

Properties of byssal threads, the chemical nature of their colors and the Veil of Manoppello

J.S. Jaworski

Faculty of Chemistry, University of Warsaw, Pasteura Street 1, 02-093 Warsaw, Poland
jaworski@chem.uw.edu.pl

Abstract

Considering the hypothesis that the Veil of Manoppello is made from byssal threads their physical and chemical properties were analyzed in relation to the possible origin of image colors seen on the Veil. Most of these colors are similar to the colors of byssal threads, either natural (brown) or after their chemical degradation (yellow, gold, pale straw, reddish). Pheomelanin was found to be the main pigment of byssal threads. Preliminary examinations of threads after bleaching by the polarized light microscopy are reported.

Keyword: byssus, bleaching, pheomelanin.

1. INTRODUCTION

The image on the Veil of Manoppello is worshipped in the Manoppello Sanctuary in the Abruzzi region, Italy, as the Holy Face of Jesus. It corresponds to the traditional presentation of the Face of Jesus and reveals some similarities with the Face of the Shroud of Turin. The similarities were well established by Schlömer and Resch [1 - 5] and encompass not only the general appearance but also some details including the marks of wounds. The documented history of the Veil in Manoppello has been known since the 16th century but its origin is uncertain. The Veil is highly transparent when strongly illuminated and the image can be seen on both sides of the Veil. The microscopic and spectroscopic studies (performed through the glass of the reliquary) did not lead to clear conclusions as to the nature of the image. Microscopic photographs show serious contaminations on the surface of the threads. Some researchers recognized them as pigments [6 - 8], while others consider the image as the *acheiropoietos* and point to its unique characteristics, especially to the fact that different details can be seen on it depending on the kind of illumination applied, and also to the fact that the technique used to paint the Veil still remains unknown.

The Veil of Manoppello is made either from linen or from byssal threads. The latter origin was claimed by Chiara Vigo from S. Antioco, near the island of Sardinia, Italy who produces byssus cloths using traditional methods known for generations. However, no experimental scientific support of that was reported. Byssus threads are non-living, completely acellular, delicate silky fibers, golden or brown in color, which are secreted by glands of some mussels in order to anchor the bivalve to the hard surfaces and to secure it against the waves. Among marine mussels, the pen shell (*Pinna*

nobilis) living offshore in the coastal regions of the Mediterranean Sea has been best known since ancient times because of its very large shell (which can grow up to 1 m). Its threads were spun to form yarn and then woven to produce exceptionally fine, valuable and rare fabric.

The dominant color on the Veil of Manoppello is brown, which corresponds to the natural color of dry byssus threads of *Pinna nobilis*. Some other properties of byssus described in popular literature, like its being fireproof appear however to be only legendary; in fact, its threads glow in red and burn away like hair.

It should be added here that byssal threads secreted by other marine mussels, in particular those from *Mytilidae* family, have been intensively investigated in recent years [9 - 17] in search of inspirations as concerns the use of biomimetic materials in engineering and also for medical purposes. The research has been focused on understanding the structure of natural fibers in order to explain their unusual properties such as the ability to secure stiffness and elasticity in different parts, their extremely high strength, great capacity of absorbing and dissipating energy and strong adhesion to different (even wet) surfaces. As a result of the above-mentioned investigations, byssal threads were found to consist of molded collagenous fibers coated with a fine (2 μm - 4 μm) protective cuticle. Each thread of *Mytilus* species can be divided into two morphologically and mechanically distinct parts of different composition: proximal (soft, elastic and crimped) and distal (smooth and stiff), with a gradual transition between them [9 - 12, 17]; the distal part has an adhesive plaque at the end. The principal protein components are three byssal collagens known as preCols: in the distal region preCol-D, in the proximal section preCol-P and uniformly presented through the

Proceedings of the International Workshop on the Scientific approach to the Acheiropoietos Images, ENEA Frascati, Italy, 4-6 May 2010

thread preCol-NG. Each of them includes a central collagen domain, variable flanking domains, and histidine-rich terminal domains. Flanking domains for preCol-D, preCol-NG and preCol-P resemble spider dragline-silk, Gly-rich plant cell wall protein and elastin, respectively. The graded distribution of preCols with different flanking domains is responsible for unique mechanical properties of the threads. Histidine and residues of dihydroxyphenylalanine (DOPA) in terminal domains form reversible coordination bonds with transition metal ions which play a role of sacrificial bonds in yield and self-healing in the distal region of the thread. On the other hand, the cuticle is hard, yet highly extensible due to its ultrastructure with nanoscale granules [13 - 16].

It should be pointed out that in recent investigations there is no information about pigments responsible for the color of byssal threads.

In this report physical and chemical properties of byssus threads from pen shell (*Pinna nobilis*), obtained from the S. Antioco region near the island of Sardinia, Italy were examined. In particular, the change of color due to chemical bleaching and the chemical analysis of the threads pigments by the high-performance liquid chromatography (HPLC) are reported. Finally, preliminary investigations of the threads using the polarized light microscopy (PLM) are discussed.

2. EXPERIMENTAL

Physical properties of natural byssal threads. Natural threads have different tints of a brown color from reddish to brownish-black, but some beige threads can also be found. Threads are lustrous, elastic, up to 7 – 9 cm long and they form irregular waves, as is illustrated in Figure 1 showing the scanning electron microscopy (SEM) image. SEM images were obtained using a LEO 435VP (Zeiss) microscope, EHT = 15 kV and samples coated with gold.

The threads have circular or slightly elliptical cross section and their diameter (or thickness), determined from SEM images, ranges from $10 \pm 2 \mu\text{m}$ to $45 \pm 5 \mu\text{m}$ but even for the same thread the thickness can change twice in different parts. The average diameter is of ca. $25 \mu\text{m}$.

Fanti reported [6] the value of $14 \pm 6 \mu\text{m}$ for threads in the Veil of Manoppello, estimated from the number of threads in a yarn and its diameter in microphotographs. Obviously, finer threads could be used for more delicate fabric. The thread surface is rather smooth (Figure 2), without any overlapping scales in the cuticle, as for human hair.

Under stronger illumination the threads are semi-transparent and the edges of the bottom thread at the crossing area are well visible under microscope (Figure 3). The complete transparency is evident for colorless threads after bleaching.

The thread surface is hydrophobic as could be seen after

attempts to cover it by water-colors: no absorption and no uniform surface coverage were obtained, as in the case of linen threads, but separate microgranules were seen on the surface in microscopic images. This behavior corresponds to the protective role of the cuticle in natural aqueous environment of mussels. Moreover, it remains in agreement with the reported results [11] claiming that strong incorporation of iron in the cuticle occurs only during the secretion of a new thread, whereas threads already formed are unable to sequester iron from the iron-supplemented seawater.

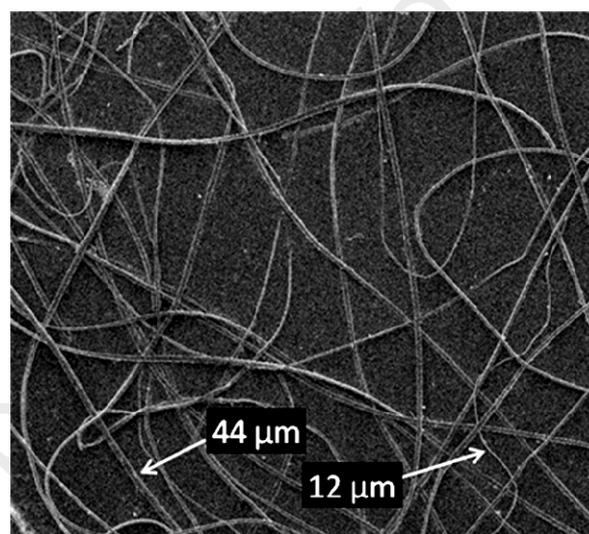


Figure 1. SEM image (x100) of byssus threads; threads of extreme thickness are shown.

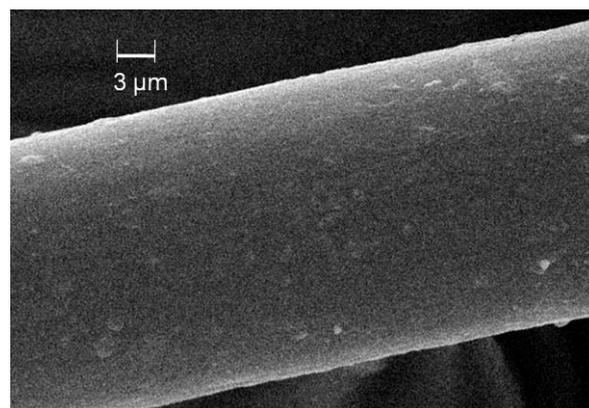


Figure 2. SEM image (x6000) of the byssus thread.

Chemical bleaching of byssus threads. The brown color of threads is unaffected by common organic solvents (e.g., methanol, acetone, ethyl acetate, toluene), diluted acids and bases, and reductants such as sodium thiosulphate, $\text{Na}_2\text{S}_2\text{O}_3$, and hydrazine hydrate $\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$, as well as temperature and UV irradiation (overnight, 254 nm or 365

Proceedings of the International Workshop on the Scientific approach to the Acheiropietos Images, ENEA Frascati, Italy, 4-6 May 2010

nm from an 8 Watt Spectroline lamp). However, the use of strong oxidants (like hydrogen peroxide, H_2O_2) in proper media can easily change the color to yellow, gold or pale straw, or even make the threads completely colorless.

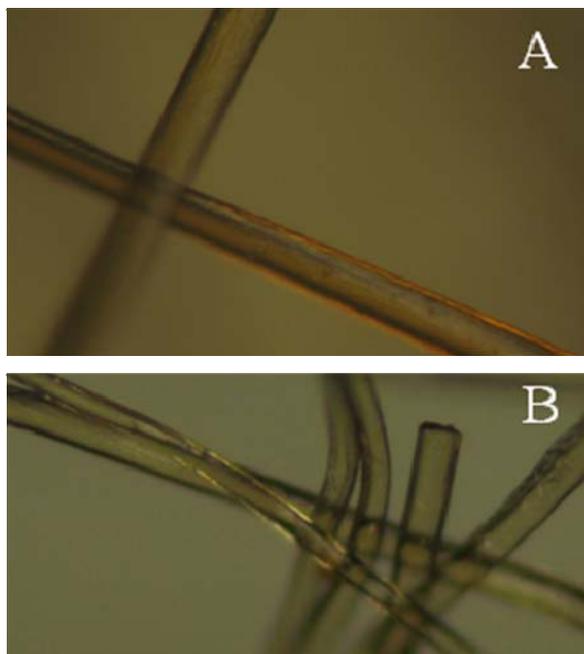


Figure 3. Microscopic photographs of byssus threads showing their transparency under strong illumination: (A) natural brown threads and (B) colorless threads after the 20-h degradation in ammonia with hydrogen peroxide.

The H_2O_2 oxidation was performed using alkaline solutions of sodium hydroxide NaOH, ammonia NH_3 or potassium carbonate K_2CO_3 . The reddish color was also obtained in parts of the thread after hydrolysis in hydriodic acid, HI. Some examples of typical bleaching results are presented in Table 1. The media used in these experiments were chosen following literature procedures proposed for the degradation of melanin pigmented tissues [18 - 22], in particular mammalian hair, in order to obtain melanin markers among fragmentation products. A complete dissolution of the keratin matrix was obtained then. However, shorter time of degradation and more diluted solutions allowed us to obtain the changes in color without substantial damage to the byssus thread. No evidence of damage was observed in SEM images, for instance there was no change in thickness of the threads after the degradation used for the HPLC determination. It should be added that the final colors as presented in Table 1 were observed in natural daylight but the change from pale yellow or gold to colorless could be seen with the change of illumination and thickness of the threads layer. It is evident from the results obtained that most of the colors that can be seen on the Veil of Manoppello (except black) correspond to the colors of byssus threads, either natural or after bleaching.

TABLE 1. Chemical bleaching of byssal threads at room temperature

Medium (volume ratio)	Conditions	Final color
1.5% H_2O_2 ^a	5 min	brown, lustrous
1 M NaOH + 1.5% H_2O_2 ^a	1) 35 min 2) 65 min 3) 4 h	1) pale gold, lustrous 2) 3) colorless, lustrous
1 M NaOH + 30% H_2O_2 (2 : 1)	50 min	colorless, lustrous
1 M NaOH + 30% H_2O_2 (20 : 1)	1.5 h stirring	colorless
24% NH_3 + 1.5% H_2O_2 ^a	1) 5 min 2) 30 min	1) brown 2) pale brown
25% NH_3 + 30% H_2O_2 (2 : 1)	30 min	pale yellow, lustrous
2 M NH_3 + 30% H_2O_2 (1 : 1)	20 h	yellow ^{b,c}
	1) 5 min	1) pale reddish-brown
	2) 10 min	2) dark yellow
1 M K_2CO_3 + 30% H_2O_2 (2 : 1)	3) 30 min	3) pale gold, lustrous
	4) 1 h	4) pale yellow
	5) 3 h	5) straw to colorless
	6) overnight	6) colorless
1) 1 M K_2CO_3 2) after cooling + 30% H_2O_2 (1 : 0.07)	1) 0.5 h 80°C 2) 5 days room temp.	pale gold
3% $KMnO_4$ + 1 M H_2SO_4 (1 : 90)	40 min stirring	colorless
ca. 57% HI + H_2O + 50% H_3PO_2 (50 : 5 : 1)	20 min ca. 100°C	red and gold fragments

^aFinal concentrations. ^bColorless under microscope.

^cProcedure used in HPLC determination.

Determination of byssus pigments. The observed change of colors of byssus threads after their degradation performed under similar conditions as described for mammalian hair suggested the presence of the same pigments – melanins – in both.

Melanins are the most popular pigments in animal world. They are responsible for the color of skin, hair, eyes and feathers. There are two types of melanins: the black to dark-brown insoluble eumelanin and the yellow to reddish-brown pheomelanin which is soluble in alkali [21,

23]. Their natural synthesis requires amino acids: L-tyrosine for eumelanin and both L-tyrosine and L-cysteine for pheomelanin. Both melanins are heterogeneous macromolecules formed from fundamental monomeric building blocks: 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid for eumelanin, and cysteinyl-dopa for pheomelanin. However, the detailed structure of melanins is still under debate [23]. Unique physical and chemical properties of melanins (like broad band UV-visible absorption, electrical conductivity and photoconductivity, paramagnetism, and redox properties) are responsible for their enormous biological importance, first of all as photoprotective and antioxidant agents [23].

It was established in recent years that in the case of black, brown and blond hair which contains approximately from 99% to 95% eumelanin, the color depends on melanin quantity rather than on the ratio of eumelanin to pheomelanin, whereas red hair contains 67% eumelanin and 33% pheomelanin [20]. Moreover, the color of hair strongly depends on the structure and dispersion of these pigments.

Chemical analysis of eumelanin and pheomelanin as recently proposed in the literature is based on chemical degradation of biological material (pigmented tissues) in strictly definite media, which results in the fragmentation of melanin macromolecules to a number of small organic molecules. Some of them are chosen as specific markers of melanins. The degradation is followed by the HPLC chromatography with the electrochemical, spectroscopic UV or mass spectrometric (MS) detection of markers.

In order to determine melanins in byssal threads the alkaline hydrogen peroxide degradation at room temperature was used following the procedure of Napolitano group [22] but with some modifications (e.g., ammonia was used instead of sodium hydroxide which was not convenient for the MS detection applied). Pyrrole-2,3,5-tricarboxylic acid (PTCA) and 6-(2-amino-2-carboxy-ethyl)-2-carboxy-4-hydroxybenzothiazole (BTCA) were used as markers for eumelanin and pheomelanin, respectively [19, 22].

HPLC equipment (Shimadzu) and a Luna C-18(2) chromatographic column (100 mm x 2.1 mm, 3 μ m; Phenomenex, USA) with a pre-column were used, with formic acid/methanol as the mobile phase at a flow rate of 0.2 ml/min. To detect both markers a mass spectrometer 3200 QTRAP (Applied Biosystems) was used. MS conditions for the multiple reaction monitoring (MRM) approach were optimized for the synthetic PTCA standard (synthesized by the oxidation of 5-hydroxyindole-2-carboxylic acid) which was supplied continuously to the spectrometer at a rate of 10 μ l/min. The MS₂ spectrum of PTCA in the negative ions mode shows four peaks at m/z = 197.9 ($[M-H]^-$ = 198), 154.1 ($[M-H-CO_2]^-$ = 154), 109.9 ($[M-H-2CO_2]^-$ = 110) and 66.1 ($[M-H-3CO_2]^-$ = 66). For MRM analysis two pairs of ions 199/110 and 199/154 were chosen. The determination of BTCA was based on the MS₂ spectrum in positive polarity of ions showing the

original signal at m/z = 283.2 ($[M+H]^+$ = 283) and the following signals of fragmentation products: 177.0, 133.2, and 89.2. The same signals were found in the red hair sample after the same degradation (283.1, 177.3, 133.1, and 89.0, as well as the additional small signal at 196.3). For MRM analysis two pairs of ions 283/89 and 283/133 were used. More details on the HPLC-MS/MS determination will be published separately.

The whole procedure used was checked first for the determination of both melanins in human hair of different colors: black, brown and red. In accordance with the literature data [19, 20], the highest amount of eumelanin marker PTCA was found in black hair, a lower amount was found in brown hair whereas in red hair the amount of PTCA was practically nondetectable. The pheomelanin marker BTCA was found in amounts following the reverse order.

For byssus threads after the same chemical degradation a substantial amount of BTCA was found but PTCA marker was under the limit of determination. Figure 4 shows a comparison of chromatograms obtained for light brown human hair (40 mg sample) and byssus threads (9.5 mg sample); the amount of BTCA marker looks comparable in both samples. BTCA found in byssus threads is an average amount for the threads collected from different mussel species, with different tints of a brown color. Thus, a more quantitative comparison is not advisable.

The results obtained show evidently the presence of pheomelanin as the main pigment in byssus threads and explain similar bleaching procedures to those established for mammalian hair.

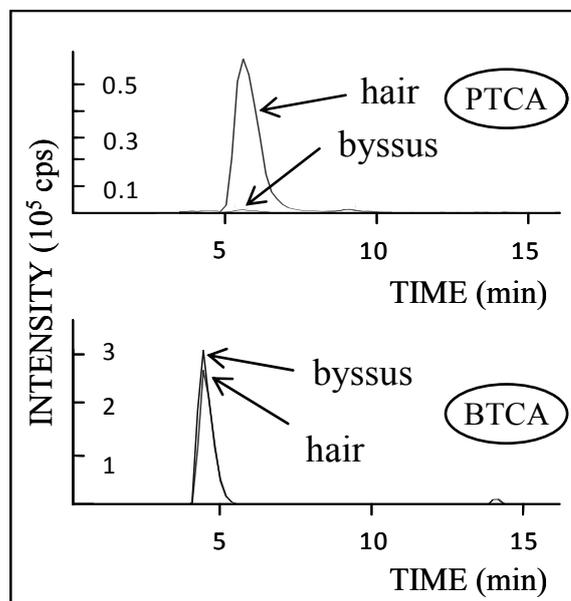


Figure 4. Comparison of HPLC chromatograms (MRM approach) for human light brown hair and byssal threads after the same chemical degradation in ammonia with H₂O₂.

It should be added here that bleaching processes cannot be fully understood unless the melanins structure is

known. Nevertheless, it was proposed [24] on the basis of the small-angle X-ray scattering that during the chemical bleaching of synthetic eumelanin the oxidative disruption of hydrogen bonds between oligomer sheets occurs, leading to deaggregation and delamination of origin stacked oligomers. A similar change can be expected during the bleaching of byssus threads.

Polarized light microscopy of byssal threads. Very recently Fanti was successful in obtaining PLM images in crossed-polarized light of some broken threads in the Veil of Manoppello [8]. They showed intense interference colors, the so-called second- and third-order colors. Very similar images are obtained from linen fibers which are built from crystalline cellulose, a birefringent material. Moreover, Fanti did not find similar colors for a sample of byssal threads and concluded [8] that the Veil was made from linen.

Byssal threads of *Mytilus* species are made in mussels from prefabricated smectic polymer liquid crystals in a process similar to the reaction injection molding [9, 10]. The thread core under the outer cuticle is a crystalline area [10]. The birefringence of the distal part of the thread parallel to its axis was reported [25] for the Mediterranean *Mytilus galloprovincialis*. In full agreement with those results, our preliminary examination of *Pinna nobilis* threads under crossed polarizers (using an Eclipse LV100 POL (Nikon) microscope in the transmission mode) showed that the threads had a light blue color between brown edges parallel to the thread axis. The image was completely different from the images of linen fibers and those found in the Veil. Nevertheless, the structure of collagens and melanins in byssus threads is complex, as was mentioned earlier, and at least for melanins it changes substantially during the bleaching process. Thus, the chemically bleached threads may appear in various interference colors, even very different from the natural one.

In PLM images obtained in the reflection mode, using an AXIO observer.A1m (Zeiss) microscope, most of the threads after bleaching according to the procedures presented in Table 1 are pale gold-yellow (Figure 5A), which is contrary to the human red hair (containing the same pigment - pheomelanin) which showed blue and red interference colors (Figure 5B).

In the transmission mode the majority of byssus threads after bleaching appeared in PLM images in light blue and red colors along the thread axis (Figure 6A). The saturation of colors is weak and the pattern of colors is also different from that observed for linen fibers, where the transition between colors is situated perpendicularly to the fiber axis (Figure 6D).

Some byssus threads after bleaching also appeared in other colors (yellow, green) in a more complicated pattern (Figures 6B and 6C), yet different from the images of linen fibers, which are most similar to PLM images obtained from the Veil [8].

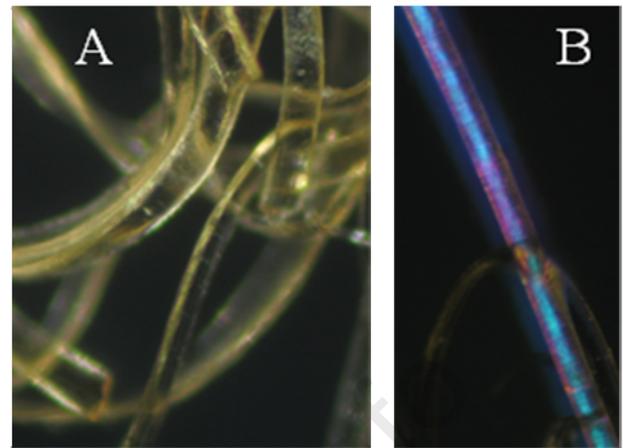


Figure 5. PLM images in reflection mode of byssus threads (A) and human hair (B) after the same bleaching in a solution of ammonia with hydrogen peroxide.

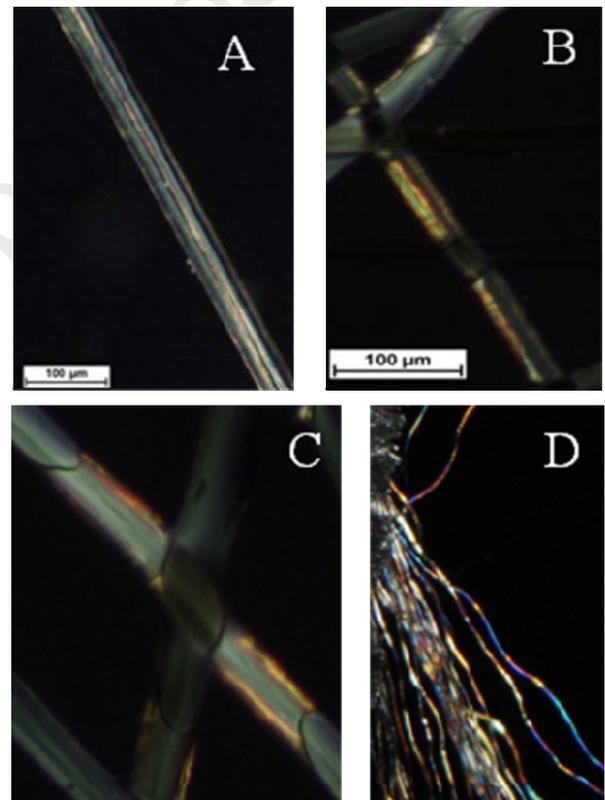


Figure 6. PLM images in transmission mode of byssus threads after bleaching:

(A) 45 h in 1 M K_2CO_3 + 30% H_2O_2 (2:1);
(B) and (C) 35 min in 1M NaOH + 1.5% H_2O_2 ;
and PLM image of linen fibers (D).

3. CONCLUSIONS

A. Considering the properties of byssus which could be related to colors and other optical characteristics of byssus

Proceedings of the International Workshop on the Scientific approach to the Acheiropietos Images, ENEA Frascati, Italy, 4-6 May 2010

fabrics the following conclusions can be drawn.

(i) It was found using chromatographic analysis that pheomelanin is the main pigment of byssal threads responsible for their natural brown color.

(ii) The original brown color of threads can be chemically changed to yellow, golden, pale straw, reddish and finally colorless.

(iii) It was confirmed that under strong illumination the bleached byssus threads are completely transparent.

(iv) Preliminary examinations revealed that after degradation byssus threads could have interference colors in PLM images with cross-polarization.

B. As regards the images seen on the Veil of Manoppello, the following conclusions can be drawn.

(i) There are some interesting properties of byssal threads, which could be related to the unique properties of the images on the Veil of Manoppello. They include the transparency of threads under a strong illumination and the possibility to obtain most of the colors seen on the Veil from the natural pigment of byssus threads after their modification, e.g., by chemical bleaching. A possibility of the change of byssus colors by other modifications of pheomelanin, e.g., by applying UV irradiation, should be verified experimentally.

(ii) The byssus hypothesis offers an additional similarity between the images on the Veil of Manoppello and on the Shroud of Turin, besides similarities in the appearance of the Face. Namely, both images appear to be similar as concerns their chemical nature, which is related to some modifications of the fabric itself: the oxidation / dehydration processes of cellulose of the linen in the case of the Shroud and bleaching processes of pheomelanin in byssal threads (at least in some parts of the image) in the case of the Veil.

(iii) PLM images of byssal threads after bleaching were different from those found by Fanti for the threads in the Veil of Manoppello. Taking into account the very complex structure of byssus threads and melanins as well as the lack of knowledge about the relations between bleaching procedures and interference colors seen in PLM images, the byssus hypothesis should be further investigated. However, at present the results obtained by Fanti [8] point to the linen as a more probable material of the Veil.

ACKNOWLEDGMENTS

The author thanks the fellows from The University of Warsaw: Dr M. Biesaga and Dr M. Donten (Faculty of Chemistry) for HPLC measurements and SEM images, respectively, Dr M. Bojanowski (Faculty of Geology) for his help with PLM images and MISMAP student K. Abramczyk for her assistance in bleaching experiments. Thanks are also due to Father C. Cucinelli from Manoppello Sanctuary for samples of byssus threads.

REFERENCES

1. B.P. Schlömer, *Der Schleier von Manoppello und das Grabtuch von Turin*, Andreas Resch Verlag, Innsbruck, 2nd ed. (2001)
2. B.P. Schlömer, *The Manoppello Veil compared with the Turin Shroud and with other grave cloth of Christ*, poster, IWSAI Workshop, Frascati (2010)
3. A. Resch, *Grenzgeb. Wissenschaft* **56**, 95-124 (2007)
4. A. Resch, *The face on the Shroud and on the Veil of Manoppello*, poster, IWSAI Workshop, Frascati (2010)
5. J.S. Jaworski and G. Fanti, *3-D processing to evidence characteristics represented in Manoppello Veil*, in: www.shroud.com (2008) and references cited therein
6. G. Fanti, *Volto Santo di Manoppello, Relazione Tecnica*, manuscript (2001)
7. R. Falcinelli, *The Veil of Manoppello: work of art or authentic relic?*, in: www.shroud.com; The Third Dallas International Conference on the Shroud of Turin, Dallas (2005)
8. G. Fanti, *How the acheiropietos images are produced?* presented at IWSAI Workshop, Frascati (2010)
9. J.H. Waite, E. Vaccaro, C. Sun, and J.M. Lucas, *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **357**, 143-153 (2002)
10. T. Hassenkam, T. Gutschmann, P. Hansma, J. Sagert, and J.H. Waite, *Biomacromolecules* **5**, 1351-1355 (2004)
11. C.J. Sun and J.H. Waite, *J. Biol. Chem.* **280**, 39332-39336 (2005)
12. M.J. Harrington and J.H. Waite, *J. Exp. Biol.* **210**, 4307-4318 (2007)
13. N. Holtén-Andersen, G.E. Fantner, S. Hohlbauch, and J.H. Waite, *Nat. Mater.* **6**, 669-672 (2007)
14. E. Carrington, *Trends in Biotech.* **26**, 55-57 (2008)
15. N. Holtén-Andersen, T.E. Mates, M.S. Toprak, G.D. Stucky, F.W. Zok, and J.H. Waite, *Langmuir* **25**, 3323-3326 (2009)
16. N. Holtén-Andersen, H. Zhao, and J.H. Waite, *Biochem.* **48**, 2752-2759 (2009)
17. M.J. Harrington, H.S. Gupta, P. Fratzi, and J.H. Waite, *J. Struct. Biol.* **167**, 47-54 (2009)
18. A.M. Kolb, E.G.W.M. Lentjes, N.P.M. Smit, A. Schothorst, B.J. Vermeer, and S. Pavel, *Anal. Biochem.* **252**, 293-298 (1997)
19. A. Napolitano, M.R. Vincenzi, P. Di Donato, G. Monfrecola, and G. Prota, *J. Invest. Dermatol.* **114**, 1141-1147 (2000)

Proceedings of the International Workshop on the Scientific approach to the Acheiropoietos Images, ENEA Frascati, Italy, 4-6 May 2010

20. C.R. Borges, J.C. Roberts, D.G. Wilkins, and D.E. Rollins, *Anal. Biochem.* **290**, 116-125 (2001)
21. K. Wakamatsu and S. Ito, *Pigment Cell Res.* **15**, 174-183 (2002)
22. L. Panzella, P. Manini, G. Monfrecola, M. d'Ischia and A.Napolitano, *Pigment Cell Res.* **20**, 128-133 (2007)
23. P. Meredith and T. Sarna, *Pigment Cell Res.* **19**, 572-594 (2006)
24. K.C. Littrell, J.M. Gallas, G.W. Zajac, and P. Thiyagarajan, *Photochem. Photobiol.* **77**, 115-120 (2002)
25. A. Bairati and L. Vitellaro-Zuccarello, *J. Submicr. Cytol.* **6**, 367-370 (1974)

www.acheiropoietos.info