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Fluorescence detection and photodynamic activity of endogenous protoporphyrin in human skin

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Abstract. Human skin shows a strong autofluorescence in the red spectral region with main peaks around 600, 620, and 640 nm caused by the porphyrin production of the gram positive lipophile skin bacterium Propionibacterium acnes. Irradiation of these bacteria reduces the integral fluorescence intensity and induces the formation of photoproducts with fluorescence bands around 670 nm and decay times of about 1 and 5 ns. The photoproduct formation is connected with an increased absorption in the red spectral region. The endogenous fluorescent porphyrins act as photosensitizers. Photodestruction of Propionibacteria acnes by visible light appears therefore to be a promising therapy. The photodynamic activity of the photoproducts is lower than that of protoporphyrin IX.

Subject terms: biomedical optics; fluorescence spectroscopy; protoporphyrin; autofluorescence; scattering; photodynamic activity.

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1 Introduction

UV radiation induces a luminescence of the human skin in the red spectral region[1] that is connected with the existence of the gram positive lipophile microorganism Propionibacterium acnes. The bacterium belongs to the normal microbial flora of the skin and is associated with the pathogenesis of acne vulgaris.[2]

Propionibacteria acnes are able to produce fluorescent porphyrins[3] that cause the red autofluorescence of the skin. The photosensitizing properties of various porphyrins have so far been studied after external application.[4–6] Energy and charge transfer from the metastable triplet state of the porphyrins results in the formation of singlet oxygen and reactive radicals. Besides cytotoxic effects, oxygen dependent photodestruction of the photosensitizer may occur. This process involves the decrease of the integral fluorescence (photobleaching) as well as the formation of photoproducts as shown, e.g., in the case of irradiation of the photosensitizer hematoporphyrin derivative.[7,8]

The purpose of the present work is to study the fluorescence behavior of the endogenous porphyrins of the human skin produced by Propionibacteria acnes upon irradiation.

2 Materials and Methods

2.1 *Objects of Investigation*

Twenty people of different ages and gender were used for in-vivo fluorescence spectroscopy on human skin. Propionibacteria acnes were isolated from sebaceous follicles and grown under dark conditions at 37°C in anaerobic jars for 5 days. Spectral behavior was investigated by measuring the colonies grown on the agar medium.

The photodynamic activity was tested on human erythrocytes (1 million cells in 1 ml NaCl solution) incubated with protoporphyrin (PP) or photoproducts of PP. Protoporphyrin IX was obtained from Porphyrin Products, Logan, Utah.

2.2 *Apparatus*

The spectrometry system based on krypton ion laser-induced fluorescence is shown in Fig. 1. Fluorescence is excited by the 406.7-nm line coupled to a 600-μm quartz fiber. The spontaneous radiation and the luminescence of the Brewster windows were separated by use of two diffraction prisms.[9,10] The fluorescent light is collected by a polished quartz fiber bundle arranged around the excitation fiber. The slit-adapted end of the bundle is fixed in front of the input slit of a polychromator. A dichroitic long-pass filter for wavelengths above 460 nm is used to reject the backscattered excitation light. Spectra are registered by the photodiode array of a multichannel analyzer.

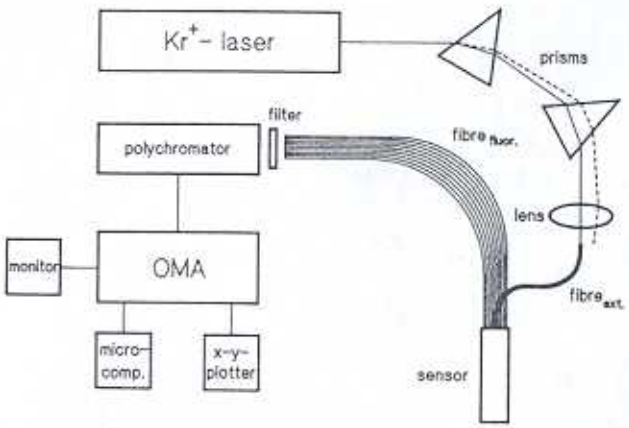

Fig. 1 Arrangement of the fluorescence detection equipment.

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Time-resolved fluorescence measurements were carried out using a novel frequency-doubled laser diode as excitation source (390 nm, 40 ps, 100 kHz) and a single photon counting unit.[11]

The photodynamic activity was determined by measurements of Mie-scattering using a cw-laser diode (780 nm) that induces the light scattering and a tuneable dye-laser that induces the photodynamic reactions (Fig. 2).[12] Scattering was measured at an angle of 12 deg to the incident light beam. Swelling of the cells was expected to increase the signal, whereas cell destruction was expected to decrease the scattered signal.

3 Results and Discussion

3.1 *Fluorescence Spectroscopy*

All those investigated with the described fiber optic sensor showed a strong autofluorescence in the red spectral region. The fluorescence was found to be located in sebaceous follicles that contain large amounts of the skin bacterium Propionibacterium acnes. The structured spectrum consisted of three main peaks around 600, 620, and 640 nm (see Fig. 3). Intensity and ratio of these spectral bands vary in dependence on the irradiated area of the face. Comparative studies on different porphyrins in a hydrophobic environment show a strong similarity with the spectral bands of zinc protoporphyrin, coproporphyrin and protoporphyrin.

Propionibacteria acnes isolated from sebaceous follicles produce species with either a 635-nm fluorescence band (protoporphyrin) or a 620-nm band (coproporphyrin) or species that emit both fluorescence bands.

Photoinduced changes during 407-nm irradiation of bacteria with a fluorescence band around 635 nm are shown in Fig. 4. The intensity at 635 and 700 nm decreases, but the fluorescence intensity around 670 nm increases. That means that in addition to a photobleaching process, a fluorescent photoproduct is also formed. This additional fluorescence increases, reaches a maximum, and slowly decreases during further light exposure.

Irradiation of solutions of protoporphyrin IX in dimethylsulfoxide (DMSO) (Fig. 5) results in the same photoinduced fluorescence modifications. A fluorescent photoproduct with an absorption maximum at 440 nm and an additional absorption band around 660 nm occurs. That means that under irradiation the endogenous protoporphyrins are partly converted to photoproducts, called photoprotoporphyrins.[13]

3.2 *Time-Resolved Fluorescence Measurements*

Fluorescence decay curves from fluorescent extrusions of some comedones (obtained by pressure extraction) are demonstrated in Fig. 6. The deconvolution of the fluorescence curve of the nonirradiated sample gives two decay times of about 1 and 6 ns. Irradiation results in a decrease of the integral fluorescence and a shortening of the decay times to 630 ps and 3.4 ns (lower curve). A classification of the decay times is difficult because the content of these comedones is a mixture of zinc protoporphyrin, coproporphyrin, and protoporphyrin that occur in monomeric and aggregated forms. However, monomeric protoporphyrin and coproporphyrin in dimethylsulfoxide show a decay time of about 15 to 20 ns, and zinc protoporphyrin a decay time of about 2.4 ns. The decay times of the photoprotoporphyrins could

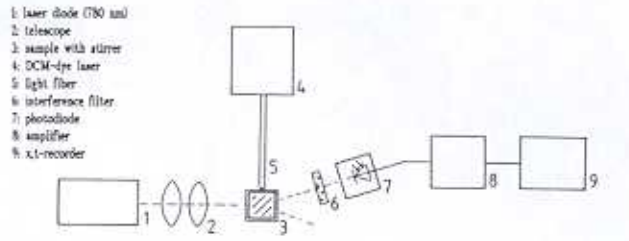

Fig. 2 Setup for scattering measurements on human erythrocytes: 1—laser diode (780 nm), 2—telescope, 3—sample with stirrer, 4—dye laser, 5—fiber, 6—interference filter, 7—photodiode, 8—amplifier, and 9—*x,t*-recorder.

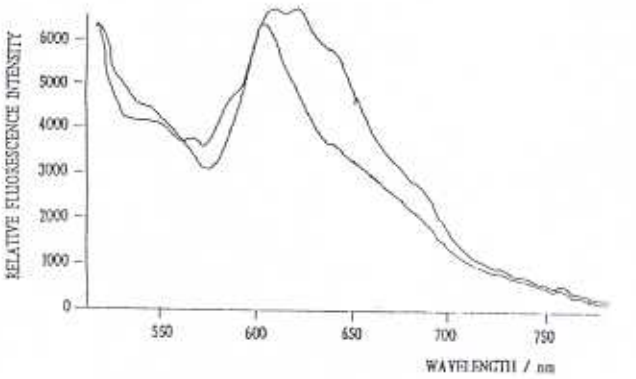

Fig. 3 In-vivo fluorescence spectrum of human skin showing at least three fluorescent species with strongly varying intensities (both curves from the same person).

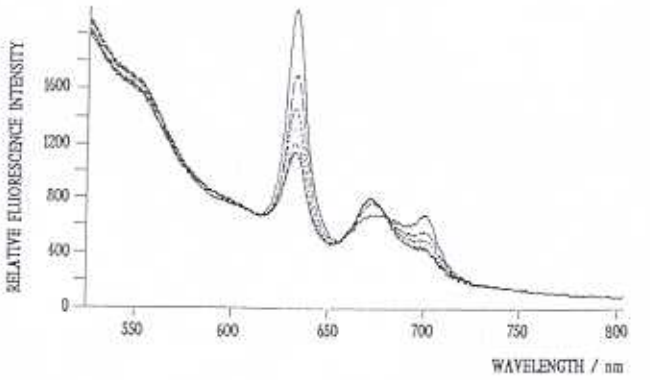

Fig. 4 Photoinduced changes of the autofluorescence spectrum of Propionibacterium acnes colonies on agar during 407-nm irradiation with increasing exposures of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 J/cm^2. Note the formation of fluorescent photoproducts at 670 nm.

be determined to be 675 ps and 4.5 ns in aqueous solution (Fig. 7) and 1.1 and 5.8 ns in epithelial cells.[14] So, it seems possible that the shortening of fluorescence decay times on irradiation may be caused by the bleaching of mainly copro- and protoporphyrin monomers (in contrast to the zinc-protoporphyrin fluorescence) as well as on a possible photoproduct formation.

3.3 *Photodynamic Activity*

Fresh and irradiated solutions of protoporphyrin (the latter contains a large amount of photoproducts) were incubated

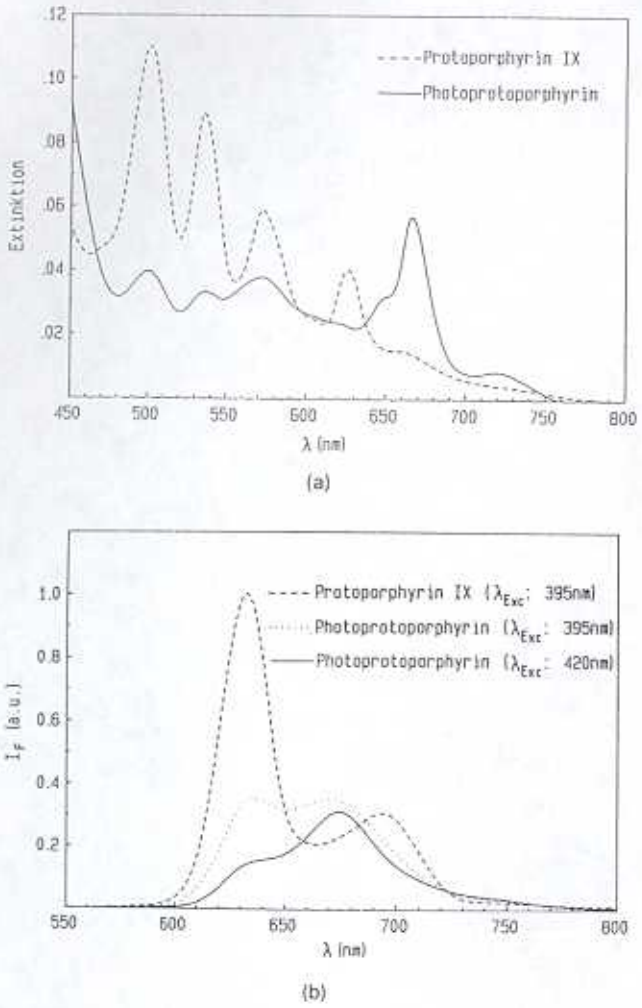

Fig. 5 (a) Absorption and (b) fluorescence spectrum of protoporphyrin and its photoproduct in dimethylsulfoxide.

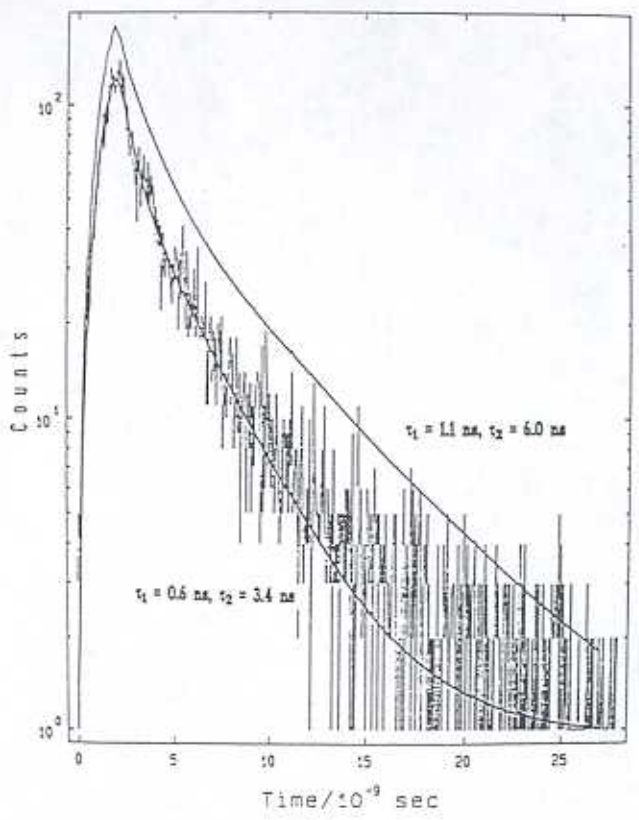

Fig. 6 Variation of the fluorescence decay properties of fluorescent comedones in semilogarithmic scale before and after irradiation (mercury lamp, 5 J/cm^2): Before $t_1 = 1.1$ ns, $t_2 = 6.0$ ns, and $\chi^2 = 1.24$; after $t_1 = 630$ ps, $t_2 = 3.4$ ns, and $\chi^2 = 1.05$.

to human erythrocytes to prove the photodynamic activity of the photoproducts. Photochemically induced morphological changes and the process of hemolysis were registered by scattering measurements (see Fig. 8). Action spectra were measured by varying the emission wavelength of the dye laser. As shown in Fig. 9, the photodynamic activity of the photoproducts was found to be dependent on the irradiation wavelength, but was lower than that of protoporphyrin. The highest activity was obtained for the fresh protoporphyrin solution at 630-nm irradiation. The irradiated solution shows at this wavelength a photodynamic effect also, but this was mainly due to the small part of protoporphyrin that is not converted into photoprotoporphyrin. A much lower photodynamic activity was measured at 670 nm where the photoproducts absorb strongly. The higher effect of the irradiated solution at this wavelength in comparison with the fresh one is caused by the small photodynamic activity of photoprotoporphyrin.

4 Conclusions and Outlook

The human skin contains fluorophores that are photodynamically active and photo-instable substances. These endogenous fluorophores, mainly protoporphyrin, are produced by Propionibacterium acnes. High amounts of these bacteria are found in closed comedones and especially in patients suffering from acne vulgaris.

It should be possible to induce a selective therapeutic effect on these patients by irradiation of large skin areas using light sources emitting radiation at wavelengths that correspond to the absorption bands of protoporphyrin. This irradiation results in a destruction of the bacteria by their endogenously produced porphyrins that act as cytotoxic photosensitizers.

Indeed, recently we found a photodynamically induced inactivation of Propionibacteria acnes by violet (407 nm) and red light[15] and Meffert et al.[16] treated 34 patients with acnes vulgaris by means of blue light. They reported an improvement of the disease. The efficiency of this treatment depends on the concentration of endogenous porphyrins and oxygen as well as on the penetration depth of the radiation.

In addition to the photoinduced cytotoxic effects, on-line fluorescence measurements should allow monitoring of the photo-oxidation reactions leading to photoinactivation of the bacteria and simultaneous destruction of the sensitizer. The latter becomes obvious as photobleaching and photoproduct formation. The corresponding changes in fluorescence thus can be used as an indicator of the therapeutic efficiency.

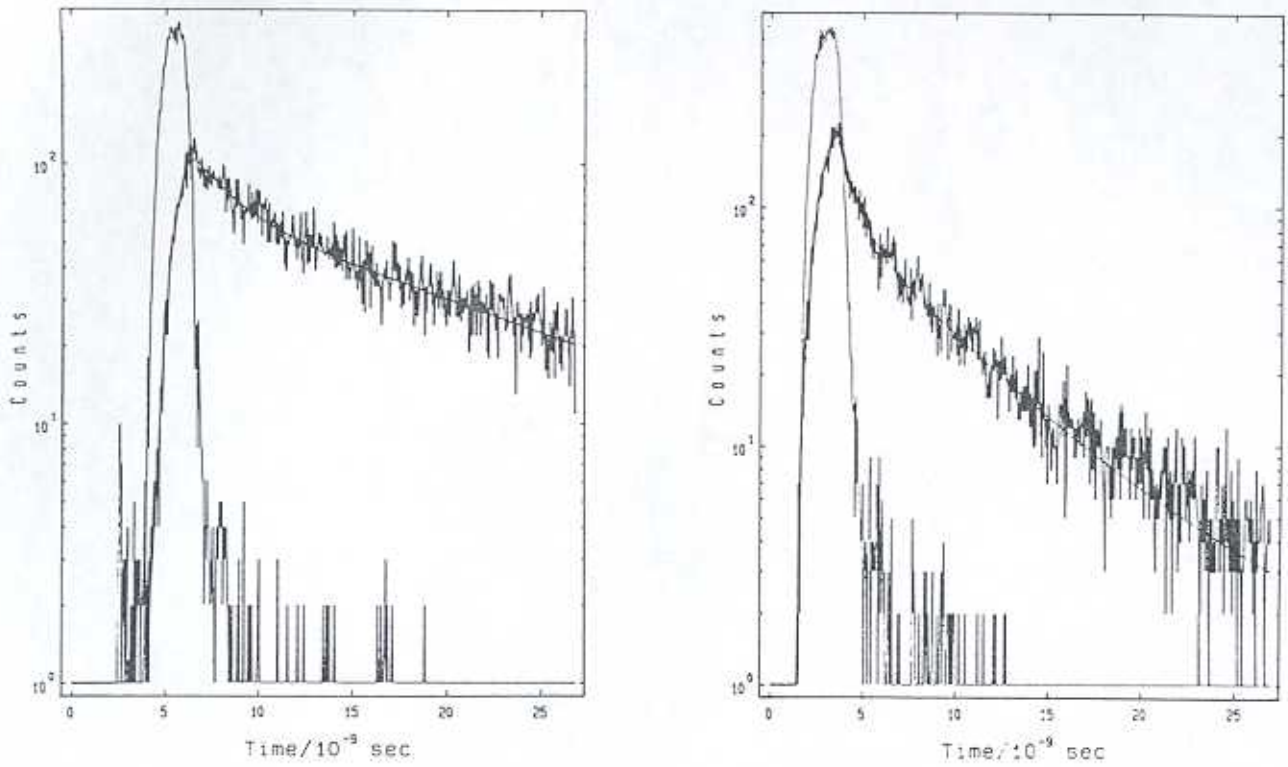

Fig. 7 Fluorescence decay in dimethylsulfoxide of, left, protoporphyrin [$t_1 = 3$ ns (11%) and $t_2 = 17$ ns (89%)] and, right, photoprotoporphyrin [$t_1 = 670$ ps (38%) and $t_2 = 4.5$ ns (62%)].

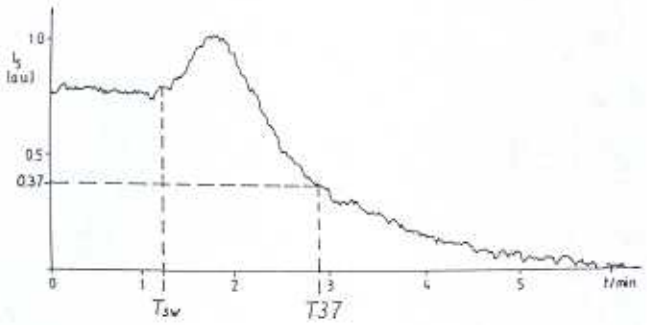

Fig. 8 Time-dependent scattered signal. The figure shows the process of swelling followed by the hemolysis (signal drops to zero).

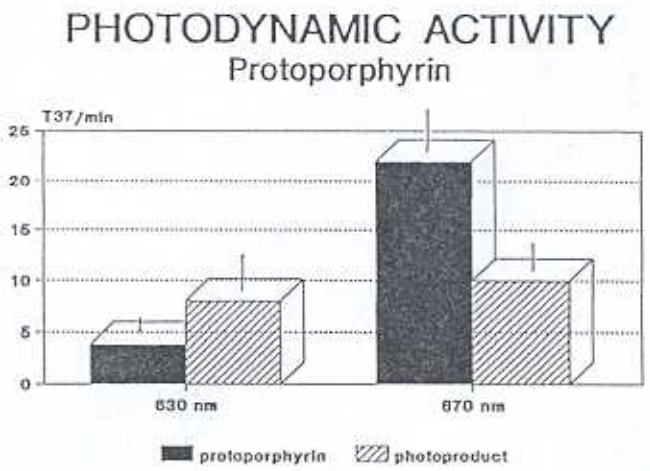

Fig. 9 Wavelength dependence of photodynamic activity. T37: time after which the fraction of living cells is reduced to 37%. Wavelength is in nanometers.

Additional irradiation around 670 nm can increase the therapeutic effect in the case of photoprotoporphyrin formation. However, the photodynamic activity of the photoproducts is low in spite of the increased absorption in the red spectral region.

Acknowledgments

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Karsten König: Biography and photograph appear with the paper "Fluorescence decay kinetics and imaging of NAD(P)H and flavins as metabolic indicators" in this issue.

Angelika Rück: Biography and photograph appear with the paper "Photodynamic cancer therapy" in this issue.

Herbert Schneckenburger: Biography and photograph appear with the paper "Fluorescence decay kinetics and imaging of NAD(P)H and flavins as metabolic indicators" in this issue.