

## Photoacoustic Absorption Measurements on Tumor Tissue Stained with the Photosensitizer Methylene Blue

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A photoacoustic absorption spectrometer using a tunable DCM-dye laser is described. The excitation radiation (600nm–700nm) is modulated by a chopper (10–1000 Hz) or by oscillating beam deflection. The latter allows the measurement of lateral absorption gradients. The samples are placed in a closed gas cell connected to a sensitive microphone. In addition to the measurements of depth-resolved absorption spectra, information on the transmission and scattering behavior can be obtained by means of a simple modification of the spectrometer. With this experimental set-up the absorption behavior of the photosensitizer Methylene Blue inside the tissue and as a thin layer above the tumor tissue was investigated. For low dye concentrations the spectra are influenced by tissue scattering. The selective uptake of Methylene Blue in tumor tissue after dye instillation into the bladder could be verified.

**KEYWORDS:** *photoacoustics, tumor tissue, Methylene Blue, photosensitization*

### INTRODUCTION

The technique of photoacoustic spectroscopy (PAS) can be used in order to obtain information on the localization, the concentration, and the diffusion behavior of dyes into tissue. This is of interest, e.g. in the case of the photochemotherapy of tumors caused on light-induced chemical reactions of special dyes (photosensitizers) and their preferential retention in malignant tissue.

PAS is based on the thermal deactivation of a sample after light absorption. The excitation with a modulated light beam induces periodic temperature changes. These changes are converted to pressure changes if the sample is placed in a closed gas cell. Variations in the gas pressure can be detected by sensitive microphones (gas-cell microphone technique).

The PAS signal depends on optical and thermal properties of the sample. It can be received from a surface layer of the thickness  $\mu_{th}$ . The thermal diffusion length  $\mu_{th}$  is given by  $\mu_{th} = (2\alpha/\omega)^{0.5}$ , where  $\alpha$  is the thermal diffusivity constant and  $\omega$  the modulation frequency. The thickness of the sample is no limitation in PAS which is opposite to the conventional absorption spectroscopy based on a transmission measurement. Optically

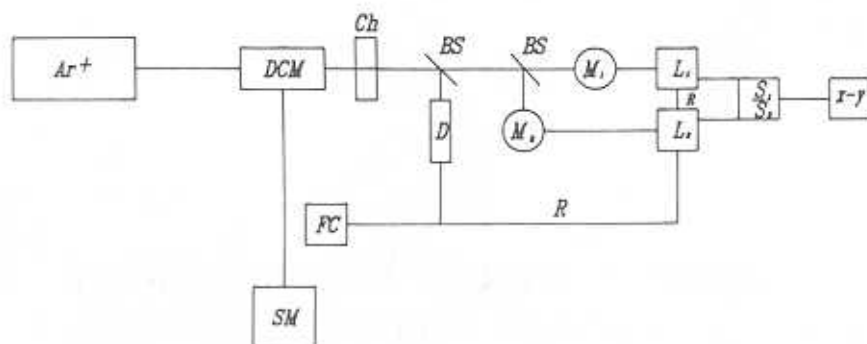


FIGURE 1 Experimental set-up of the photoacoustic absorption spectrometer. Ar<sup>+</sup>: argon-ion laser DCM: dye laser with the active medium CH: chopper BS: beam splitter D: photodiode M: photoacoustic chamber with microphone L: lock-in R: reference signal S: photoacoustic signal S1/S2: ratio module x-y: recorder SM: step motor.

opaque samples can be measured if the thermal diffusion length is smaller than the absorption length ( $\mu_{th} < \mu_a$ ).

Variation of the modulation frequency and scanning of the light beam allow information on the lateral as well as the depth distribution of the absorbing dye molecules to be obtained. Photoacoustic absorption spectra can be received by variation of the excitation wavelength.

In the literature there are only few photoacoustic measurements on dye-containing biological tissue reported. Kölmel *et al.* [1] investigated the diffusion behavior of sunscreen drugs into the skin. Boucher *et al.* [2] reported on chromophore distribution measurements in the retina; Burgi and Dovichi [3] carried out measurements on stained histological samples by means of a photothermal microscope. An application concerning the photosensitizer HpD is described in reference [4] where highly concentrated aqueous solutions were studied.

This paper deals with a photoacoustic spectrometer working in the red spectral range. It was used to investigate the absorption behavior of Methylene Blue stained biological tissue and to study the dye accumulation in bladder tumors. The dye is a known photosensitizer with a high yield of singlet oxygen [5,6]. A preferential uptake by tumor tissue was observed [7]. Combining these facts we proposed the use of Methylene Blue for a selective photochemotherapy of tumors, especially in the case of bladder tumors [8,9,10].

## EXPERIMENTAL ARRANGEMENT AND METHOD

The scheme of the set-up of the photoacoustic absorption spectrometer is shown in Figure 1. An argon ion laser pumped DCM-dye laser is used as light source (300 mW maximal power) emitting radiation in the spectral range 600nm–700nm. The laser is tunable by a step-motor driven birefringent filter. The beam is modulated by a mechanical chopper (10–1000 Hz) whereas the intensity is nearby given by:  $I(t) = (I_0/2) (1 + \cos \omega t)$ . For the measurement of lateral absorption gradients the beam is oscillating laterally (see Fig. 2). For this purpose the beam hits a deflection mirror fixed on a loudspeaker membrane. The membrane is driven by a RC-generator.

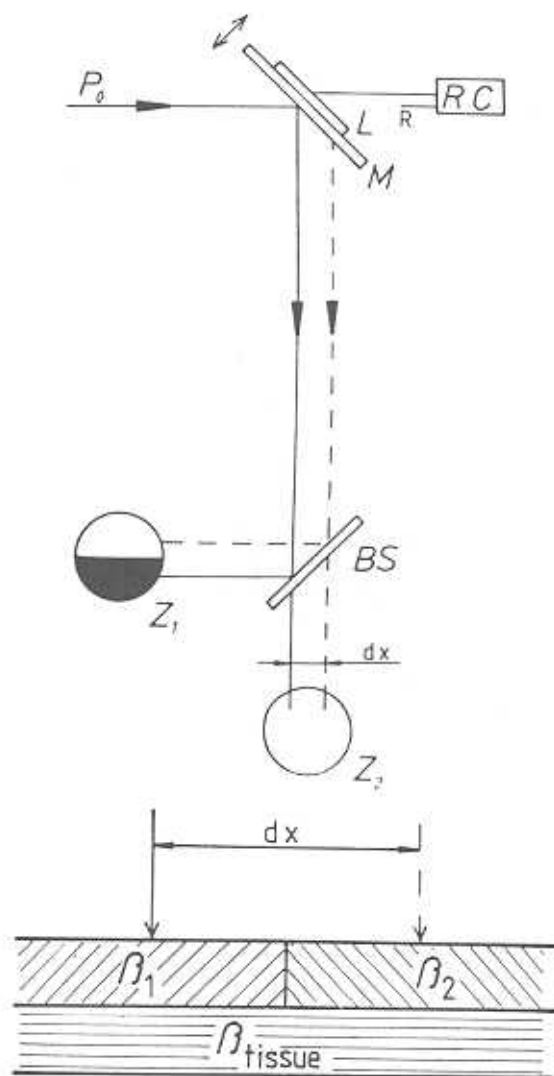


FIGURE 2 Method of oscillating beam position. Top: scheme of the modified set-up. Bottom: Concentration differences along the path  $dx$ ;  $P_0$ : incident power L: loudspeaker membrane M: mirror Z1: reference chamber Z2: chamber for sample R: reference signal  $\beta$ : absorption coefficient RC: RC-generator

The deflection  $dx$  on the sample depends on the voltage  $U$ ; the irradiation spot can be described by:  $x(t) = x_0 + dx(U)\exp(i\omega t)$ . A lateral absorption gradient results periodic gas pressure changes which can be detected.

The vibration-damped photoacoustic chambers (Fig. 3) consist of a brass-body holding the sensitive capacitor microphone. The air filled chamber (height: 2 or 4 mm, diameter: 32 mm) is tightly closed by the entrance window and the sample-holding window.

The photoacoustic signal measured by the microphone depends on the thermal and optical properties of the sample as well as on the modulation frequency and the phase-shift with

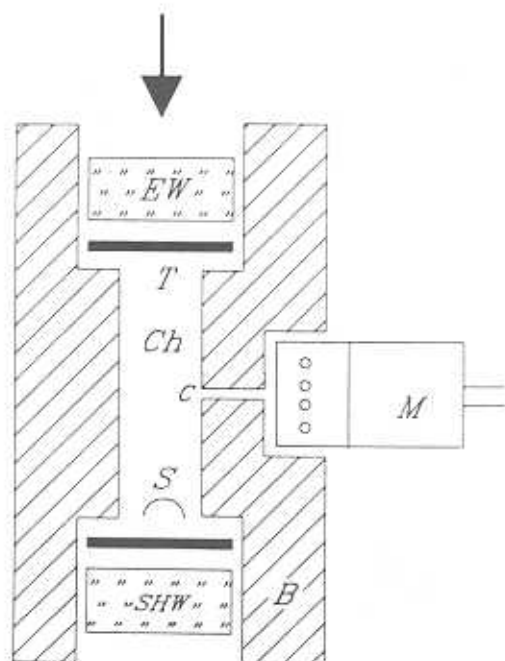


FIGURE 3 The photoacoustic chamber. M: microphone B: brass body C: air canal T: teflon EW: entrance window SHW: sample holding window S: sample.

respect to the excitation radiation. The obtained signal is directly proportional to the absorption coefficient.

The second chamber serves as a reference chamber containing a sample with a photoacoustically thick layer ( $\mu_{th} \gg \mu_1$ ). Thus, the chamber works in the region of saturation and the signal is proportional to the light intensity (power meter).

Dividing the signal amplitudes of the lock-in outputs results in corrected spectra which can be registered by a x,y-recorder. The photodiode serves as reference source for the lock-in and as frequency meter.

The additional possibility of measurement of transmission and scattering spectra is given by a simple modification of the entrance window. This one works as a surface absorber with a high absorptivity and low remission. The mirror has a small transparent opening (beam entrance). Thus, the absorbance area  $A$  of this mirror plays the role as a wavelength-independent detector area for incident scattered radiation.

In the case of a sample inside the chamber the signal can be interpreted as the sum of the absorption and scattering signal. In the case of a sample fixed outside only the scattered radiation will be measured. See Figure 4.

## INVESTIGATION OF METHYLENE BLUE MARKED TISSUE

At first the photoacoustic absorption (PAA-) spectrum of the photosensitizer Methylene Blue (Mb) in an aqueous solution (PBS, pH = 7.4, sample thickness:  $d = 200 \mu\text{m}$ ) was measured as shown in Figure 5, left. Aqueous Methylene Blue solution consists of a mixture

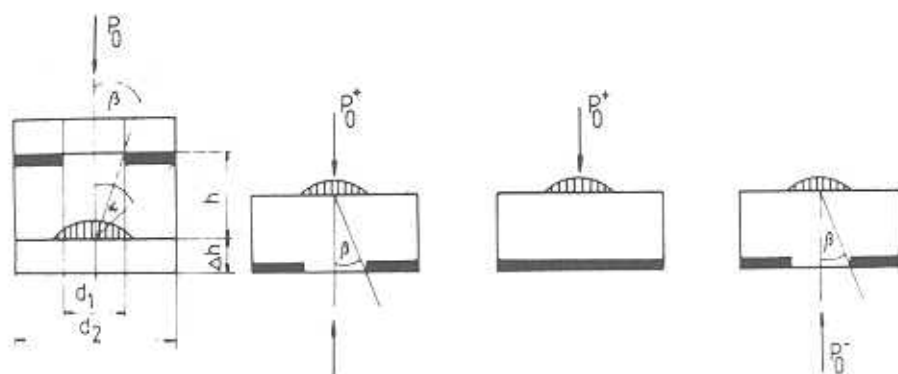


FIGURE 4. Modification of the photoacoustic chamber for the measurement of scattered light.  $P_0$ : incident power  $d_1$ : diameter of the beam opening  $d_2$ : chamber diameter dark field: absorption area  $A$ .

of dimers and monomers. The two absorption maxima around 612nm and 667 nm of the 0.3 mM solution depict the different absorption behavior of the dimers and monomers.

In the case of solutions with a lower Mb-concentration the PAA-signal decreases but the decrease is, in addition, a function of the change of the monomer-dimer ratio.

Figure 6 shows the dependence of the PAA-signal  $U$  on the concentration  $c$  using the wavelength 645nm (nearly isobestic point). The ratio  $U/c$  is roughly constant. Only for the high concentrations of 30 mM does a deviation appear due to saturation effects ( $\mu_{th} \ll \mu_1$ , see Table I). In this case a  $\omega^{-1}$ -dependence of the signal occurs in contrast to the  $\omega^{-1.5}$ -dependence of unsaturated conditions. This is in accordance with the theory [11,12].

It can be derived from Figure 6, that Mb-concentrations down to 0.005 mM (an absorbance of around  $10^{-3}$ ) can be detected. This value verifies the PAA-detection limits of liquids reported in the literature [13].

In Figure 7 PAA-spectra of Mb-marked tumor tissue (solid Ehrlich carcinoma) are shown in comparison with unmarked tissue (lowest curve). The PAA-amplitude of tissue depends on the hydration (factor of 2 between wet and dry tissue) because of variations of the values of specific heat and density as well as of the scattering coefficient  $s$ .

The middle curve describes the case of a local Mb-administration on the tumor. This kind of drug application is of interest in the photochemotherapy of bladder tumors (instillation followed by a dye-accumulation into the bladder tissue). In this measurement the dye is localized in a thin layer with a thickness of about  $200\mu\text{m}$  above the tumor tissue. The spectrum recorded immediately after the administration is comparable with that of Figure 5. Indeed, this fact is due to the case of a thermally thick sample. The spectrum reflects the absorption behavior of the upper part of the dye film only. However, a small difference between the curves in Figure 5 and Figure 7 in the signal amplitude (factor: 1.2) is obtained. This can be due to the scattering properties of the tissue which reflects a part of the transmitted radiation ( $T = 0.80$ , transmission through the dye layer). This behavior results in an increase of the photon flux in the  $\mu_{th}$ -layer (layer of the thickness  $\mu_{th}$  at which the signal is generated).

Curve 3 in Figure 7 shows the absorption behavior of the sample one hour after the local application of a thinner solution. Higher signals are obtained. This fact can be explained by a higher concentration and dimer accumulation in the upper tissue parts as well as an increased influence of tissue scattering. The layer contains both now, tissue and dye. Dye concentra-

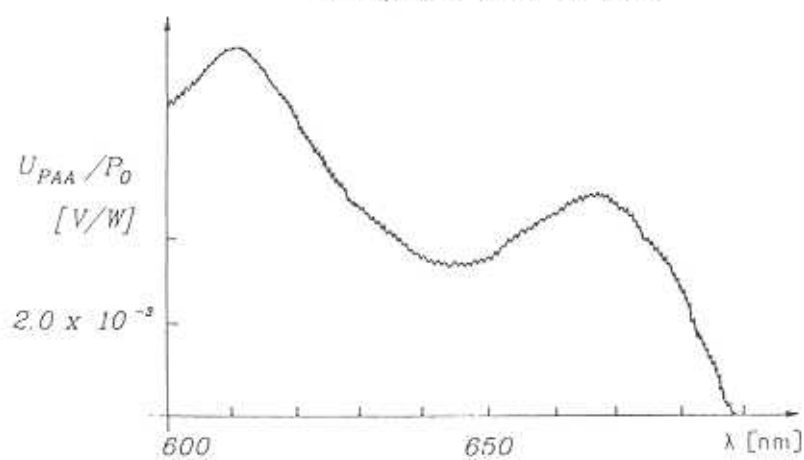
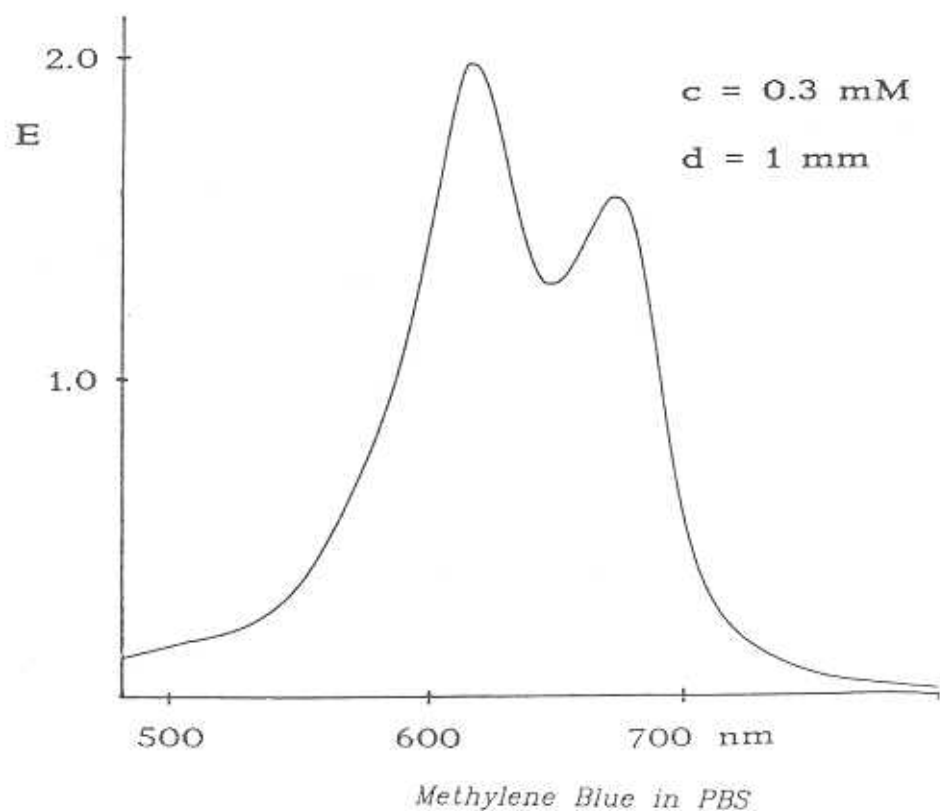


FIGURE 5 PAA-spectrum of an aqueous Methylene Blue solution (PBS,  $c = 0.3$  mM,  $f = 31$  Hz), bottom, in comparison with the absorption spectrum obtained by standard transmission measurement, top.

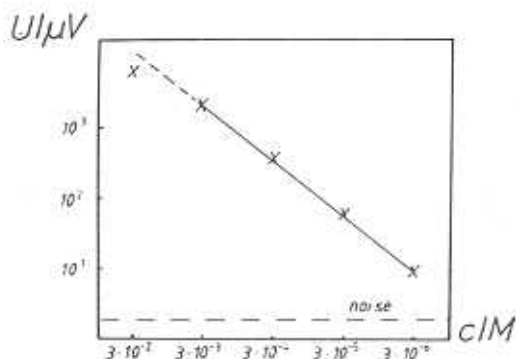


FIGURE 6. Dependence of the PAA-signal (tension) on the Mb-concentration

tions of the injection solution down to 0.1 mM could be detected in this case (dye inside the tissue).

In order to prove the property of a selective accumulation into bladder tumors the PAA-spectra of peritumoral tissue and the tissue of a bladder tumor of a 60 year old patient were compared. Before the resection he was given a 30 minute bladder instillation of a highly concentrated Mb-solution followed by an additional water washing. Whereas in the case of peritumoral tissue spectra like curve 3 in Figure 7 could be measured, the dye-concentration in the case of the bladder tumor was so high that the signal was saturated in the short-wavelength range.

This region of saturation can be reduced by higher modulation frequencies (see Fig. 8). Using the modulation of oscillating beam position this concentration difference between the Mb-content of peri- and tumoral tissue could be verified.

The PAA-signal is a function of the absorptivity of the sample, however, the influence of scattered radiation cannot be excluded. In the case of biological tissue the propagation of radiation in the red spectral range is determined by the process of multiple scattering whereas the tissue absorptivity plays a minor role ( $s \gg \beta$ ). The influence of absorbed scattered light by the chamber walls and by the detection area of the microphone is small compared to the additional tissue and dye absorption due to the increase of the photon flux into the  $\mu_{th}$ -layer. In order to get an idea of the part of remission radiation the modified chamber was used for transmission and scattering measurements. In the case of irradiated tumor tissue (650 nm,  $d = 400\mu\text{m}$ ) a remission  $R$  (backscattered radiation in the solid angle  $2\pi sr$ ) of about  $0.30 \pm 0.02$  could be determined. This scattered light includes direct reflection from the boundary air/tissue. However, this contribution is small in the case of an

TABLE I  
Thermal diffusion length  $\mu_{th}$  and absorption length  $\mu_a$  of Methylene Blue in PBS (for comparison:  $\mu_{th}$  of tissue)

$\omega/2\pi$ /s <sup>-1</sup>	$\mu_{th}$ / $\mu\text{m}$		$C_{Mb}$ /mM	$\mu_a$ / $\mu\text{m}$ (645nm)
	Mb	tissue		
10	67	47	0.03	3000
31	39	24	.3	300
100	21	15	3	20

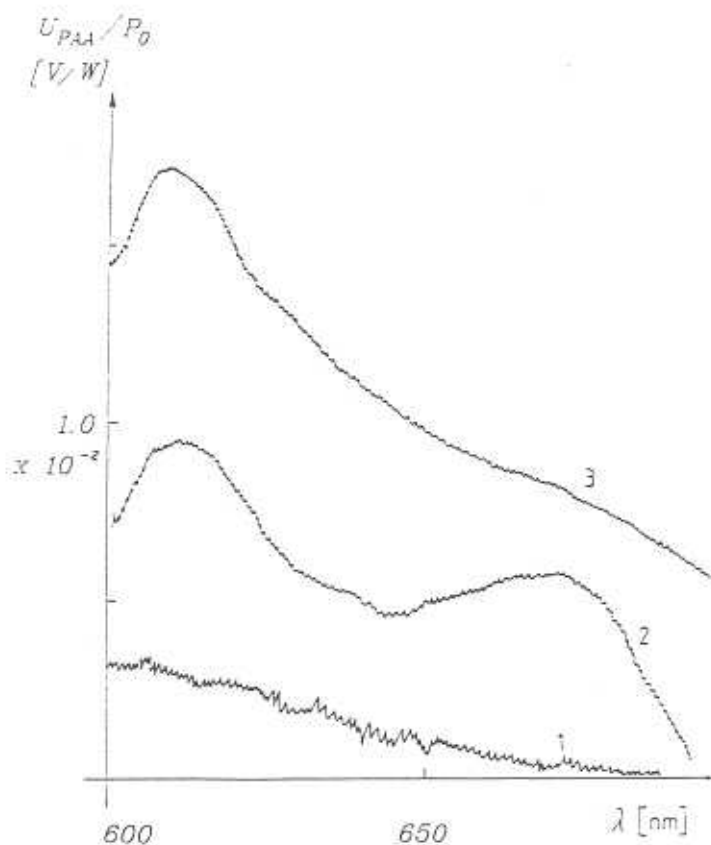


FIGURE 7 PAA-spectra of Mb-stained tumor tissue in comparison with unmarked tissue 1: tissue without Mb 2: tissue with a thin Mb-film,  $c_{\text{injection}} = 0.3 \text{ mM}$  3: Mb inside the tissue (one hour after local administration and washing,  $c_{\text{injection}} = 0.17 \text{ mM}$ )

index of refraction  $n$  with  $1.33 < n < 1.55$  (value depends on the hydration of tissue). The  $U/P_0$ -values of the recorded structureless remission spectra of the tumor tissue decreases with decreasing wavelength according to the near minimum around 580nm (absorption band of hemoglobin).

## DISCUSSION

PAS can be used to obtain information about the localization and concentration of the dye applied to biological tissue. This method is of interest especially where dyes with a low fluorescence quantum yield are applied and additional measurements on the absorption and diffusion behavior are required. The PAA-spectrometer used is of the gas cell-microphone type which allows investigation of different kinds of samples (liquids, amorphous samples with a rough surface) in a non-contact manner. This is in contrast to other photothermal techniques like a calorimetric or piezoelectric measurement (contact method) or thermal



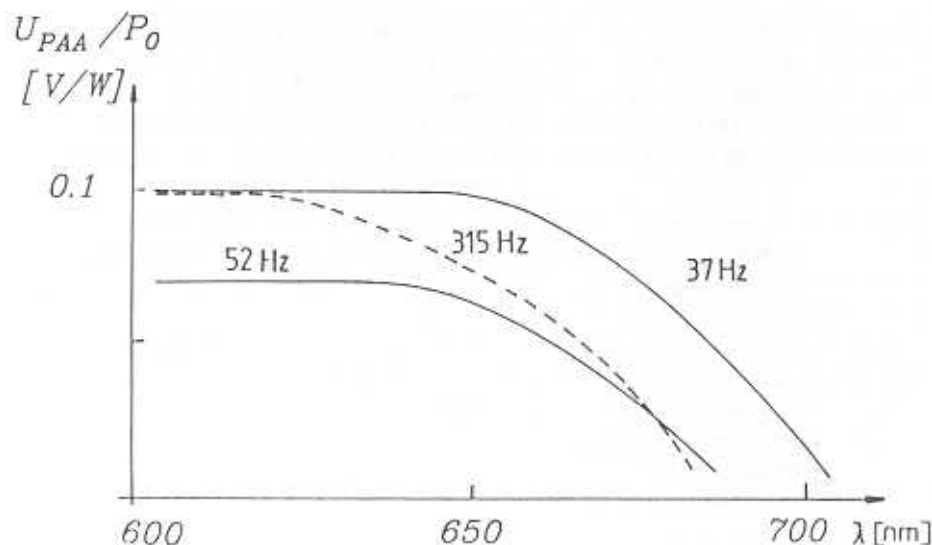


FIGURE 8 PAA-signal of a Mb-stained bladder tumor versus modulation frequency.

lensing- and beam reflection methods (planar sample surface required) [12]. In vivo investigations are possible in principle, too [1].

The PAA-measurements carried out on the photosensitizer Methylene Blue show that the dye can be detected in the concentration range  $0.005 \text{ mM} < c < c_{\text{sat}}$ , where  $c_{\text{sat}}$  is the value for the case of a photoacoustically opaque sample (saturation) which depends on the modulation frequency and the irradiation wavelength.

If the dye Methylene Blue accumulates above tumor tissue (exterior administration like the Mb-instillation into the bladder), the sample can be interpreted as a two-layer system consisting of a thin dye layer of the thickness  $l$  and the tissue layer. If the modulation frequency is chosen high enough, the nonsaturated case occurs and the  $\mu_{\text{th}}$ -layer lies completely in the upper part of the dye film ( $\mu_{\text{th}} \ll l$ ). That means, the measured absorption spectra will be similar to that of the injected solution.

However, the part of remitted radiation which occurs due to the multiple scattering of the (through the dye-layer) transmitted light must be considered. The absorption of this additional photon flux results in an increase of the signal as well as in a possible influence of the spectra by intensity modification (increase of small absorption bands in opposite to regions with a stronger absorptivity). This is due to the wavelength dependence of the remitted radiation  $P_{\text{R}} = P_{\text{O}} \exp(-\beta l)R$  with the remission  $R$ , the power  $P_{\text{O}}$  of the incident beam and the absorption coefficient  $\beta$  of the dye layer of the thickness  $l$ . The influence of scattered light must also be considered with respect to the time lag between instillation and irradiation. As a result of diffusion the dye accumulates into the tissue. The light propagation in the  $\mu_{\text{th}}$ -layer is, in the case of small dye concentration and red light, mainly determined by the scattering behavior of the tissue. However, Mb inside the tissue could be detected down to 0.1 mM of the applied solution. Simple modification of the photoacoustic chamber containing detector areas with a photoacoustically thick layer allows the measurement of the absorbed, the transmitted, as well as both the forward and the backscattered light of the sample. The obtained value  $R$  of approximately 30% remission of the incident 645nm light in the case of the solid EC-tumor seems to be typical for thick, low pigmented tissue. It reflects the high contribution of multiple scattering.

The measurement concerning the lateral distribution shows concentration differences between stained bladder tumor tissue and peritumoral tissue after Mb-instillation and water washing. This result verifies the studies of a Japanese group [7] who determined a correlation between Mb-staining and the grading of bladder carcinoma by means of histological methods. That means the photosensitizer Methylene Blue possesses tumor-localizing properties.

Because of the additional property of high yield of singlet-oxygen formation of excited Mb [5,6] an irradiation of the whole bladder should induce photochemical tumor destruction. The topical administration (instillation) of the dye has the advantage of low side effects, like no photosensitivity of the skin.

Mb can be detected in the upper part of the tissue surface layer (around 20  $\mu\text{m}$  for modulation frequencies around 35 Hz) and the absorption behavior can be determined. This measurement is of interest for the optimal irradiation wavelength. Light and dye distribution in the tissue determine the efficiency of the phototreatment. Further investigations on the dye penetration into deep-lying tissue layers in dependence on the solvent are necessary. For that case PAS can also be used to investigate histological sections.

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