Journal of Fluorescence, Vol. 10, No. 3, 2000

Fluorescence Studies of Melanin by Stepwise Two-Photon Femtosecond Laser Excitation

Klaus Teuchner,[1,3] Jürgen Ehlert,[1] Wolfgang Freyer,[1] Dieter Leupold,[1] Peter Altmeyer,[2] Markus Stücker,[2] and Klaus Hoffmann[2]

Received September 28, 1999; revised January 21, 2000; accepted February 2, 2000

Fluorescence of synthetic melanin in the solvents H_2O, KOH, ethylene glycol monomethyl ether, and dimethyl sulfoxide has been excited by two-photon absorption at 800 nm, using 120-fs pulses with photon flux densities of $\geq 10^{27}$ $cm^{-2}s^{-1}$. Compared to the one-photon (400-nm)-induced fluorescence of melanin, the overall spectral shape is red-shifted and shows a strong environment sensitivity. The decay of the two-photon-induced fluorescence (TPF) of melanin is three-exponential, with a shortest main component of about 200 ps. The results of the TPF studies in line with the unique light absorption property of melanin of a monotonously decreasing absorption spectrum between the near UV-region and the near infrared region indicate that the TPF is realized via stepwise absorption of two 800-nm photons. In comparison to the simultaneous absorption of two photons, the stepwise process needs lower photon flux densities to get a sufficient population of the fluorescent level. This stepwise process offers new possibilities of selective excitation of melanin in skin tissue in a spectral region where there is no overlap with any absorption of another fluorescent tissue component. The first results with different samples of excised human skin tissue (healthy, nevus cell nevi, malignant melanoma) suggest that fluorescence excited in this way yields information on malignant transformation.

KEY WORDS: Melanin; two-photon excited fluorescence; femtosecond laser excitation; malignant melanoma; skin cancer diagnostics.

INTRODUCTION

Melanins as heterogeneous polymers complexed with proteins are the main pigments of the human skin. Melanins are synthesized in melanosomes of the epidermal melanocytes by enzymatic oxidation of tyrosine that leads to two types of melanin in human skin: eumelanin and pheomelanin. Eumelanin is predominant in the human skin. Several synthetic approaches have been applied to obtain "synthetic" melanin [1]. Enzymatic and chemically oxidized as well as autooxidative melanins using different starting materials have been prepared [2, 3]. Melanins produced by different methods have been investigated by ESCA or XPS [4]. Results were compared to the natural melanin from *Sepia officiales*. Synthetic eumelanins show a change in the overall elemental composition compared to their starting materials used, indicating a modified eumelanin structure which was unknown in detail up to now. First proposals concerning this structure were discussed by Swan [2]. Only enzymatic and autooxidized DOPA eumelanin can be compared to *Sepia* eumelanin in elemental composition. However, purification of natural eumelanin by acid or alkali is also expected

[1] Max-Born-Institut für Nichtlineare Optik und Kurzzeitspektroskopie, Max-Born-Str. 2a, D-12489 Berlin, Germany.
[2] Department of Dermatology, St. Josef Hospital, Ruhr-University, D-44791 Bochum, Germany.
[3] To whom correspondence should be addressed. Fax: +49 30 6392 1309. e-mail: teuchner@mbi-berlin.de

1053-0509/00/0900-0275$18.00/0

to alter the chemical composition. In all, synthetic melanin seems to be at least an approach to mimic natural eumelanins.

Though the basic monomer building blocks of these pigments are known (5,6-dihydroxyindole for eumelanin and cysteindopa in the case of pheomelanin), the detailed structure of the final products of melanin biosynthesis is still unknown [1,5,6]. With respect to the results of sunlight absorption, melanin has been characterized as a two-edged sword [7]. On one hand (and probably due mostly to eumelanin), it is beneficial to the organism because of its sunscreen-like behavior and, hence, its photoprotective action against skin cancer. On the other hand (and probably originating mostly in pheomelanin), however, it is deleterious due to the ability to generate free radicals that may activate carcinogenic processes. It depends on the balance of these processes [8] whether the beneficial action of melanin is predominant or, in the worse case, malignant transformation occurs.

The complex response of melanin to sunlight is mediated via the fluorescent excited state. If the characteristics of melanin fluorescence, i.e., the spectral shape, quantum yield, and lifetime(s) of the fluorescence, depend sensitively on the local environment—as shown below—one essential prerequisite is fulfilled, that *selectively* detected melanin emission from the epidermal region of the human skin could reflect these complex processes initiated in the excited state. This could finally result in fluorescence criteria to differentiate healthy skin tissue, the intermediate steps of melagonesis, and malignant melanoma. But because of the overlap of the absorption regions of other skin fluorophores (e.g., β-carotene, NADH) with the melanin absorption and because of the extremely low fluorescence quantum yield of melanin, with conventional one-photon excitation (OPE) in the ultraviolet/visible (UV/VIS) region this selective melanin fluorescence detection is practically impossible [9–12].

Recently we proposed a new approach for selective excitation of melanin fluorescence in human skin tissue by stepwise two-photon excitation (TPE) in the near-infrared (NIR) region [13]. In that study, where we concentrated on pure (synthetic) melanin dissolved in dimethyl sulfoxide (DMSO) and excised samples from healthy human skin tissue, melanin fluorescence was induced by TPE at 800 nm using 120-fs pulses [13]. This new approach is based on the unique light absorption behavior of melanin, with its monotonously decreasing absorption spectrum between the near-UV and the NIR (cf. Fig. 1), which is completely different from the absorption of other organic fluorophores showing spectra with discrete absorption bands. In the NIR region of melanin absorption one can find spectral regions where there are no interfering absorptions of other fluorophores in the skin (e.g., around 800 nm) and where sufficient absorption exists [approximately 0.1 of the optical density (OD) at 400 nm] to excite the melanin emission by stepwise excitation with two NIR photons.

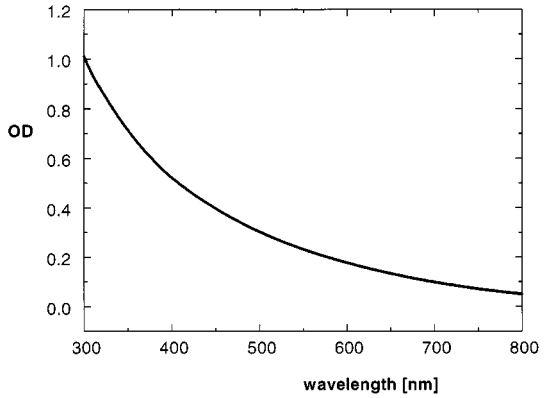

Fig. 1. Absorption spectrum of synthetic melanin, dissolved in DMSO; sample cell pathlength, $d = 1$ cm.

Extending our mentioned previous studies [13], we now have included other solvents in the TPF investigations of synthetic melanin. Furthermore, the first TPF results with different human skin tissues (healthy, nevus cell nevi, and malignant melanoma) are presented. They suggest that fluorescence excited in such a way indeed may yield information on malignant transformation.

MATERIALS AND METHODS

Chemicals. Synthetic melanin prepared by oxidation of tyrosine with hydrogen peroxide was purchased from Sigma. For further purification the pigment was treated with 3% HCl at room temperature several times and collected by centrifugation. The black precipitate was first washed with water (seven times) and successively with acetone (five times) and ether (three times). The black, amorphous powder was dried in vacuum at 50°C. To avoid long-term changes of the pigment, several small samples were sealed off into ampoules under an atmosphere of argon. In general, the solubility of purified synthetic melanin in organic solvents is very small. In a variety of appropriate organic solvents the solubility of the purified pigment is not enough for our measurements. Therefore we have chosen a few selected solvents such as DMSO (UVASOL, spectrometric grade; Merck), ethylene glycol monomethyl ether (EGE; for analysis; Merck), and water (bidistilled) with different polarities which are suitable for our investigations. DMSO and EGE contain a maximum of 0.1% water. The water content was drastically reduced by drying over a molecular sieve. In DMSO, however, synthetic melanin has a considerably enhanced

solubility [16] compared to other solvents [17,16]. The maximum solubility was found for an OD per 1-cm optical pathlength $OD_{1cm} = 3$ at 500 nm. Solutions of melanin in EGE were prepared by sonification at 40°C for 5 min under an atmosphere of argon. The slightly opaque solution was then filtered and showed a sufficient solubility (maximum value for $OD_{1cm} = 1$ at 500 nm). For preparation of a KOH solution, melanin was dissolved in 1 *M* KOH at room temperature. The solubility is high enough in getting an $OD_{1cm} = 2$ at 500 nm. The solubility in water is extremely low. By sonification, (20 min) at 40°C, an $OD_{1cm} = 0.2$ at 500 nm was obtained. For all measurements only freshly prepared solutions were used. Absorption and fluorescence spectra of these melanin solutions do not differ when solutions are bubbled with dry argon.

Human Skin Tissue. Samples of human skin tissues were excised from patients and longitudinal sections parallel to the skin surface (area, $\approx 0.5 \times 0.5$ cm^2; thickness, 0.1–0.2 cm) were prepared. To conserve these samples they were kept in a NaCl solution (9 g/L). For fluorescence studies the so-prepared skin pieces in the NaCl solution were inserted into standard quartz cuvettes (d = 0.2 cm).

Absorption and Fluorescence Measurement. Absorption spectra were recorded on a Lamda-19 spectrophotometer (Perkin–Elmer). Fluorescence spectra were measured with a Fluorolog FL-112 (Spex Industries). Fluorescence quantum yields were determined relative to the fluorescence quantum yield of 9,10-diphenylantracene in cyclohexane ($\phi = 0.90$ [18]). The solutions were excited at 400 nm ($OD_{1cm} = 0.03$) and the corrected emission spectra were integrated taking into account the reabsorption and the refractive index of the solutions.

The equipment for the measurement of two-photon excited fluorescence (TPF) spectra is depicted in Fig. 2. Excitation was performed with a Clark regeneratively amplified Ti:sapphire laser (repetition rate, 1 kHz; excitation pulse duration, 120 fs; center wavelength, 800 nm; maximal used pulse energy, 5 μJ). The melanin solutions (optical pathlength, 1 cm) were directly excited by means of lens L1 (focal length, 150 mm) and the fluorescence was measured at a right angle to the excitation. In the case of the fluorescence studies with excised skin tissue, an additional mirror (M1) was inserted in the excitation beam. To probe the melanin-containing epidermal region of the skin, the fluorescence was excited normal to the skin surface (mirror M2, lens L1). With an *xy*-positioning system an appropriate site of the tissue was adjusted for illumination. Additionally, the illumination spot was monitored by a CCD camera to check the status of the sample. The emission was observed at an angle of about

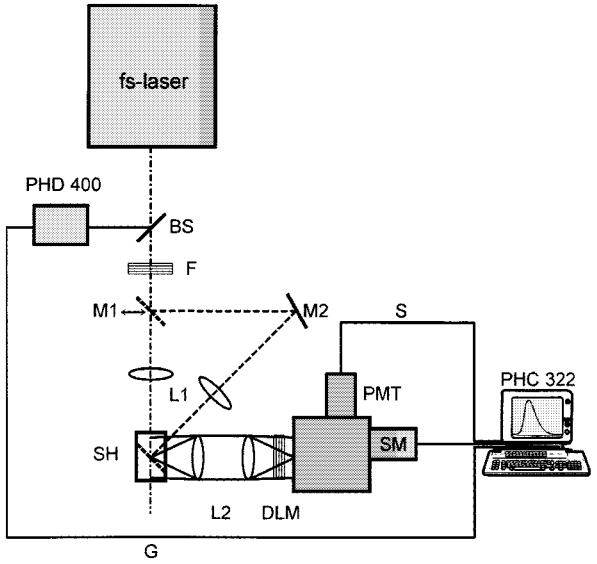

Fig. 2. Experimental setup for the TPF studies of synthetic melanin in solution and of excised samples from human skin tissue (for explanation see text).

30° to the excitation beam. In both cases the fluorescence was imaged (lenses L2) to the entrance slit of a spectrometer consisting of a monochromator M (type, λ-Scanner, Max-Born-Institute Berlin; aperture, 1:3, wavelength range, 300–800 nm) fitted with a PHC 322 gated photon counting module (Becker & Hickl, Berlin) and a high-speed PMH-100 PMT detector head (Becker & Hickl), which is equipped with a Hamamatsu H5783P-01 photosensor module with an extended red photocathode. To get a gating pulse for photon counting, a small part of the excitation light was separated by a beam splitter (BS) and directed to a PHD-400 photodiode (Becker & Hickl). Especially in the investigations with excised skin tissue, a dielectric laser mirror with a high reflectance at 800 nm and a high transparency in the spectral region of interest (400–700 nm) was applied immediately in front of the entrance slit of the emission monochromator to suppress scattered excitation light. The emission spectra were spectrally corrected.

RESULTS AND DISCUSSION

Synthetic Melanin in Solution

One-Photon Excitation of the Fluorescence (OPF)

OPF is the conventional activation of fluorescence emission of organic molecules (with the precondition of a sufficient one-photon absorption cross section). The fluorescent excited state is populated via the Franck–

Condon (FC) state occupied directly by the absorption transition. Therefore the fluorescence is generally Stokes-shifted to longer wavelengths in comparison to excitation.

Basic investigations of OPF of melanin were carried out by Kozikowski *et al.* [16] and Gallas and Eisner [17]. They found emission spectra that revealed several bands dependent on the excitation wavelength. The appearance of distinct emission bands for different excitation wavelengths is in contrast to the structureless, unspecified spectral absorption behavior (cf. Fig. 1) and represents a striking indication of heterogeneity. Previously we have studied the emission characteristics of synthetic melanin in DMSO [13]. As mentioned above the solubility of synthetic melanin in organic solvents is very low, and among a variety of solvents, DMSO showed the best one. However, only three further solvents were found to have a sufficient solubility for synthetic melanin and were therefore suitable to extend our previous investigations: KOH, EGE, and H_2O (cf. Materials and Methods).

In Fig. 3 the OPF spectra of synthetic melanin in these solvents are collected for an excitation wavelength $\lambda_{exc} = 400$ nm (this was chosen with respect to the TPE at 800 nm described below). Reabsorption effects were widely eliminated by using low optical densities ($OD_{1cm} < 0.05$ at 500 nm). The fluorescence quantum yield ϕ_F of synthetic melanin is very low. In the case of $\lambda_{exc} = 400$ nm it amounts to 1×10^{-3} for DMSO and KOH and is further reduced for H_2O ($\phi_F = 6 \times 10^{-4}$) and EGE ($\phi_F = 2 \times 10^{-4}$). In aqueous solution, EGE and KOH synthetic melanin exhibits relatively homogeneous OPF spectra. The emission spectrum in H_2O peaks at about 510 nm; in EGE it is only slightly shifted to the red but is noticeably broader; in KOH, however, the maximum is shifted to 530 nm. In contrast, the emission spectrum in DMSO is essentially broader and much more structured. Beside shoulders at about 500 and 650 nm, respectively, it peaks at 575 nm (it should be mentioned that for an excitation wavelength $\lambda_{exc} = 337$ nm, the shoulder at about 500 nm disappears [13]). For a better characterization of the origin of the emission, fluorescence excitation spectra at several emission wavelengths were measured for all solvents used. They show the same trend. As representative examples, in Fig. 4 such spectra for the DMSO solution are depicted. The shape of the excitation spectrum changes with variation of the emission wavelength, and crossing points of the individual curves indicate strong heterogeneity, that is, different absorbing species contribute to the emission. As a consequence, strong changes in the emission spectrum (spectral shape and shifts) occur dependent on the excitation wavelength.

The time-resolved fluorescence characteristics of melanin exhibits multiexponential decay [19,13]. Especially for synthetic melanin in DMSO at $\lambda_{exc} = 337$ nm, we previously found three exponentials by global deconvolution analysis in the emission range 500–800 nm: $\tau_1 \leq 200$ ps, $\tau_2 = 1.9$ ns, and $\tau_3 = 7.9$ ns [13]. The weight factors (amplitudes) are dependent on the emission wavelength and confirm very strongly the heterogeneity of the sample.

Two-Photon Excitation of the Fluorescence TPF

As already discussed in Ref. 13, two principles are possible to realize TPF, namely, *simultaneous* and *stepwise* absorption of two photons. Simultaneous TPE takes place instantaneously without any intermediate energy

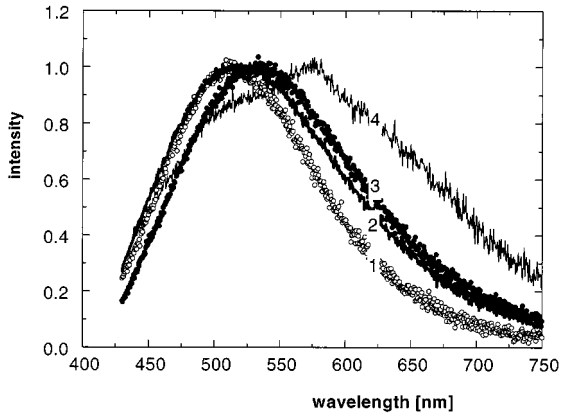

Fig. 3. Fluorescence spectra of synthetic melanin in different solvents ($OD_{1cm} < 0.05$ cm^{-1} at 500 nm, $\lambda_{exc} = 400$ nm): (1) H_2O; (2) EGE; (3) KOH; (4) DMSO.

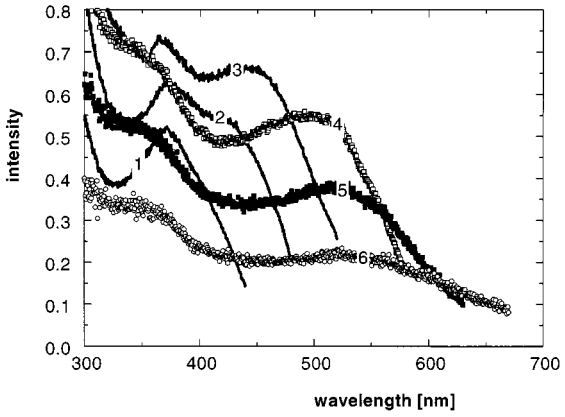

Fig. 4. Fluorescence excitation spectra of synthetic melanin in DMSO ($OD_{1cm} = 0.03$ at 500 nm) for different emission wavelengths λ_{em}: (1) $\lambda_{em} = 460$ nm; (2) $\lambda_{em} = 500$ nm: (3) $\lambda_{em} = 540$ nm; (4) $\lambda_{em} = 600$ nm; (5) $\lambda_{em} = 650$ nm; (6) $\lambda_{em} = 700$ nm.

level, that is, the one-photon absorption cross section at the excitation wavelength is vanishingly small, whereas that of two-photon absorption is typically of the order 10^{-48}–10^{-50} cm^{-4} · s [20]. Stepwise TPE, on the other side, requires a real intermediate energy level, that is, a noticeable one-photon absorption at about half the energy of the transition from the ground to the fluorescing state. Simultaneous TPE needs a two or more order of magnitude higher excitation intensity to get the same population density of the fluorescing energy level compared to the stepwise one.

The normalized population densities of the excited fluorescing energy levels for simultaneous and stepwise TPE can be given analytically [see Eqs. (1) and (2)], if the following preconditions hold: (i) Gaussian pulse shape $i(t)$, with τ the pulse duration (FWHM) and, I_{max} the peak intensity at time t_0, (ii) slow relaxations of the populated excited states in comparison to the pulse duration, and (iii) a low population of the excited energy levels.

$$n_A(I_{max}, t_0) = 0.38\sigma_{12}^{(2)}\tau\, I_{max}^2 \quad (1)$$

$$n_B(I_{max}, t_0) = 0.14\sigma_{12}\sigma_{23}\tau^2\, I_{max}^2 \quad (2)$$

Equation (1) is valid for simultaneous TPE in case of a two-level system (level 1, ground state; level 2, fluorescent energy level; $\sigma_{12}^{(2)}$, two-photon absorption cross section). It results immediately by solving the rate equation [Eq. (3)] of the fluorescing level (population density $n_A = n_2$, normalized according to $n_1 + n_A = 1$):

$$\frac{\partial}{\partial t} n_A = \sigma_{12}^{(2)} I^2 n_1 \quad (3)$$

with $I = I_{max}\, i(t)$ and $n_1 \approx 1$.

Equation (2) belongs to the stepwise TPE process in the case of a three-level system (level 1, ground state; level 2, intermediate energy level; level 3, fluorescent energy level; σ_{12}, one-photon absorption cross section for the first absorption step between level 1 and 2; σ_{23}, one-photon absorption cross section for the second absorption step between level 2 and level 3—both values are typically of the order 10^{-16}–10^{-17} cm^{-2}) and results by solving, first, the rate equation for the population density n_2 of the intermediate energy level and, then, with this result, the rate equation for the population density $n_B = n_3$ (normalized according to $n_1 + n_2 + n_B = 1$) of the fluorescing level:

$$\frac{\partial}{\partial t} n_2 = \sigma_{12} I n_1 \quad (4)$$

$$\frac{\partial}{\partial t} n_B = \sigma_{23} I n_2 \quad (5)$$

with $I = I_{max} i(t)$ and $n_1 \approx 1$.

As can be seen from Eqs. (1) and (2), the population densities n_A and n_B (which are directly proportional to the corresponding fluorescence intensity) are dependent on the squared excitation intensity. From these equations it is also obvious that one needs considerably smaller excitation intensities in the stepwise-excitation case compared to simultaneous excitation to get the same population density of the fluorescent level.

Because of the long absorption tail toward the NIR range, melanin has a sufficient absorption at twice the wavelength of the spectral region for activation of OPF, e.g., at 800 nm (cf. Figs. 1 and 5). So, TPF of melanin should be realizable by stepwise absorption of two photons in the NIR at 800 nm. However, no TPF for nanosecond or picosecond excitation could be observed, but 120-fs excitation was successful, showing an intensity-squared dependence, which confirms the two-photon nature of the excitation process [13]. The results are given below.

Figure 5 shows the TPF spectra of synthetic melanin for the used solvents upon 800-nm/120-fs excitation in comparison to their OPF spectra at $\lambda_{exc} = 400$ nm. The TPF spectra exhibit an enhanced environment sensitivity, that is, a more complex structure and a stronger shift to the red spectral region in the series $H_2O \rightarrow$ EGE $\rightarrow$ KOH $\rightarrow$ DMSO as can be seen compared to the OPF spectra. In aqueous solution the TPF spectrum is slightly shifted, to ≈540 nm, and contrary to the OPF spectrum, a new emission band occurs at about 630 nm, which is more increased in KOH. In EGE, however, the short-wavelength part of the TPF spectrum decreases and the overall emission maximum peaks at 580 nm, i.e., around 70 nm, toward longer wavelengths as the corresponding OPF spectrum. For DMSO the long-wavelength part of the TPF spectrum, with a maximum at about 620 nm, predominates. Because the spectra of synthetic melanin in DMSO and EGE are identical to those obtained using specially dried solvents, water content is not responsible for the shoulder at about 500 nm in DMSO.

The TPF decay is similar to the OPF, showing multiexponential time behavior. For the overall emission in DMSO at $\lambda_{exc} = 800$ nm, three components, with $\tau_1 = 200$ ps, $\tau_2 = 1.5$ ns, and $\tau_3 = 5.8$ ns were measured [13].

The strong differences between OPF and TPF spectra together with the sufficient absorption in the NIR range at 800 nm and the intensity-squared dependence of TPF support the stepwise nature of the TPE process for melanin. An exact method to decide between simultaneous and stepwise TPE results from Eqs. (1) and (2), namely, fluorescence studies with excitation pulses of the same photon flux density but different pulse durations in the femtosecond range. In the case of stepwise TPE the emission should have a squared dependence on the excita-

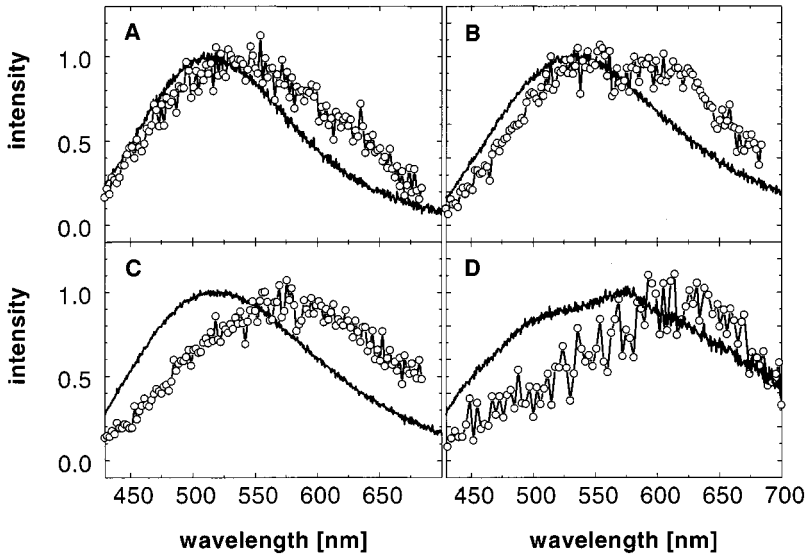

Fig. 5. TPF spectra of synthetic melanin in different solvents in comparison to one-photon-excited emission ($OD_{1cm} < 0.05$ at 500nm). (—) UV excitation at 400 nm; (—○—) NIR excitation at 800 nm): A, H_2O; B, KOH; C, EGE; D, DMSO.

tion pulse duration; otherwise, a linear one. Such investigations are currently in preparation.

Human Skin Tissue

First, investigations of TPF, predominantly with excised samples from healthy human skin tissue, were already discussed in Ref. 13. They exhibit a remarkable difference between the spectral shape of the UV one-photon (400 nm) excited steady-state fluorescence spectrum and the TPF spectrum excited with 120-fs pulses with a center wavelength at 800 nm (similar to the experiments with synthetic melanin in solution described above). The TPF spectrum is clearly broader compared to the OPF spectrum and is shifted a little to longer wavelengths. This behavior, with increased emission at the redmost side of the TPF emission band, is similar to the TPF spectra of synthetic melanin in solution. Because of the strong environment sensitivity of the two-photon excited melanin fluorescence, differentiation between a healthy, pigmented skin tissue and malignant melanoma can be expected.

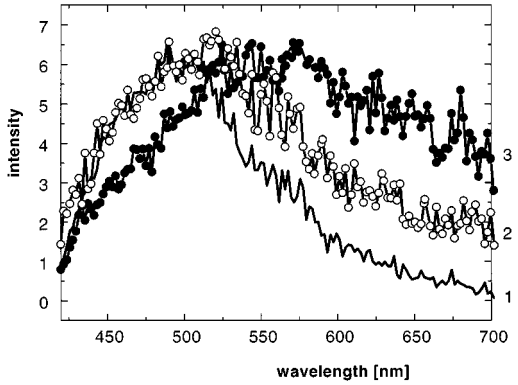

Fig. 6. TPF spectra of excised samples of human skin tissue: characteristic examples of healthy skin tissue (1), common nevus cell nevus (2), and malignant melanoma (3). The spectra are normalized.

The results for excised samples of skin tissue given in Fig. 6 support this assumption. Characteristic TPF spectra are depicted for healthy skin tissue, common nevus cell nevus, and malignant melanoma. They show characteristic differences, especially a distinct bathochromic shift of the maximum (from ≈500 nm for healthy skin tissue to about 550 nm for malignant melanoma) and a drastic enhancement of the intensity in the long-wavelength part of the TPF spectrum in the case of malignant melanoma. In contrast to this, the OPF spectra do not show any characteristic feature for malignant melanoma. A detailed discussion, in particular of medical aspects, will be published elsewhere [21].

CONCLUSIONS

The fluorescence of melanin has a much higher information content than its optical absorption. This includes the substructure as well as the sensitivity to the microenvironment. The peculiarities are clearly enhanced

in the case of two-photon excitation of this fluorescence. With respect to melanin in skin, stepwise two-photon excitation has the additional advantage of elimination of fluorescence of other skin fluorophores. The ultimate goal of the application of TPF to skin tissue could be a new method of noninvasive, early detection of black skin cancer; this possibility is currently under investigation.

ACKNOWLEDGMENTS

We gratefully acknowledge the collaboration with the fs-spectroscopy application lab of the Max-Born-Institute (Dr. F. Noack). The technical assistance of R. Lendt, K. Herrmann, and F. Moldenhauer is appreciated. Financial support by the Deutsche Forschungsgemeinschaft (grant Le 729/6-1) is acknowledged.

REFERENCES

1. N. Kollias, R. M. Sayre, L. Zeise, and M. R. Chedekel (1991) *J. Photochem. Photobiol. B Biol.* **9**, 135–160.
2. G. A. Swan (1974) *Fortschr. Chem. Org. Naturst.* **31**, 521–582.
3. A. Napolitano, M. G. Corradini, G. Prota (1985) *Tetrahedon Letters* **26**, 2805–2808.
4. M. B. Clark, J. A. Gardella, T. M. Schultz, D. G. Patil, and L. Salvati (1990) *Anal. Chem.* **62**, 949–956.
5. C. Linge (1996) *Cancer Surveys* **26**, 71–87.
6. L. Zeise, M. R. Chedeckel, and T. B. Fitzpatrick (eds.) (1995) *Melanin: Its Role in Human Photoprotection*, Valdenmar, Overland Park.
7. H. Z. Hill (1996) *Photochem. Photobiol. (Spec. Iss.)* **63**, 41S–42S.
8. T. G. Salopec, K. Yamada, S. Ito, and K. Jimbow (1991) *Pigment Cell Res.* **4**, 172–179.
9. H. J. C. M. Sterenborg, M. Motamedi, and R. F. Wagner (1994) *Lasers Med. Sci.* **9**, 191–201.
10. A. Pradhan, P. Pal, G. Durocher, L. Villeneuve, A. Balassy, F. Babai, L. Gaboury, and L. Blanchard (1995) *J. Photochem. Photobiol. B Biol.* **31**, 101–112.
11. H. J. C. M. Sterenborg, S. Thomson, S. L. Jacques, and M. Montamedi (1995) *Melanoma Res.* **5**, 211–216.
12. Yu. P. Sinichkin, S. R. Utts, I. V. Meglinskii, and E. A. Pilipenko (1996) *Opt. Spectrosc.* **80**, 383–389.
13. K. Teuchner, W. Freyer, D. Leupold, A. Volkmer, D. J. S. Birch, P. Altmeyer, M. Stücker, and K. Hoffmann (1999) *Photochem. Photobiol.* **70**, 146–151.
14. A. R. Young (1997) *Phys. Med. Biol.* **42**, 789–802.
15. T. M. Cooper, S. T. Schuschereba, R. D. O'Connor, D. L. Bolton, and D. J. Lund (1986) Letterman Army Inst. Res., Presidio of San Francisco, LAIR-216, Order No. AD-A168677/3/GAR.
16. S. D. Kozikowski, L. I. Wolfram, and R. R. Alfano (1984) *IEEE J. Quant. Electr.* **QE-20**, 1379–1382.
17. J. M. Gallas and M. Eisner (1987) *Photochem. Photobiol.* **45**, 595–600.
18. D. F. Eaton (1988) *J. Photochem. Photobiol. B Biol.* **2**, 523–530.
19. R. Cubeddu, F. Docchio, R. Ramponi, and M. Boulton (1990) *IEEE J. Quant. Electr.* **26**, 2218–2225.
20. C. Xu, W. Zipfel, J. B. Shear, R. M. Williams, and W. W. Webb (1996) *Proc. Natl. Acad. Sci. USA* **93**, 10763–10768.
21. K. Hoffmann, M. Stücker, P. Altmeyer, K. Teuchner, and D. Leupold (1999) submitted for publication.