monochromator and acting as a low band pass filter allowed harmonic rejection greater than 10^{-3} with total transmission greater than 70 %.
The measurements were carried out in vacuum (10^{-5} mbar) in order to minimize the absorption of air for the sulphur XRF signal.

Results

- Physiological variability

For all the chosen samples we have collected sulphur and calcium mapping images. The sulphur distribution is, as expected, quite uniform, on the contrary the calcium distribution across hair section is highly non-uniform and highly variable from one person to another. We have interpreted these observations as due to the existence of several calcium sites (in press, Biochim. et Biophys. Acta):

C. Mérigoux [a] , F. Briki [a], F. Sarrot-Reynauld [b], M. Salomé [c], B. Fayard [c], J. Susini [c], J. Doucet [a*]
"Evidence for several calcium sites in human hair shaft revealed by sub-micrometer X-ray fluorescence"
Abstract: *"New information about calcium status in human scalp hair shaft, deduced from X-ray micro-fluorescence imaging, including its distribution over the hair section, the existence of one or several binding-types and its variability versus people, is presented. The existence of two different calcium types is inferred. The first one corresponds to atoms (or ions) easily removable by hydrochloric acid, located in the cortex (granules), in the cuticle zone and also in the core of the medulla, which can reasonably be identified as calcium soaps. The second type consists of non-easily removable calcium atoms (or ions) that are located in the medulla wall, probably also in the cuticle, and rather uniformly in the cortex; these calcium atoms are probably involved in Ca^{2+}-binding proteins, their concentration is fairly constant from one subject to another. In addition to its non-uniform distribution across the hair section, the second striking feature of the first type calcium content is its high variability from one subject to another, up to more than ten times. This information will be probably useful for analysing into more detail and understanding the relationship of hair calcium concentration to environmental and medical parameters."*

- Disease-induced modifications

A very striking effect was observed according to calcium metabolism. People with a high calcium content in blood, like in case of heart diseases and hypercalcemia display a very low calcium content across hair, whilst people with low content in blood exhibit a large calcium content in hair. We are preparing another manuscript on the calcium status in hair of people in relation with those diseases, however this medicine-oriented work requires a lot of care and prudence.

Conclusion

The long-term project was in our opinion quite a success and thanks to the support from the ID13 and ID21 staff the data collection were successful. The interpretation of the results is of course not yet completed, it will require time due to the huge quantity of data and information collected. Let us mention that similar projects are presently being carried out in Canada (diffraction), Sweden (fluorescence) and Russia (diffraction and fluorescence). There is now some competition in this topic which is quite promising and should give rise to new and simple prognostic methods, for which new proposals at the ESRF will be submitted.