The Possibility of Measuring Thermal Protein Denaturation by an Optoacoustic Method

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ABSTRACT

The coupling of proteins to a chromophore allows in principle to achieve a high temperature for a short time in a highly confined microvolume. On the one hand this can be used for the study of very rapid thermal denaturation, which may happen in microseconds. On the other hand it is possible to produce very precise damage to special cellular and subcellular structures by heating a microscopic volume. Calculations show that extremely photostable absorbers with picosecond relaxation time have to be used, a condition which can not be fulfilled by dye molecules.
Nevertheless submicron absorbing solid-state particles such as melanin granules can be used due to their higher absorption cross-section and better photostability.
Preliminary optoacoustic experiments were conducted to explore the possibility of detecting denaturation of proteins in such conjugates by their volume change, which is associated with the change of the tertiary structure. As expected, the melanin granules proved to be efficient photon-to-heat converters. Nevertheless, the volume change due to protein denaturation may be too small compared to the thermal expansion of water to allow quantitative evaluations.

1. INTRODUCTION

1.1 Thermal Denaturation

The kinetics of thermal damage in biological tissue is important in many medical applications of lasers such as coagulation, cutting, tissue welding, and laser hyperthermia. Although the denaturation kinetics in many experiments can be described as a rate process having an exponential dependence of the rate constant on the temperature in limited time and temperature intervals,[1 - 3] this relation seems not to hold over a time scale from microseconds to seconds. Retinal photocoagulation of rabbit eyes using microsecond laser pulses for example resulted in the same ophthalmoscopic visible lesions at lower temperatures compared to coagulations using a pulse width between milliseconds and seconds (see data in Ref. 4). This is in contradiction to a simple rate law of the denaturation process. Knowledge of the denaturation kinetics may also help to assess more precise exposure limits for the eye in the case of repetitive laser pulses.[5]
Since it is assumed that thermal damage of cells is correlated with the denaturation of proteins or enzymes in the cell,[6,7] knowing the denaturation kinetics of proteins in different time domains may help to understand thermal effects on tissue. Thermal denaturation which takes place in times of 0.1 sec and longer has already been investigated.[8,9,10,11] On the other hand denaturation experiments with very short temperature pulses in the order of microseconds are difficult to set up. A rapid heating of a certain volume is easily possible with a short laser pulse, whereas the time for cooling down of the heated volume depends on its size and is proportional to the square of its radius r. The typical thermal relaxation time τ_r for this process can be estimated by

$$\tau_r = \frac{r^2}{6D} \qquad (1)$$

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where D is the thermal diffusivity of the medium. In water, whose thermal properties are similar to tissue, a volume with a radius of 1 μm cools down in approximately 1 μsec. Therefore coupling of proteins to a small chromophore in principle allows to achieve a very short temperature rise and fall, since only a microvolume is heated which cools down very quickly. Another interesting aspect of heating microvolumes by small absorbing particles is the possibility to confine thermal damage to cellular and subcellular structures adjacent to the absorber.[12,13]

Of the several physical parameters which can be used to detect the denaturation of a protein (e.g. change of absorption, fluorescence, heat capacity or electrochemical properties)[10] we choose the change of volume. The protein denaturation is a result of a change of its tertiary structure which is associated with a molecular volume change[10] in the order of $5 \cdot 10^4$ Å^3. A rapid volume change should cause an acoustic signal which, in principle, could be detected by laser-induced optoacoustic spectroscopy (LIOAS).[14]

1.2 Laser-Induced Optoacoustic Spectroscopy

In solution usually after absorption of pulsed radiation by a chromophore the absorbed energy is partly or entirely converted to thermal energy, which causes a local heating and expansion of the solvent. A rapid volume change creates an acoustic wave which can be measured by a piezodetector. For a simple evaluation the height of the first maximum of the measured electrical signal is taken as the optoacoustic (OA) signal H_S. A linear relation between H_S and the absorbed energy has been shown in theory and experiments.[14,15] An additional volume change by a photoinduced reaction contributes as well to the OA signal. In water a separation of both signals is possible because its thermal expansion coefficient $\beta(T)$, which is proportional to the OA signal, varies largely with the temperature and becomes zero at approximately 4°C. Usually a simple additivity of the thermal volume change ΔV_{Th} and the reaction volume ΔV_R is assumed[14,16,15]

$$H_S = k'(\Delta V_{th} + \Delta V_R)$$

with (1)

$$\Delta V_{th} = \beta(T)\, \alpha \frac{E_a}{c_p \rho}$$

E_a being the energy absorbed in the sample volume; α is the conversion efficiency of absorbed energy to heat, c_p the heat capacity, and ρ the mass density of the solvent. Equation (1) has been derived for a cylindrical geometry neglecting effects of thermal diffusion and the temperature dependence of β. Measuring the OA signal H_R of a reference substance which has a known quantum yield α_{Ref} of heat production and no molecular volume change, should provide a signal according to

$$H_R = k'\, \beta(T)\, \alpha_{Ref} \frac{E_a}{c_p \rho} \tag{2}$$

The ratio H_S/H_R allows to cancel the constant k' which depends on various unknown parameters of the experimental set-up

$$\frac{H_S}{H_R} = \frac{\alpha}{\alpha_{Ref}} + \Delta V_R \frac{c_p \rho}{E_a\, \alpha_{Ref}\, \beta(T)} \tag{3}$$

2. THEORETICAL CONSIDERATIONS

For the denaturation of a protein in microseconds we assume that a temperature increase of more than 100 K in a volume of about 5 nm diameter is needed. Myoglobin,[17] for example, has the approximate dimensions of 2.5 × 4.4 × 4.4 nm^3. The radiant flux density which is necessary to heat a certain volume can be calculated by solving the differential equation of heat diffusion.[18]

$$\frac{\partial T}{\partial t} - D\Delta T = \frac{w}{\rho c_p}$$

with T: Temperature c_p: Specific heat (4)
t: Time D: Thermal diffusivity
ρ: Mass density w: Thermal energy produced per time and volume

The parameters of tissue are approximated by those of water, and photons with a wavelength of 500 nm are assumed throughout the following calculations. At first, let us consider a dye molecule coupled to a protein, which can be modeled by an infinitesimal small source of heat. Solving equation 4 for a rectangular laser pulse of a duration τ leads to[18,19]

$$T(r,t,\tau) = T_1(r,t) - T_1(r,t-\tau)$$

$$\text{with} \quad T_1(r,t) = \begin{cases} \dfrac{w}{4\pi D r c_p \rho} \operatorname{erfc}\left(\dfrac{r}{\sqrt{4Dt}}\right) & t > 0 \\ 0 & t \le 0 \end{cases} \tag{5}$$

where erfc(x) is the complementary error function and r is the distance from the heat source. The highest temperature rise for a given amount of thermal energy, which corresponds to a certain pulse energy, is obtained by depositing all energy $E_0 = w\tau$ in a very short time, i.e. $\tau = 0$. The maximal temperature reached at a distance r is then given by

$$T_{max} = \frac{E_0}{8\rho c_p (\pi e/6)^{2/3}} \frac{1}{r^3} \tag{6}$$

The number of photons needed to heat a point at a given distance can be obtained by this equation. At 1 nm distance 0.14 photons, at 5 nm 18 photons, and 140 photons at 10 nm distance give an increase of temperature by 1 K. It is obvious, that a dye molecule must absorb more than 100 K × 18 photons / K to denature a protein of 5 nm size. It can be expected that photodegradation of the dye limits the amount of photons being absorbed and hence the possible temperature increase. Using longer laser pulses even reduces the maximal attainable temperature per photon since thermal energy is lost by heat diffusion. Fig. 1 shows the calculated temperature reached at the end of the laser pulse depending on the pulse width. A constant irradiation of 1 GW/cm^2 and an extinction coefficient $\varepsilon = 10^5$ $dm^3/(mol\ cm)$ were assumed. A nearly constant temperature increase of 10 K is reached at 5 nm distance with a pulse width of more than 10 nsec. The top axis shows the

number of photons absorbed. Although a high irradiance of 1 GW/cm^2 is used a denaturation is not possible. For a temperature increase of 100 K the dye has to absorb 10^5 photons in 10 nsec which would require a thermal relaxation time below 100 fsec. A temperature increase of more than 100 K at a distance of 5 nm seems to be impossible because of the high photostability, the high irradiance, and the short relaxation time needed.

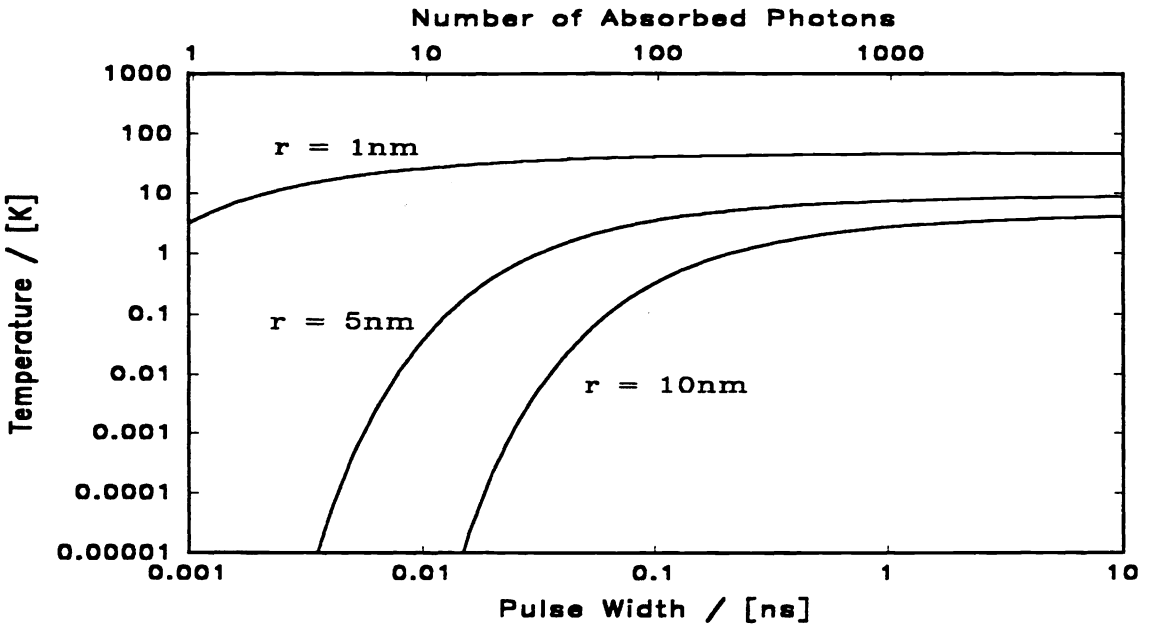

Fig.1. Heating of a micro-volume by a dye molecule. Calculated temperature increase at the end of the laser pulse at 1, 5 and 10 nm distance from the surface. A constant irradiance of 1 GW/cm^2 and a molar extinction coefficient of $\varepsilon = 10^5$ dm^{3}mol^{-1} cm^{-1} are assumed.

Therefore dye molecules are not suitable as heat sources for thermal denaturation. Nevertheless submicron solid state particles such as Melanin granules can combine very short time of thermalisation of absorbed energy with good photostability and an absorption cross-section which is several orders of magnitude higher than that of dyes.[20,21,22] Using geometrical optics and the assumption of a spherical shape with the radius R, the absorption cross-section σ_{Mel} of a melanin granule can be calculated:

$$\sigma_{Mel} = \int_{\text{cross-section}} (1 - \exp(-\mu_{Mel} z(df)))\, df = \frac{4}{3}\pi R^3 \mu_{Mel} \quad \text{for} \quad R << \frac{1}{\mu_{Mel}} \tag{7}$$

The integral is taken over the whole geometrical cross-section and z(df) is the distance that a photon travels through the granule in the infinitesimal part of area df. μ_{Mel} is the macroscopic extinction coefficient of melanin which can be estimated as $\mu_{Mel} = 1.6 \cdot 10^4/cm$.[13] A correction according to the Mie theory of scattering, which is expected to change the cross-section, is neglected.[23] Calculated cross-section, equivalent extinction coefficient ε, and the thermal relaxation time are shown in table 1 for two melanin samples (SepiaMelanInk™ and MelanInk™) used in the LIOAS-experiments.

	Radius / [nm]	σ_{Mel} / [cm^2]	ε_{Mel} / [dm^{3}mol^{-1}cm^{-1}]	t_r / [nsec]
MelanInk™	75 nm	$3 \cdot 10^{-11}$	$7 \cdot 10^9$	6
SepiaMelanInk™	17 nm	$3 \cdot 10^{-13}$	$7 \cdot 10^7$	0.3

Table 1: Radius, absorption cross-section σ_{Mel}, equivalent molar extinction coefficient ε_{Mel}, and thermal relaxation time τ_r of the melanin samples used in the experiments.

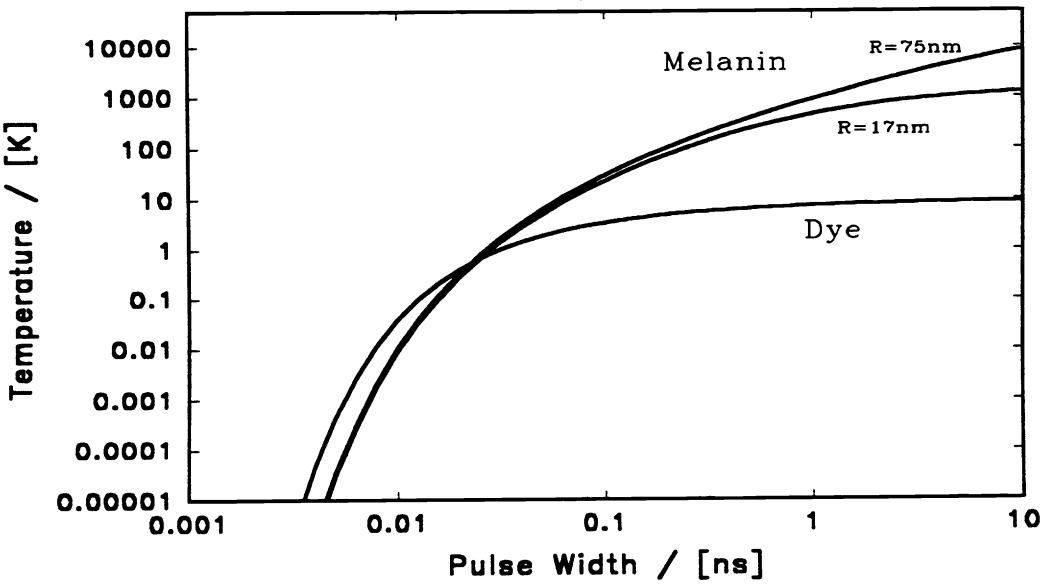

Fig. 2. Heating of a microvolume by melanin granules. Calculated temperature increase at the end of the laser pulse at 5 nm distance from the surface. A constant irradiance of 1 GW/cm^2 is assumed. For comparison the temperature increase by dye molecule ($\varepsilon = 10^5$ dm^{3}mol^{-1} cm^{-1}) at 5 nm distance from the surface is plotted.

The temperature increase (fig. 2) of the melanin granules at the end of a laser pulse of constant irradiance was calculated using a model of a sphere with constant heat production embedded in a medium of the same thermal properties (see problem II in reference 19, p. 348f). At 5 nm distance a three orders of magnitude higher temperature increase is possible

with the 75 nm melanin granules compared to the dye molecule (fig. 2). Therefore an increase of 100 K with a 10 nsec laser pulse can be achieved with a radiant exposure of only 100 mJ/cm^2 . A further advantage of solid state particles is a much less dependence of the temperature increase on the distance.

3. ESTIMATION OF THE OPTOACOUSTIC SIGNALS

In order to detect the denaturation by LIOAS the optoacoustic signal due to a volume change of the proteins has to be large enough compared to the signal caused by the thermal expansion of water. Using the approach of eq. (1) the ratio between both signals can be expressed by the ratio of the volume changes. With the supposition of certain volume change v_{Den}, which occurs when a temperature T_{Den} is reached, the ratio of the OA signals can be estimated. Differences in the thermal properties of water and melanin are neglected.

$$\frac{H_{Den}}{H_{Th}} = \frac{\Delta V_{Den}}{\Delta V_{Th}} = \frac{1}{\beta(T)} \frac{v_{Den} \ \gamma \ N}{4/3 \, \pi R^3 \, T_{Den}} \tag{8}$$

Where N is the number of proteins coupled to one absorber and γ is the ratio between the temperature reached at the location of the protein and the temperature $T = E_0 \mu_{Mel} / (c_p \rho)$ at the surface of the granule, neglecting losses due to heat diffusion. This estimation and equation (1) are a great simplification since they neglect effects due to heat diffusion and temperature dependence of β, and assume a cylindrical geometry. During a denaturation process the situation is different. At the beginning a high temperature and therefore high thermal expansion coefficient exist in very small statistically distributed spherical regions. After a certain time the temperature peaks level off in the large irradiated sample volume and an elevation of temperature below one Kelvin remains. Although theoretical calculations of the OA signal for varying geometry with different assumptions exist, this special case has not been treated. Since in most calculations the OA signal increases with shorter pulse width and smaller irradiated volumes, a strong contribution of the hot spherical regions to the OA signal of the whole volume is expected. The ratio H_{Den}/H_{Th} at two different temperatures may give an estimation. In principle H_{Den}/H_{Th} can be made arbitrarily high by choosing a temperature near to the value at which $\beta(T)$ becomes zero, but practically the temperature may be controlled only with in 10 - 100 mK. Table 2 shows the ratio H_{Den}/H_{Th} at two values corresponding to 5°C and 100°C for $T_{Den} = 100\,K$ and $v_{Den} = 5 \cdot 10^4$ Å^3. Calculations of γ for 9 nsec laser pulse width result in 0.3 and 0.03 for MelanInk and SepiaMelanInk respectively.

	H_{Den}/H_{Th}	
	MelanInk, R = 75 nm $\gamma = 0.3$	SepiaMelanInk, R = 17 nm $\gamma = 0.03$
T = 5°C, $\beta(T) = 16 \cdot 10^{-6}$ 1/K	$5.3 \cdot 10^{-3} \cdot N$	$4.5 \cdot 10^{-2} \cdot N$
T = 100°C, $\beta(T) = 750 \cdot 10^{-6}$ 1/K	$1.1 \cdot 10^{-4} \cdot N$	$9.7 \cdot 10^{-4} \cdot N$

Table 2: Estimation of the ratio between the OA signals H_{Den} caused by the protein denaturation and H_{Th} caused by thermal expansion of the solvent. N is the number of proteins coupled to a granule.

Even at a temperature between 4°C and 5°C the detection of a denaturation signal could only be possible by coupling hundreds of proteins to the absorber. However the effect of an increased temperature during denaturation which changes β for a short time in a small volume is not considered in equation (8) and the OA signal is expected to be higher. H_{Den}/H_{Th} at a temperature of 100°C is a upper limit of the ratio for a local temperature rise of 100 K. LIOAS experiments were conducted to further elucidate the effect of a temperature dependence of β.

4. MATERIALS AND METHODS

4.1 Optoacoustic Set-up

The optoacoustic set-up is described in detail elsewhere.[24] In short, a Q-switched frequency doubled Nd:YAG laser (Spectron Laser Systems SL8000) with 8 nsec pulse width at 1064 nm was used to irradiate the sample (fig. 3). With a variable neutral density filter the radiant exposure of the laser pulses was adjusted between 0 and 200 mJ/cm^2. These high radiant exposures were necessary to produce a high local temperature at the granules. An aperture in front of the sample limited the beam diameter to 1 mm which corresponds to an effective acoustic transit time of 670 nsec in water. The transit time determines the time resolution of the measurements. All processes faster than this time contribute equally to the OA signal.

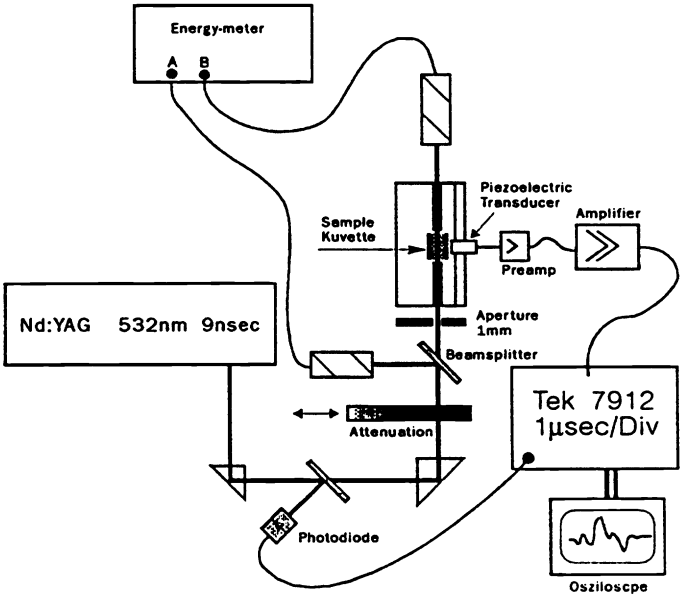

Fig. 3. Set-up for the optoacoustic measurements.

The sample solution was placed in a 1 cm square quartz cuvette which was hold firmly in a temperature-controlled metallic block. The cuvette was not removed during all the measurements. The temperature was controlled between 0°C and 100°C with an accuracy of 0.1 K using a PT100 thermoelement placed directly into the sample solution. The ceramic piezodetector was mounted in a stainless steel housing which was in direct contact to the cuvette. Due to the low resonance frequency of the piezodetector the temporal shape of the signal was determined by the detector. In order to improve the signal-to-noise ratio the electric signal of the detector was amplified and averaged 100-200 times. The signals were

digitised and stored by a transient digitiser (Tektronix 7912), which was linked to a RISC workstation (DEC-station 5000/200). The energies of the incoming and the transmitted laser pulse were measured with a pyroelectric energy meter (Polytec RJ 7620).

4.2 Samples

Two melanin suspensions with different particle size, which were made from the ink of cephalopods, were used. Melan-Ink™ (Lot M226L011, particle diameter 150±15 nm) and SepiaMelanInk™ (Lot M137L021, particle diameter 35±15 nm) were purchased from MeL-Co, CA, USA in a 10% (wt/Vol) suspension. The melanin was diluted with phosphate buffered saline (PBS, pH 7.4) until an optical density of 0.14 at 532 nm was reached. Because of the strong scattering of the particles absorption was measured with a special photometer (Perkin Elmer 356 UV/VIS). The photomultiplier of the photometer was located directly beside the sample cuvette, in order to measure as much scattered light as possible. Bromocresol purple in PBS (pH 7.4) with the same absorbance at 532 nm was used as a calorimetric reference.[14]

5. RESULTS OF THE LIOAS EXPERIMENTS

At a temperature of 6°C and 20°C the optoacoustic signals of the smaller melanin granules (SepiaMelanInk™) show exactly the same dependence on the radiant exposure as the reference sample bromocresol purple (fig. 4). Because of the salts in PBS the temperature at which β becomes zero was shifted from 4°C to a value between 2.5°C and 3.5°C. At lower temperatures a negative OA signal is observed, i.e. absorption of the laser pulse results in a contraction of the sample volume (fig. 4). In this temperature range the slopes of the experimental curves of SepiaMelanInk and the reference showed a slight difference.

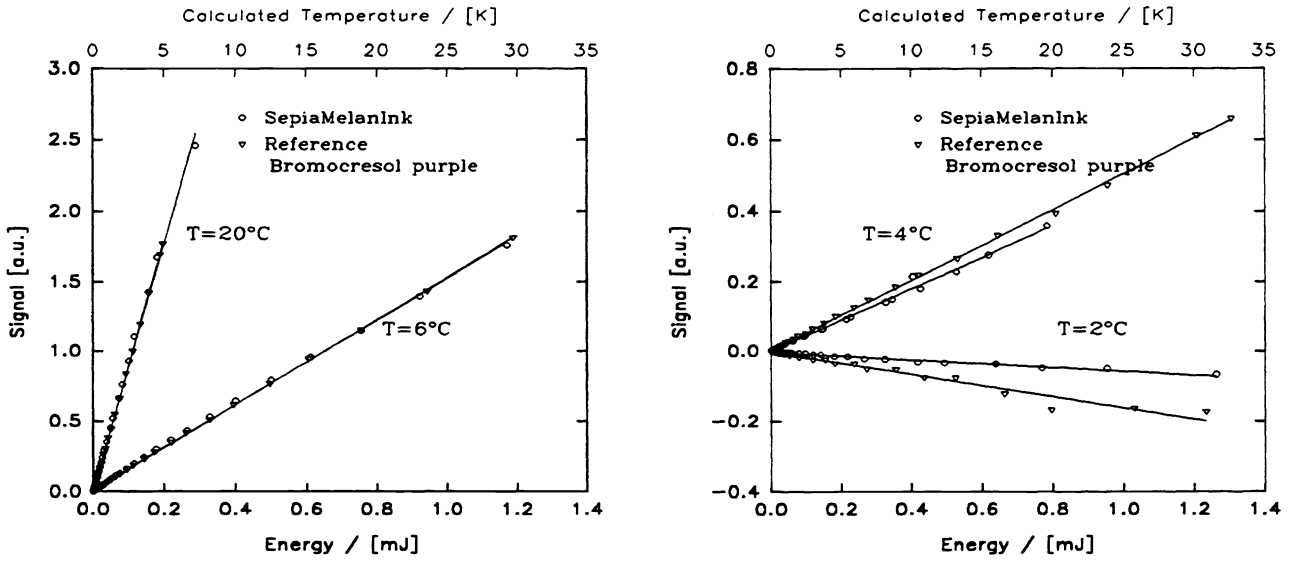

Fig. 4. Optoacoustic signal versus irradiating pulse energy for SepiaMelanInk and the reference bromocresol purple at different temperatures. The scale at the top shows the calculated temperature increase at the surface of the SepiaMelanInk granules.

Since the signals became very small between 2.5°C and 4°C considerable variations of the signal were observed at high radiant exposures, which may be attributed to experimental artifacts. However a slight deviation from the linear relation between H_S and E_A.was seen in some measurements (not shown).

With the bigger melanin granules (MelanInk) a strong non-linear behaviour of the OA-signal was observed at 6°C (fig. 5). At low pulse energy the energy dependence of the MelanInk signals coincides with SepiaMelanInk and the reference bromocresol purple. At higher energies the signal increases strongly. Together with the non-linear increase a slight change of the shape of the OA-signal was observed.

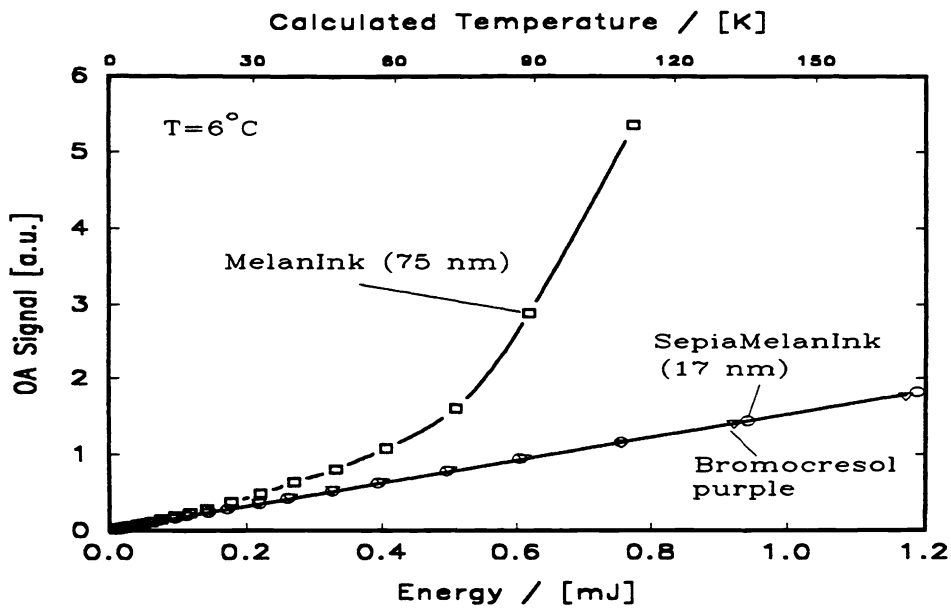

Fig. 5. Optoacoustic signal versus irradiating pulse energy for MelanInk, SepiaMelanInk and the reference bromocresol purple at 6°C. The scale at the top shows the calculated temperature increase at the surface of the MelanInk granules.

6. DISCUSSION

At the high temperatures, 20°C and 6°C, the OA-signal should be dominated by the thermal expansion of water. From the linear relation between signal and pulse energy, which is identical with the reference, it can be concluded that all absorbed energy is converted to heat within a time less than the acoustic transit time (670 nsec). Neither the reference nor SepiaMelanInk showed any non-linear effect up to a radiant exposure of 150 mJ/cm^2 which corresponds to an irradiance of 19 MW/cm^2. The difference of the slopes near 3°C, at which β is very small, may be explained by a different thermal expansion of water and melanin or by experimental artifacts. The MelanInk sample, which has an absorption cross-

section, eighty times higher shows a very strong deviation from linearity even at 6°C. At a calculated temperature increase of about 110 K the observed signal is more than 4 times larger than the linear signal. Since the reference converts all absorbed energy into heat, the reason for a higher signal must be a change in the process, which generates the acoustic wave. At the moment it is not clear whether the strong increase of the signal is due to the temperature dependence of $\beta(T)$ and the high temporary temperatures or due to an effect of melanin granules, which may be destroyed by the high temperature. Nevertheless the non-linear increase of the signal would strongly interfere with the signal caused by a volume change. Therefore quantitative measurements of protein denaturation would probably be difficult with an optoacoustic detection. Since the experimental set-up did not allow to get radiant exposures producing similar high temperatures with SepiaMelanInk, no predictions of the non-linearity of the signal can be made for this sample.

7. CONCLUSIONS

Calculations have shown that dye molecules are not suitable for heating a microvolume of 5 nm since extreme short relaxation times and an extreme photostability are needed. In addition an irradiation of more than 1 GW/cm^2 would be required. Melanin granules and other submicron solid state particles should be able to generate temperature increases of more than 100 K at a distance of 5 nm. Since their absorption cross-section can be orders of magnitude higher than that of a dye molecule the necessary irradiance is greatly reduced. Exact calculations of the resulting temperatures may be difficult since the involved parameters are somewhat uncertain and the Mie theory has to be applied.

The preliminary optoacoustic measurements have shown that the melanin granules used are able to convert all the absorbed energy into heat in a very short time. No saturation of the absorption was observed with an irradiance up to 19 MW/cm^2. Assuming a linear dependence of the OA-signal on the absorbed energy and hence neglecting the influence of a change of $\beta(T)$ a separation of the signals resulting from thermal expansion and protein denaturation should be possible if the granules can be densely covered with hundreds of proteins. Nevertheless non-linear contributions to the OA signal have to be expected and have been measured with the 75 nm melanin granules. Further experiments and calculations of the OA-signal which take heat diffusion and a dynamic change of the thermal expansion coefficient into account have to show whether the optoacoustic detection of protein denaturation is possible.

8. REFERENCES

1. R. Birngruber, "Thermal Modeling in Biological Tissues" in *Lasers in Biology and Medicine*, E. Hillenkamp, R. Pratesi, C.A. Sacchi (ed.), pp. 77 - 97, 1980.

2. A. J. Welch, "The Thermal Response of Laser Irradiated Tissue", *IEEE J. QE*, **QE20**, pp. 1471 - 1481, 1984.

3. S.L. Jacques, S.A. Prahl, "Modeling Optical and Thermal Distributions in Tissue During Laser Irradiation", *Lasers in Surg. Med.*, **6**, pp. 494 - 503, 1987.

4. J. Roider, N.A. Michaud, T.J. Flotte, R. Birngruber, "Response of the Retinal Pigment Epithelium to Selective Photocoagulation", *Arch. Ophthalmol.* , **110**, pp. 1786-1792, 1992.

5. D. Sliney, M. Wolbarsht, *Safety with Lasers and Other Optical Sources*, pp. 244, Plenum Press, New York and London, 1980.

6. B. Rosenberg, G. Kemeny, R.C. Switzer, T.C. Hamilton, "Quantitative Evidence for Protein Denaturation as the Cause of Thermal Death", *Nature*, **232**, pp. 471 - 472, 1971.

7. J.R. Lepock, K.-H. Cheng, H. Al-Qysi, J. Kruuv, " Thermotropic lipid and protein transitions in Chinese hamster lung cell membranes: relationship to hyperthermic cell killing", *Can. J. Biochem. Cell Biol.*, **61**, pp. 421 - 427, 1983

8. Y. Yang, A.J. Welch, H.G. Rylander III "Rate Process Parameters of Albumen", *Lasers in Sur. Med.*, **11**, pp. 188-190, 1991.

9. F.M. Pohl, "Temperature-Dependence of the Kinetics of Folding of Chymotrypsinogen A", *FEBS Lett.*, **65**, pp. 293-296, 1976.

10. M. Joly, *A Physico-chemical Approach to the Denaturation of Proteins*, Academic Press, London and New York, 1965.

11. F.H. Johnson, H. Eyring, B.J. Stover, *The Theory of Rate Processes in Biology and Medicine*, John Wiley & Son, 1974.

12. R.R. Anderson, J.A. Parrish, "Selective Photothermolysis: Precise Microsurgery by Selective Absorption of Pulsed Radiation", *Science*, **220**, pp. 524 - 527, 1983

13. J. Roider, F. Hillenkamp, T. Flotte, R. Birngruber, "Microphotocoagulation: Selective Effects in Biological Tissue Using Repetitive Short Laser Pulses", submitted to *Proc. Natl. Acad. Sci. USA*, 1993.

14. S.E. Braslavsky, G.H. Heibel, "Time-Resolved Photothermal and Photoacoustic Methods Applied to Photoinduced Processes in Solution", *Chem. Rev.*, **92**, pp. 1381- 1410, 1992.

15. A.C. Tam, "Applications of photoacoustic sensing techniques", Rev. of mod. Phys., **58**, 381 - 431, 1986.

16. G.L. Indig, D.G. Jay, J. Grabowski, "The efficiency of malachite green, free and protein bound as a photon-to-heat converter", *Biophys. J.*, **61**, pp. 631-638, 1992.

17. A. Ansari, J. Berendzen, S.F. Bowne, H. Frauenfelder, I.E.T. Iben, T.B. Sauke, E. Shyamsunder, R.D. Young, "Protein states and proteinquakes", *Proc. Natl. Acad. Sci. USA*, **82**, pp. 5000 - 5004, 1985.

18. R. Birngruber, "Choroidal Circulation and Heat Convection at the Fundus of the Eye", in *Laser Applications in Medicine and Biology*, **Vol. 5**, pp. 277-361, M. Wolbarsht (ed.), Plenum Press, New York, 1991.

19. H.S. Carslaw, J.C. Jaeger, *Conduction of Heat in Solids*, p.261, 2nd ed., Oxford at the Clarendon Press, 1959.

20. P.R. Crippa, C. Viappiani, "Photoacoustic studies of non radiative relaxation of excited states in melanin", *Eur. J. Biophys.*, **17** ,pp. 299, 1990.

21. P.R. Crippa, V. Horak, G. Prota, P. Svoronos, L. Wolfram, "Chemistry of Melanins", in *The Alkaloids*, **Vol. 36**, pp. 253 - 323, Ac. Press , 1989.

22. M.R. Chedekel, "Photochemistry and Photobiology of Epidermal Melanins" *Photochem. Photobiol.*, **35**, pp. 881 - 885, 1982.

23. M. Born, E. Wolf, *Principles of Optics*, pp. 656-664, Pergamon, 1970.

24. M. Rohr, W. Gärtner, G. Schweitzer, A.R. Holzwarth, S.E. Braslavsky, "Quantum Yields of the Photochromic Equilibrium between Bacteriorhodopsin and Its Bathointermediate K. Femto- and Nanosecond Optoacoustic Spectroscopy.", *J. Phys. Chem.*, **96**, pp. 6055 - 6061, 1992.