Phthalocyanines: from dyes to photosensitizers in diagnostics and treatment of cancer.

Spectroscopy and Raman imaging studies of phthalocyanines in human breast tissues.

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Introduction

Cancer has become a major cause of mortality. In 2012 there were an estimated 14.1 million new cases of cancer in the world [1], that’s why there is a considerable interest in the developing new techniques allowing noninvasive diagnosis and tracking of the progress of therapies used to treat a cancer. Cancer is a multi-factorial disease that demonstrates various mechanisms and phases of pathogenesis. Therefore, selection of diagnostics and treatment methods, selection of markers informing about the development of cancer diseases must be reliable to improve patient survival rates. The World Health Organization estimates that around 30% of all cancers can be cured if identified earlier and more precisely.[1]

Presently a histological analysis, being the standard procedure for cancer diagnostics, including human breast cancer, is highly invasive, sometimes can be inconvenient, time consuming, and in a number of cases can lead to ambiguous results prone to human interpretations. This evidently demonstrates a need to develop new methods that would enable an unequivocal diagnosis within a short time with high precision.
Current diagnostic and imaging methods are often limited by inadequate sensitivity, specificity and spatial resolution.[2] Spectroscopic methods are ideally suited to study abnormalities, including cancer, in biological tissues and their use can be enhanced by photosensitizers. Optical methods offer several significant advantages over the routine clinical imaging methods including: a) non-invasiveness through the use of safe, non-ionizing radiation, b) display of contrast between soft tissues based on optical properties of the tissue, c) high spatial resolution (less than 0.5 micron lateral resolution in the visible range).

Among spectroscopic methods Raman imaging (RI) based on Raman scattering due to molecular vibrations is an emerging field that has generated a lot of interest both for the label free Raman methods and the biofunctionalized and targeted methods using unique properties of phthalocyanines.[3-6] RI has reached now a level of sophistication in terms of sensitivity, selectivity and specificity that makes it competitive with more classical methods of the confocal fluorescence microscopy [5-15]. RI provides direct biochemical information because vibrational fingerprint features reflect chemical composition of cells, their organelles and tissues. The structural fingerprinting is very effective owing to narrow and highly resolved Raman bands (0.1 nm compared with a typical bandwidth of 10–50 nm for fluorescence). Resonance Raman effect and Surface Enhanced Raman Scattering are able to amplify the probe's signal to the point that it can be detected in crowded biological structures. RI combined with contrast enhancing probes active in Raman spectroscopy and able to cross cell membranes may identify specific gene products, DNA sequences, metabolic products and intracellular processes.[5,16].

In biomedical applications we require: high spatial resolution far below the diffraction limit, strong signal enhancement and high temporal resolution enabling monitoring dynamics of photosensitizers in biological systems.
The most promising method to study evolution in time is the femtosecond pump-probe transient absorption spectroscopy. [17-18]

Phthalocyanines are important photosensitizers in medical photodiagnosics and therapy (PDD and PDT). The unique physical and chemical properties of phthalocyanines such as the similarity in structure to the biological molecules (chlorophyll, haemoglobin), their diversity regarding the central metal, substituents, intrinsic capability to self-assemble have activated the interest in this group of chemical compounds for many years and are important for applications in optics and medicine.[19-40]

Photodynamic therapy (PDT) is a promising cancer treatment that involves the combination of visible light and a photosensitizer. Each factor of this therapy (photosensitizer, light) is harmless by itself, but when combined with oxygen, can produce lethal cytotoxic agents that can destroy cancer cells. The most important factor in PDT is selectivity towards diseased tissue as only those cells that are simultaneously exposed to the photosensitizer, light and oxygen are exposed to the cytotoxic effect. The selectivity of PDT is produced also by a preferential uptake of the photosensitizer by the diseased tissue and the ability to confine activation of the photosensitizer to this diseased tissue by restricting the illumination to that specific region. Therefore, PDT allows the selective destruction of cancers leaving normal tissue intact. [41]

The detailed mechanism of photodynamic reactions in the tissues is not completely understood yet; however, three mechanisms can take place, and the predominance of one of these mechanisms depends on the concentration of oxygen in tissues, Type I, II and III.

In the Type II of PDT, the excited triplet state of the photosensitizer ($^3$PS*) is reached through the intersystem crossing (ISC) from the first excited singlet state ($^1$PS*), derived from the ground state ($^1$PS), by the absorption of laser light. Due to the sufficiently long lifetime of the triplet state ($^3$PS*), a photosensitizer can interact efficiently with the environment of the
tissue. The $^3\text{PS}^\ast$ state of the photosensitizer reacts directly with the triplet state oxygen molecule ($^3\text{O}_2$), generating highly toxic singlet oxygen ($^1\text{O}_2^\ast$) and effectively destroying tumor tissue. This process competes with the radiant (fluorescence, phosphorescence) and radiationless (internal conversion) deactivation of the $^3\text{PS}^\ast$ state of the photosensitizer.

Phthalocyanines have been studied by various spectroscopic and theoretical methods for many decades.[20-38] Although the studies provide extensive information about these systems, a number of problems still remain to be resolved: the mechanisms of communication between different states such as metal-centered, ligand-centered, charge-transfer states as well as the reactions induced by light, the early intermediates upon photon excitation, mechanisms of photodissociation, radicals, protonated isomers, adduct or peroxo compound formation, mechanisms of macrocycle disruption, selective accumulation in biological systems[17,18].

In this chapter we discuss some fundamental properties of phthalocyanines in solutions, films and at biological interfaces of human cancerous and noncancerous tissues using conventional and new emerging methods of molecular spectroscopy.

We will introduce also some fundamental principles of Raman imaging as a new, emerging and noninvasive technology of tissues mapping to monitor distribution and localization of photosensitizers in live cells and human tissues. The applications of the femtosecond pump-probe transient absorption spectroscopy of phthalocyanines will also be discussed.

The properties of phthalocyanines will be discussed based on results obtained in our laboratory for zinc and aluminum phthalocyanines.[5,17,18,39]

We will demonstrate how the combination of Raman spectroscopy, IR spectroscopy, femtosecond spectroscopy and Raman imaging techniques can be used to obtain unique insight into vibrational features of cell/tissue and intracellular processes occurring on the time
scale from femtoseconds to nanoseconds in normal and cancerous human tissues as well as localization of photosensitizers.

**Synthesis, Materials and Experimental Methods**

**Synthesis**

Zinc tetrasulfonated phthalocyanine tetrasodium salt (ZnPcS₄) and aluminum tetrasulfonated phthalocyanine, tetrasodium salt (AlPcS₄) (Fig. 1) have been synthesized according to the following procedure: phthalocyanines have been prepared by the process similar to that described by Griffiths and co-workers for the case of zinc phthalocyanines [35]. Thus, for aluminum phthalocyanine the mixture of dry 4-sulphophthalic anhydride (5 g, Sigma Aldrich), urea (4 g), ammonium chloride (0.34 g), ammonium molybdate (0.06 g), boric acid (0.06 g), anhydrous aluminium chloride (0.85 g, dissolved in 5 cm³ sulfolane) was introduced to 10 cm³ of sulpholane, slowly heated to 200–210 °C and kept for 1–2 hrs. After cooling the excess of sulpholane was removed and the residue was dissolved in 200 cm³ of hot water, filtrated after addition of activated carbon and precipitated by addition of 10 cm³ of 30% hydrochloric acid. The crude dye was dissolved in 25 cm³ of distilled water, neutralized by sodium carbonate to pH = 7.0 and precipitated by ethanol (1:1). Finally, 0.7 g of chromatographically pure dye was obtained. The same procedure was used for zinc phthalocyanine synthesis.

Figure 2 illustrates the second method used in our experiments for synthesis of AlPcS₄ (salt) by condensation of sulphophthalic anhydride. This method provides a mixture of a reduced number of regioisomer, because the position of the sulphonyl group in the substrate of the reaction is fixed.
Aluminum phthalocyanine chloride tetrasulphonic acid was purchased from Frontier Scientific, Inc. (AlPcS-834), aluminum phthalocyanines chloride, was purchased from Sigma-Aldrich (362530) and they were used without further purification.

**Fig.1.** Structure of: (A) zinc tetrasulfonated phthalocyanine tetrasodium salt (ZnPcS₄), (B) aluminum tetrasulfonated phthalocyanine tetrasodium salt (AlPcS₄-salt) and (C) aluminum phthalocyanine chloride tetrasulfonated acid (AlPcS₄-acid).


For solution preparation a distillated water was used, purified on Millipore filters and characterized by electrolytic conductivity equal to 0.17–0.2 MΩcm (5–6 μS/cm).

Patients and samples

All procedures were conducted under a protocol approved by the institutional Bioethical Committee at the Medical University of Lodz, Poland (RNN/45/14/KE/11/03/2014). We have studied ductal and lobular carcinoma (in situ and infiltrating) as well as various benign changes including benign dysplastic and neoplastic lesions. The total number of patients was 250. For each patient the two types of tissues: the tissue form the safety margin and the tissue from the tumor mass were analysed. Thousands of spectra for biological samples were recorded using spectroscopic methods. All tissue samples were snap frozen and stored at -80 °C. One part of each type was cryosectioned with a microtome (Microm HM 550, Sermed) into 6 μm-thick sections for Raman analysis. The thin cryosectioned tissue samples (without staining and paraffin embedding) have been examined by Raman imaging, UV-Vis
or IR spectroscopy. After spectroscopic analysis these sections were stained and histologically examined. The adjacent part of the tissue was paraffin embedded and also cut into 6 μm-thick sections for typical histological analysis.

**Raman spectroscopy and imaging**

Raman scattering is inelastic scattering, and measuring the difference between the energy of the incident photons and scattered photons one can obtain the information about vibrational energy and frequencies (Fig.3). The energy of the scattered photon can be shifted to lower frequencies (Stokes component) when the incident photon gives part of its energy to the environment bath (mainly vibrations) or to higher frequencies (anti-Stokes component) when the incident photon gets the energy from the bath.

The levels denoted as \( E_0 \) and \( E_1 \) represent electronic energy levels while the levels numbered with the vibrational quantum number \( \nu \) represent the vibrational energy levels. If the sample is illuminated with photons of energy, smaller than the resonance energy, all of the photons that interact with the sample are not absorbed, but cause the potential energy of the interacting molecules to be raised to virtual state, above the ground state. Almost immediately most molecules return to the ground state through the emission of photons of the same energy as the incident photons. This elastic scattering is called the Rayleigh scattering. A small fraction of the molecules drops back to the first excited vibrational state \((\nu = 1)\) instead to the ground state. Since the energy of the incident and the scattered photons are different, the scattering is inelastic and the process is known as Stokes Raman scattering with the scattered radiation observed at lower energy. Molecules that are already in the excited vibrational state \((\nu = 1)\) will undergo analogous effect when illuminated with a laser light. When the excited molecules drops back to the ground vibrational state \((\nu = 0)\), the scattered radiation will be observed at higher energy. This scattering is known as anti-Stokes Raman scattering, The
frequency $\omega_{\text{vib}}$ denotes the frequency of a given vibrational mode of the molecule. To describe Raman scattering a fully quantum-mechanical theory is required but some intuitive description can be also obtained from a classical picture.

The electric field drives the electron displacements that induce the polarization P in a medium modulated in time that in turn generates a wave at the same frequency $\omega_L$ (Rayleigh scattering). When the dipole oscillations are modulated additionally by the molecule vibrations at frequency $\omega_{\text{vib}}$ the waves at $(\omega_L - \omega_{\text{vib}})$ (Stokes Raman scattering) or $(\omega_L + \omega_{\text{vib}})$ (anti-Stokes Raman scattering) are generated.

Raman scattering can be observed microscopically using instrumentation presented in Figure 4, which is very similar to the laser fluorescence microscopy, but instead of the fluorescence signal, Raman scattering signal is detected. Light from a laser enters through a small pinhole and expands to fill the pupil area of a microscope objective lens. The incident light is focused on the biological sample by means of a high numerical aperture NA of objective lens to the resolution corresponding to the diffraction limit. The diffraction limit for lateral and axial spatial resolution, $\delta_{\text{lat}}$ and $\delta_{\text{ax}}$, are determined by

\( \delta \text{lat} = 0.61 \, \lambda / \text{NA} \) \quad \delta \text{ax} = 2 \, \lambda \, n / (\text{NA})^2 \quad (1) \)

where \( \lambda \) is the wavelength of exciting light, \( n \) is the refractive index, and \( \text{NA} \) is the numerical aperture of the objective. Light scattered back from the illuminated spot on the sample, is collected by the objective, propagates through the dichroic mirror and is directed to a pinhole placed in front of the spectrometer. As it was mentioned, placing a pinhole aperture in the emission light path at a conjugate location of the focal volume in the specimen allows to obtaining 3-D resolution. Indeed, only photons generated inside this volume will be focused at the pinhole aperture and can be transmitted to the detector in contrast to photons from outside of this focal volume, which are defocused at the aperture plane and will be blocked. This ability to reject light from above or below the focal plane enables the confocal microscope to perform depth discrimination and provides a solution for optical tomography. Scanning of the specimen is achieved by moving the laser beam along the specimen’s surface or a moving of the microscope table in a raster-pattern. A true 3D image can be processed by taking a series of confocal images at successive planes into the specimen. Scanning along the x-y axes provides mapping of the sample, while scanning along the z axis, provides the image sectioning of the sample, without the need to section it physically. In the non-confocal Raman image, a specimen plane outside the focal plane deteriorates the information of interest from the focal plane. In the confocal image, specimen details blurred in non-confocal imaging become distinctly visible, and the image throughout is greatly improved in contrast. [5]
Raman spectra and images were obtained with an alpha 300 RA (WITec, Ulm, Germany) model equipped with an Olympus microscope coupled via the fiber of a 25 μm core diameter with an UHTS (Ultra High Throughput Spectrometer) spectrometer and a CCD Camera Andor Newton DU970N-UVB-353 operating in standard mode with 1600x200 pixels at -60 °C with full vertical binning. The incident laser beam (doubled SHG of the Nd:YAG laser (532 nm)) of alpha 300 RA was focused on the sample through a 40x dry objective (Nikon, objective type CFI Plan Fluor C ELWD DIC-M, numerical aperture (NA) of 0.60, and a 3.6–2.8 mm working distance) to the spot of 200 nm. The average laser excitation power was 10 mW, with an integration time of 0.3 s (Raman imaging) and 0.1 s (fluorescence imaging). Rayleigh scattered light was removed using an edge filter. The samples were irradiated by a laser at 532 nm at a dose 79 J/cm² (fluorescence imaging) and 318 J/cm² (Raman imaging). A piezoelectric table was used to record Raman images. Spectra were collected at one acquisition per pixel and a 1200 lines/mm diffraction grating. Prior to the basis analysis, each spectrum was processed to remove cosmic rays, increase the signal-to-noise ratio via spectral smoothing (Savitzky-Golay method), subtract a signal arising from the (CaF₂) substrate and
correct for biological autofluorescence. The large number of spectra collected in this study required the use of automated removal method for all of the spectra, which is critical to remove sources of variability arising from autofluorescence and substrate contamination. After baseline removal, the dominant remaining source of distinction between spectra is the intensity of the Raman features, arising from the variable amount of biological material within the sample. Data acquisition and processing was performed using WITec Project 2.10. The 2D array images of tens of thousands of individual Raman spectra were evaluated by the basis analysis method. In this method, each measured spectrum of the 2D spectral array is compared to basis spectra using a least squares fit. Such basis spectra are created as the average spectra from different areas in the sample. The weight factor at each point is represented as a 2D image of the corresponding color and mixed coloring component. The color code of Raman maps were based on the integrated Raman intensities in specific regions (sum option in the filter manager in the Witec project Plus 2.10). Using a lookup table, bright yellow colors indicate the highest intensities, whereas brown colors indicate the lowest intensities of the chosen region.

**Pump-probe transient absorption spectroscopy**

Approach that has a big impact on biological research is the development of femtosecond laser technologies providing unprecedented temporal resolution to monitor dynamics.

Femtosecond laser spectroscopy is a spectroscopic technique that uses ultrashort laser pulses for study the dynamics on extremely short time scales. In this technique most often based on pump-probe experiments, the pump pulse is used to excite a molecule and the second pulse (probe pulse) , delayed with respect to the pump pulse, is used to obtain an absorption spectrum of the sample at various times after excitation. The main idea of pump-probe spectroscopy is presented in Figure 5.
In our experiment the source of femtosecond pulses was mode-locked titanium sapphire femtosecond laser (MIRA, Coherent, 800 nm, 76 MHz, 9 nJ, < 200 fs) pumped with diode-pumped solid-state laser (VERDI V5, Coherent, 532 nm). The fundamental beam was amplified with Ti: Sapphire regenerative amplifier (Coherent Legend USP, 800 nm, 1 kHz, 3 mJ, 50 fs). The regenerative amplifier was pumped with diode-pumped Nd: YLF laser (JADE, Thales Laser, 527 nm, 1 kHz, 20 mJ, < 200 ns). The pulse was split in two and further amplified in a dual single pass amplifier (Coherent Elite-Duo, 800 nm, 1 kHz, 2 × 4.5 mJ, 50 fs). This amplifier was pumped by high power Nd:YLF laser (Evolution, Coherent, 527 nm, 50 mJ, < 200 ns). The output of the laser system was split and two 1 mJ laser pulses were used to pump two optical parametric amplifiers (OPA, model TOPAS from Light Conversion). These OPA combined by frequency conversion modules generate femtosecond pulses tunable in 300 nm and 2600 nm range. The energy of the pump pulse was adjusted to 2 μJ in water experiments and 700 nJ or less in experiments with DMSO. The energy of the probe pulse was at least hundred times lower than energy of the pump pulse. The pump and probe pulses were overlapped on 1 mm optical path of cell containing the AlPcS₄ or ZnPcS₄ solutions. Each solution was circulating in the cell by magnetic stirrer in order to minimize the thermal lensing and photo-quenching effects. The time delay between the pump and the probe pulse
was adjusted by motorized translation stage that has a spatial resolution of 1.5 μm. Transient absorption signals was measured with two silicon photodiodes (Thorlabs, TDS-1000) placed before and after the sample which measure initial ($I_0$) and the transmitted intensity of the probe beam ($I_t$) respectively. The $I_0$ was normalized with respect to the intensity of the pump beam, which was measured with the same type of the photodiode. The absorption signal $S(t)$ was calculated as $\log(I_0/I_t)$ The transient absorption signal $\Delta A(t)$ was computed by subtracting the absorption signal measured with and without the pump pulse. For that reason the pump beam was chopped at 40 Hz. The typical measurement error of $\Delta A$ was better than $10^{-3}$. Figure 6 presents the experimental setup.

**Fig.6.** The schematic presentation of pump-probe experimental setup.

**Steady-state UV-Vis absorption measurements**

UV-Vis absorption electronic spectra were measured with Varian Cary 5E spectrophotometer at 293 K for the solutions, films and for the thin sections of 6 μm of human breast samples of the cancerous and the noncancerous tissues stained with AlPcS₄.
**Infrared spectroscopy**

IR spectra were recorded using Specord M80, Germany. Specord M80 is a double beam spectrometer recording in the range of 4000 to 200 cm⁻¹ (2.5-50 µm) with accuracy changing from ± 0.8 cm⁻¹ till ± 0.3 cm⁻¹ depending on the spectral range. The spectra were scanned with the step of 4 cm⁻¹ and recorded in the range 800-4000 cm⁻¹ at 293K.

**Results and discussion**

**UV-Vis absorption spectroscopy of phthalocyanines**

The aggregation has a direct influence on photophysical behavior of phthalocyanines, rendering normally active photosensitizers inactive through self-quenching. UV-Vis absorption spectroscopy is ideally suited to control the aggregation of photosensitizers, a crucial factor for photodynamic phthalocyanines activity. Therefore, it is very important to discuss absorption properties of phthalocyanines.

It is well know that phthalocyanines have two characteristic, strong and broad electronic bands: the Soret band (π→π*) in the near UV, and the Q band (n→π*) in the red side of the spectrum.[39]

Figure 7 shows the electronic absorption spectra of zinc tetrarsulphonated phthalocyanine tetrasodium salt (ZnPcS₄) as a function of concentration in dimethyl sulphoxide (DMSO) and H₂O solutions, respectively.
**Fig. 7.** The electronic absorption spectra of tetrasulphonated zinc phthalocyanine tetrasodium salt ZnPcS$_4$ in the spectral range of 250-800 nm and 500-800 nm as a function of concentration in DMSO (A, B) and aqueous solutions (C, D). Reprinted from: Beata Brożek-Pluska, Arkadiusz Jarota, Krzysztof Kurczewski, Halina Abramczyk, J. Mol. Struc. 924-926, (2009) 338-346. Copyright 2009 Elsevier.

One can see from Fig. 7 that ZnPcS$_4$ has two typical major absorption bands at 350 nm (B or Soret band) and the Q band between 600-700 nm typical for all phthalocyanines. The Q band consists of at least three bands: at 613 nm, 652 nm, 679 nm (in DMSO), and at 583 nm, 633 nm, 674 nm (in H$_2$O). The bands at 613 nm (in DMSO) and at 583 nm (in H$_2$O) have been assigned to a vibronic band (VB) in the Q transition. They are related to the frequency of 1586 cm$^{-1}$ (in DMSO) and correspond quite well to the frequency of the $\nu_3$ vibrational mode at 1512 cm$^{-1}$ of zinc phthalocyanine (ZnPc) corresponded to the stretching vibration of the macrocycle.[24,39] The other Q subbands at 652 nm, 679 nm (in DMSO) and at 633 nm, 674 nm (in H$_2$O) have been assigned to dimers and monomers, respectively.[24,39] We have considered also other possible origins of the 652 nm band in DMSO, for example, an electronic transition associated with vibrational manifold of monomeric species. We have checked both the ratio of the intensities at maximum and the ratio of the integral intensities of the bands at 652 and 679 nm. If the both bands are
associated with the monomer transitions, the ratios should be constant with concentration changes. However, the ratios change significantly with concentration, which confirmed that the band at 652 nm in DMSO and the band at 633 nm in H\textsubscript{2}O represent the dimer species. The dimerisation equilibrium constants and the molar extinction coefficients have been calculated for ZnPcS\textsubscript{4} based on the results presented in Fig. 7 for the concentrations ranging from 10^{-4} to 10^{-6} M using 0.1 cm quartz cell.

To calculate the dimerization constant K we have assumed that the dilute solutions (under 10^{-4} M) contain no higher aggregates than dimers. [39] In this case the dimerization constant K for monomer - dimer equilibrium can be expressed as:

\[ K = [d]/[m]^2 \]  \hspace{1cm} (2)

where \([d]\) and \([m]\) represent the concentration of dimer and monomer respectively. Simultaneously concentrations of monomers and dimmers are related to the total concentration \(c_t\) according to the formula:

\[ c_t = 2[d] + [m] \]  \hspace{1cm} (3)

and the absorbance (A) measured in the experiments can be expressed finally as:

\[ A = (\varepsilon_m [m] + 2\varepsilon_d [d])l \]  \hspace{1cm} (4)

where \(l\) is the optical length of the cell, \(\varepsilon_m\) and \(\varepsilon_d\) are the molar extinction coefficients of monomer and dimer (in dm\(^3\) cm\(^{-1}\)/mol), respectively. Combining and rearranging the equations (2)- (4) we get:

\[ A = (\varepsilon_d c_t + (\varepsilon_m - \varepsilon_d)[-1 + \sqrt{(1 + 8K_d c_t)}] / 4K_d)l \]  \hspace{1cm} (5)

The experimental data for absorbance as a function of the total concentration \(c_t\) have been fitted with three parameters: \(\varepsilon_m\), \(\varepsilon_d\), and \(K\) to get the best agreement with the experimental data according to the standard mean square deviation procedures.

Table 1 shows the results for K obtained from the eq.(5) for ZnPcS\textsubscript{4} solutions presented in Fig.7.
Table 1. The dimerization constants $K$ and the molar extinction coefficients $\varepsilon_m$, $\varepsilon_d$ for ZnPcS$_4$ phthalocyanine.[39]

The comparison of the results from Table 1 and Fig. 7 confirms evidently that the tendency to form dimers by phthalocyanines is stronger in H$_2$O than in DMSO, which support earlier results obtained in literature.[24,39]

Figure 8 shows the electronic absorption spectra of aluminum phthalocyanines recorded at 293 K for H$_2$O solutions ($c = 10^{-5}$, $10^{-4}$, and $10^{-3}$ M) and in the hydrated films of aluminum phthalocyanines for controlled humidity of the samples. All absorption spectra for aluminum phthalocyanines exhibit, like for zinc phthalocyanines, the characteristic B and Q bands. For aluminum phthalocyanines the maximum peak positons have been recorded at around 644 nm and 675 (677) nm due to the $\pi\rightarrow\pi^*$ electronic transitions, the band at around 610 nm has been also identified.[39]

Like for others phthalocyanines, the structure of the absorption bands for aluminum phthalocyanines arise from the following factors: (a) the electronic transitions associated with the vibrational manifold of monomeric species (0-0, 0-1, and 0-2 vibrational transitions at 677 nm, 644 nm, and 610 nm, respectively)[42]; (b) exciton splitting into Qx and Qy components in the dimer structures (644 nm and 677 nm) and 0-1 vibrational transition of monomeric species at 611 nm;[43] and (c) splitting due to axial ligation of the central metal atom by water that decreases molecular symmetry [44].

Detailed inspection into Fig. 8 shows also that the absorption spectra of AlPcS$_4$ (both acid (I) and salt (II)) are independent of concentration in a wide range up to $10^{-5}$ M in a contrary to the
zinc phtahlocyanine (Fig.7.). This finding confirms that AlPcS4 does not aggregates in a broad concentration range. For higher concentrations the contribution of aggregates becomes more important and one can notice bands at 644 nm and at 610 nm.

**Fig.8.** Electronic absorption and emission spectra of aqueous solutions of AlPcS4 as a function of concentration: (A) Absorption spectra of AlPcS4 (acid (I)), (B) emission spectra of AlPcS4 (acid (I)), (C) absorption spectra of AlPcS4 (salt (II)), and (D) emission spectra of AlPcS4 (salt (II)). Reprinted from: Arkadiusz Jarota, Marc Tondusson, Geoffrey Galle, Eric Freysz, and Halina Abramczyk, J. Phys. Chem. A 2012, 116, 4000−4009. Copyright 2012 American Chemical Society.

The contradicting reports can be found in the literature about the aggregation of tetrasulphonated aluminum phthalocyanines [45-47]. Some reports suggest that that AlPcS4 does not form aggregates even at high concentrations of 10^{-4} M [47]. Simultaneously Yoon et al. have been attributed a red-shift of absorption and emission spectra of phthalocyanines at high concentrations to the dimers formation.[45] However, Dhami et al. has changed this conclusion and explained the observations by the reabsorption of fluorescence by ground state molecules of phthalocyanine. [46] Analyzing the dimerization of AlPcS4 one should remember that the dimerization of this phthalocyanine can be prevented by axial ligands acting as steric inhibitors.
It has been suggested also that the doublet band structure evident in the fluorescence emission and absorption spectra of many metallophthalocyanines in various solvents can be attributed to the splitting of the Q-band into the x and y components [48,49], and the splitting of the Q-band in the aluminum tetrasulphonated phthalocyanine is caused by the ligation of water molecules to the aluminum atom which decreases the molecular symmetry.

Some important conclusions can be drawn also by the comparison of the electronic absorption spectra of AlPcS₄ in solutions and in a solid state (in films).

Figure 9 presents the electronic absorption and emission spectra of AlPcS₄ in solutions and in a solid thin film respectively.

**Fig.9.** Electronic absorption and emission spectra of: (A) AlPcS₄ (acid(I)) in aqueous solution c = 10⁻⁵ M; (B) AlPcS₄ (salt(II)) in aqueous solution c = 10⁻⁴ M; and (C) AlPcS₄ (salt(II)) in the solid phase film at different humidities of water (H₂O), 0%, 23% (obtained by using saturated aqueous solution of CH₃COOK), 75% (obtained by using saturated aqueous solution of NaCl), and 100% (pure H₂O). Reprinted from: Arkadiusz Jarota, Beata Brozek-Pluska, Wojciech Czajkowski, and Halina Abramczyk, J. Phys. Chem. C 2011, 115, 24920–24930. Copyright 2011 American Chemical Society.
The comparison of the electronic absorption spectra of AlPcS$_4$ in solutions and in a solid state (in film) shows that instead of a sharp, intense Q-band structure with a clearly resolved substructure that can be observed in H$_2$O solution (Figs. 9A, 9B) for a solid state of aluminum phthalocyanine a broad Q-band can be noticed with red-shifted components (Figure 9C). Moreover, the absorption profile in a solid phase strongly depends on humidity. The components Qx and Qy of the Q-band are significantly red-shifted from 677 and 644 nm in the H$_2$O solution to 821 and 753 nm in the solid thin film. The vibrational band maximum position does not depend on the content of water in the film and is observed at 610 nm like in the aqueous solution.

Summarizing, one can conclude that the electronic properties of solutions and liquids cannot be directly extrapolated to the solid phases.

The steady-state absorption spectra of AlPcS$_4$ have been recorded in our laboratory not only for water solution or in film on the glass support, but also at biological interfaces of the noncancerous and cancerous human breast tissues. [5] The results at biological interfaces are shown in Figure 10.

![Absorption spectra of AlPcS$_4$ in: noncancerous human breast tissue (A), cancerous human breast tissue (B), pure thin film (C) and aqueous solution c=10$^{-3}$ M (D).](image)

One can see from Fig. 10 that in contrast to the solutions, the absorption spectra at biological interfaces of the tissues are very broad and are similar to the films of pure AlPcS₄ on the glass substrate. These spectra are also structureless in the region characteristic to the absorption of monomers, dimers, and higher order aggregates.

Assuming the thickness of the AlPcS₄ layer equal to 100 μm and the absorption coefficient the same as in solution we have estimated the concentration of the photosensitizer in the tissues as high as 10⁻² M, which indicates that the effects related to the aggregation of AlPcS₄ discussed in this chapter earlier [18,50-52] are likely very important.

**IR spectroscopy of phthalocyanines films**

Water is the most important medium in which chemical and biological processes take place. That’s why interactions of phthalocyanines with water molecules are crucial to understand interactions of photosensitizers in human tissues. Interactions of phthalocyanines macrocycles and water molecules can be easily and directly analyzed using IR spectroscopy.[53,54]

Figure 11 presents the IR spectra of the hydrated films of AlPcS₄ (acid (I)) in different environment humidities of water H₂O=0, 23, 75, 100 %.
Fig.11. IR spectra of OH stretching vibration of water in AlPcS$_4$ (acid (I)) film for various contents of H$_2$O: 100% (a), 75% (b), 23% (c), 0% (d), Raman spectrum of bulk H$_2$O (e). Reprinted from: Arkadiusz Jarota, Beata Brozek-Pluska, Wojciech Czajkowski, and Halina Abramczyk, J. Phys. Chem. C 2011, 115, 24920–24930. Copyright 2011 American Chemical Society.

The comparison for the results obtained for different humidities shows that pronounced differences can be seen in the region at around 3000- 3700 cm$^{-1}$. Analyzing the OH bands in the region 3000- 3700 cm$^{-1}$ for phthalocyanines-water complexes for various humidities one have to remember that all bands noticed in this spectral range correspond to the mixture of different types of water: bulk and interfacial water.

One can see form Fig. 11 that the structure of the OH bands for water-phthalocyanine films is dominated by the peaks at 3234 cm$^{-1}$ and 3465 cm$^{-1}$, which have been assigned to the symmetric and asymmetric like stretching vibrations of water respectively. OH groups of water are of course involved in H-bond interactions (O-H···O) with the oxygen atoms of the sulphonyl substituents. The additional band from this region at 3087 cm$^{-1}$ can be assigned to the C-H stretching vibrations of the macrocycle [55,56]. Moreover, Fig. 11 shows evidently that for the interfacial water the contribution from the asymmetric like OH stretching vibration becomes much smaller than that from the symmetric one, in contrast to the features observed at 100 % humidity, where the bulk water interactions dominate. At 0% humidity we should assume that there is no bulk water and OH bands represent only the interfacial water interacting with the metal phthalocyanine macrocycle. Detailed inspection into Fig. 11 shows a blue-shift of the maximum peak of the asymmetric vibration from 3410 cm$^{-1}$ (observed in the pure state of bulk water) to 3465 cm$^{-1}$ (water in AlPcS$_4$ (acid (I)). In contrast, OH stretching vibration at 3234 cm$^{-1}$ is red-shifted with respect to the symmetric OH stretching band of bulk water observed at 3258 cm$^{-1}$ in the pure state.

Figure 12 shows the IR spectrum of the OH stretching vibration of water in AlPcS$_4$ (salt (II)) films.
The main vibrational features in the O-H region of water are similar for those characteristic for the acid form of phthalocyanine (Fig. 11). The main difference between the results presented in Fig. 11 and Fig. 12 is related to weaker dependence on water humidity for the salt form. The blue-shift from 3410 cm\(^{-1}\) to 3465 cm\(^{-1}\) with respect to the bulk water in the pure state of the asymmetric like band is also observed for AlPcS\(_4\) (salt (II)). The detailed analysis based on the results in Figs. 11 and 12 is very difficult, because the spectral region of 3100-3600 cm\(^{-1}\) is much more complex compared to that of the bulk water. The ambiguity in the assignment of most vibrational transitions in the OH water stretching region is due to the large number of H-bond interactions of the phthalocyanine macrocycle exhibited in a rather narrow frequency range, where they overlap each other.

The phthalocyanines macrocycle can interact with water molecules in different ways. This interactions may comprise: interaction with the central metal atom (Al), H- bond interactions (O-H⋯N) between the water molecule and the bridging nitrogen atoms as well as H-bond interactions (O-H⋯O) between the water molecule and the sulphonyl substituent.[57]
To identify the O-H stretching vibrations of water involved in the H-bond interactions with a central atom (aluminum) and/or with the pyrole nitrogen atoms and the bridging nitrogen atoms (O-H···N) we have compare the IR spectra of the sulphonated and non-substituted metal complex phthalocyanine. The results are presented in Fig. 13.


Fig. 13 shows that the IR spectrum of the non-sulphonated AlPc differs markedly from that of the sulphonated derivatives of AlPcS₄ (acid (I)). For the sulphonated phthalocyanine the only band which does not disappear arises at 3344 cm⁻¹. To identify the origin of this band we have recorded the IR spectra as a function of water content in the non-sulphonated phthalocyanine films. Fig. 14 presents the IR spectra of the non-sulphonated AlPc as a function of the external humidity.
Because no dependence on the water content in the film is observed, in contrast to the results presented so far for the sulphonated AlPcS₄, it must suggest that water in AlPc films can be attributed to more restricted environments rather than to water forming H-bond interactions with the pyrole nitrogen atoms and the bridging nitrogen atoms of phthalocyanines. It has been shown [58,59] that the splitting of the Q-band in aluminum tetrasulphonated phthalocyanine is caused by the ligation of water molecules to the aluminum atom which decreases the molecular symmetry and it has been proposed that ligating water molecules interacts with the aluminum atom whereas the water hydrogens interact with the pyrole nitrogen atoms. This structure has slightly lower energy than the structure with the water hydrogens aligned with the bridging nitrogen atoms. Such a structure would be completely insensitive to the external humidity of the environment, which is supported by the results presented in Fig. 14.

**Raman spectroscopy of phthalocyanines**

Raman spectroscopy can provide detailed information on the chemical bonds present in samples (e.g. hydrogen bonding or any other intermolecular interactions) which gives
simultaneously structural information about the analyzed system. This is extremely important as those interactions play a crucial role in biology.[53]

The Raman line shape study can yield meaningful information about the phase transitions, vibrational dynamics and structural disorder in the liquid phase, crystals and glasses.[60-62]

Rama spectroscopy can be easily used for phthalocyanines studies. Figures 15 and 16 show the emission spectra of ZnPcS₄ in DMSO as a function of temperature in a range of 293-77K, for c=1x10⁻⁵ M and c=1x10⁻² M, respectively.

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**Fig.15.** The emission and Raman spectra of ZnPcS₄ in DMSO as a function of temperature in a temperature range of 293-77K for c=1x10⁻⁵ M. Reprinted from: Beata Brożek-Pluska, Arkadiusz Jarota, Krzysztof Kurczewski, Halina Abramczyk, J. Mol. Struc. 924-926, (2009) 338-346. Copyright 2009 Elsevier.

**Fig.16.** The emission spectra of ZnPcS₄ in DMSO as a function of temperature in a temperature range of 293-285K (left) and 275-77 K (right) for c=1x10⁻² M. Reprinted from: Beata Brożek-Pluska, Arkadiusz Jarota, Krzysztof Kurczewski, Halina Abramczyk, J. Mol. Struc. 924-926, (2009) 338-346. Copyright 2009 Elsevier.
One can see from Figs. 15, and 16 that in DMSO and in H$_2$O a strong emission of phthalocyanine in the spectral range of 600-800 nm in Raman spectra is noticed. The observed emission has been assigned to the Q transition typical for phthalocyanines. The observed Q emission band has at least two components with the maxima strongly depending on concentration and solvent. The precise values of the maxima position are given in Table 2.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Concentration [mol/dm$^3$]</th>
<th>$\lambda_{\text{monomer}}$ (nm)</th>
<th>$\lambda_{\text{dimer}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>1x10$^{-5}$</td>
<td>689</td>
<td>751</td>
</tr>
<tr>
<td></td>
<td>1x10$^{-2}$</td>
<td>726</td>
<td>757</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>1x10$^{-5}$</td>
<td>686</td>
<td>752</td>
</tr>
<tr>
<td></td>
<td>1x10$^{-2}$</td>
<td>708</td>
<td>767</td>
</tr>
</tbody>
</table>

Table 2. Maximum peak position of the Q emission bands for monomer and dimer of ZnPcS$_4$ at 293K.[39]

Figure. 17 presents the emission spectra of ZnPcS$_4$ in H$_2$O as a function of temperature in a temperature range 293-77K for c=1x10$^{-5}$ M.

![Emission Spectra](image)

**Fig.17.** The emission spectra of ZnPcS$_4$ in H$_2$O as a function of temperature in a range of 293-275 K and 250-77K inset for c=1x10$^{-2}$ M. *Reprinted from: Beata Brożek-Pluska,*
Comparison for Figs. 15 and 16 as well as Fig. 17 shows that the bands characteristic for emission at lower wavelength corresponds to monomer whereas the bands at around 760 nm correspond to dimers (Table 2). The results in Figs. 16 and 17 show also that the relative intensity of the emission of dimer with respect to monomers in H₂O decreases in contrary to that for dimers in organic solvent - DMSO for the same concentration. Moreover, the dimerization constant K estimated based on the results of the absorption measurements (Table 1) is about 100 times higher in H₂O. Such big differences in emission intensity observed for H₂O compared to DMSO have to indicates that there are exist additional nonradiative energy dissipation channels for dimers in H₂O, which are very effective in this solvent. This additional channel of energy dissipation can be monitored by the low temperature emission spectra. Indeed one can see from Figs. 15-17 that the strong fluorescence of the Q band which can be observed between 293-275 K (liquid and undercooled phases) decreases dramatically at lower temperatures when the sample becomes frozen and begins to increase again with temperature decreasing in the region assigned to the monomer emission. Table 3 shows the emission maxima of the bands for ZnPcS₄ in H₂O and DMSO at 77K.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Concentration [mol/dm³]</th>
<th>λ_max (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>1x10⁻⁵&lt;br&gt;1x10⁻²</td>
<td>696&lt;br&gt;711, 789</td>
</tr>
<tr>
<td>H₂O</td>
<td>1x10⁻⁵&lt;br&gt;1x10⁻²</td>
<td>Not observed&lt;br&gt;711</td>
</tr>
</tbody>
</table>

Table 3. Maximum peak position of the emission bands of ZnPcS₄ in H₂O and DMSO at 77K.[39]
It is obvious that this emission must be assigned to transient species generated by light excitation, species stabilized by the low temperature. At room temperatures the recombination processes are likely too fast to observe the emission of the transient species. At lower temperatures the molecular motions become slower resulting in arising of the band at around 711 nm (Table 3). Trying to explain the nature of the observed species we have to take into account the photoredox dissociation processes. The visible light can induce such a reactions in zinc tetrasulphonated phthalocyanines. Photoredox dissociation can be described by the equation below:

$$[\text{Zn}^{II} \text{PcS}^{-4}]_2 \rightarrow \text{hv}=514\text{nm} \rightarrow \text{Zn}^{II} \text{PcS}^*^{-5} + \text{Zn}^{II} \text{PcS}^*^{-3}$$ (6)

The photoredox dissociation (6) leads to the electron transfer between the adjacent molecules resulting in formation of oxidized and reduced ligand radicals. The photodissociation mechanism can rationalize the lower intensity of dimers in H$_2$O (Fig. 17) than in DMSO (Fig. 15, 16) where the photodissociation channel is less effective (the intensity of emission in DMSO is much lower than that in H$_2$O). Thus, the low temperature emission for ZnPcS$_4$ at 711 nm in aqueous solution can be assigned to the emission of the oxidized (or/and reduced) ligand radical.

To answer if the transient species represent the ligand radicals (eq.6) or the transient products of the radicals associated with formation of the adduct or peroxo compound suggested in literature for some phthalocyanines

$$\text{Zn}^{II} (\text{PcS}4^*)^{-5} + \text{O}_2 \rightarrow \text{Zn}^{II} (\text{PcS}4 - \text{O}_2^*)^{-5}$$ (7)

we have recorded the emission spectra by the low temperature Raman spectroscopy for degassed samples. The results of these experiments for ZnPcS$_4$ in H$_2$O and DMSO are presented in Figs. 18 and 19.
One can see from the insets of Figures 18 and 19 that like in nondegassed samples in H₂O solutions the band at 711 nm is still observed. This observation confirms that the transient species cannot represent the peroxo compounds (7). The tremendous influence of the oxygen in contrast to the band at 711 nm, oxygen has been observed for the emission at around 540 nm and the Q band emission at around 700 nm at higher temperatures 293-275 K for liquids and undercooled liquids. One can see that in the presence of oxygen the intensities of both bands in H₂O are comparable in contrast to the intensities in degassed samples where the band at 540 nm evidently dominates. Although extensively studied the origin of the emission at 540 nm of phthalocyanines is still ambiguous. This emission in the literature was attributed to: reduced form of phthalocyanines (MePc)⁺ [25], S₂→S₀ emission for the Q band [63], Tₙ→T₁ emission [33], charge transfer (CT) between the central metal (3d states) and the ligand phthalocyanine states [51], π*→n emission [25] or the emission for the B band [52].
Raman imaging of phthalocyanines in human breast tissue

In biomedical applications we require high spatial resolution, sometimes far below the diffraction limit, strong signal enhancement enabling monitoring metabolites, the genetic and immunological responses in biological systems.

Raman spectroscopy and Raman microscopy offers many advantages over fluorescence microscopy: a) Raman spectroscopy needs no external labelling, b) biochemical signatures of the molecules are richer as each component of the tissue provides its own pattern of vibrational behaviour, including nucleic acids, lipids, biological chromophores, proteins, c) spatial resolution of Raman microscopes (TERS) goes far beyond diffraction limit and is comparable with fluorescence microscopy STED [64], d) Raman (SERS with nanoplasmons) has better sensitivity and multiplexing capabilities. This new Raman modality-nanoparticle approach has promise for enabling more accurate breast tumor diagnostics and imaging.

Raman imaging is ideally suited for the early detection of epithelial diseases, including most cancers, and for the assessment of tumor margins and therapy response. Vibrational spectroscopy may very soon replace standard but unsatisfying medical procedures of clinical diagnostics and conventional medical imaging. The most advanced of these medical applications of Raman spectroscopy could be ready for the market in less than five years.[65]

Raman and IR imaging combined with nonlinear laser spectroscopy have brought revolution in cancer detection.[66] High spatial and temporal resolution of these methods allows to detect a single cancerous cell in vivo and monitor molecular events that may contribute to cancer development. We have shown that Raman imaging allows for guidance of intraoperative tumor resection in real surgery time, and a histological correlation validated that Raman imaging is capable accurately delineate the breast tumor margins (optical biopsy).[5,7-10] Therefore, the Raman imaging is clinically of great importance, but it also focuses on issues that may open up new horizons and opportunities for research, because one
of the fundamental goals of biophysics, biochemistry and molecular biology is to understand the complex spatio-temporal interactions of molecules at biological interfaces. In the last ten years, various Raman based schemes have been developed to increase the spatial resolution of the microscope beyond the diffraction limit.

The medical applications of Raman imaging are a rapidly developing area of molecular biospectroscopy that create new possibilities in human cancer diagnostics [5-15].

Cancer diagnosis requires better screening of early stages of pathology and monitoring patient responses to treatment. Current technologies in clinical sector based on PCR amplification or immunofluorescence staining are expensive, sophisticated and time consuming. In this part we describe a powerful alternative: multi-modality: IR-Raman imaging-femtosecond spectroscopy approach which may bring revolution in cancer detection and treatment. The approach is ideally suited to explore cancer by monitoring the biochemistry/morphology/mechanics of cells necessary for survival, proliferation, differentiation, cell death, and expression of many specific functions.

Simultaneous combination of Raman imaging methods and basis of photodynamic methods (PDT, PDD) can open a new era in cancer detection and treatment. Current clinical strategies and future views in photodynamic methods have been discussed recently,[67-73]

In this part we will demonstrate that combining Raman and fluorescence imaging we can create a powerful method that can be useful in photodynamic therapy (PDT) of cancer,[73,74].

The other mechanism (Type I) occurs when the oxygen concentration in the environment of the tissue is limited. In this case, photooxidation reactions dominate with the formation of radicals in a tumor tissue. As a result of hydrogen or electron transfer between the excited photosensitizer and the tissue, ion radicals are formed, which destroy cancer cells. Alternatively, the photosensitizer may also react directly with the target (Type III).
As important as the mechanisms of photoreactions upon light irradiation is the localization of the photosensitizer. Conceptually, photosensitizers accumulate in both types of tissues, normal and cancerous, but the faster clearance from the normal tissue allows for a relatively selective accumulation and selective PDT. The resulting differences in accumulation allow for the selective destruction of cancer cells and reduce the damage to normal tissue. The ratio of the photosensitizer concentration in the tumor to normal tissue depends on the type of photosensitizer and the type of tumor tissue [75]. The initial subcellular localization of each photosensitizer depends on hydrophobic and hydrophilic properties, protein binding affinity and charge. The hydrophobic and hydrophilic properties of the photosensitizers are related to their water solubility, which regulates aggregation and the efficiency of singlet oxygen production. Taking into account the solubility of photosensitizers, one can distinguish hydrophobic photosensitizers that tend to accumulate in lipid-containing cellular structures (e.g., cell membrane, endoplasmic reticulum) and hydrophilic photosensitizers that accumulate in water-rich regions (e.g., lysosomes in the cell). Amphiphilic photosensitizers accumulate in both water- and lipid-rich regions. The charge determines the anionic, cationic, amphiphilic or neutral characteristics of photosensitizers and plays an important role in the cellular uptake and photodynamic efficacy of photodynamic therapy (PDT).[76,77] Some studies have also demonstrated that a water-soluble tetrasubstituted cationic aluminum phthalocyanine (AlPcN(4)) efficiently bound to phospholipid membranes behaved similarly to anionic tetrasulfonated aluminum and zinc phthalocyanine complexes. [78] Thus, the binding of tetrasulfonated metallophthalocyanines to phospholipid membranes has been suggested to be primarily determined through metal-phosphate coordination.[78] Among the hydrophilic photosensitizers, anionic derivatives of metal complexes of phthalocyanines with sulpho substituents, such as zinc tetrasulfonated phthalocyanine (ZnPcS₄) or AlPcS₄, are some of the best targets for the generation of novel photosensitizers. Metal phthalocyanines have become
popular in PDT since Ben-Hur [79] reported the anticancer properties of phthalocyanine aluminum chloride. The efficacy of phthalocyanine derivatives as photosensitizers in recent years was significantly enhanced by employing short-pulse irradiation with a femtosecond laser, which increases selectivity by reducing the photothermal effect on normal cells, and targeted photodynamic therapy, which increases specificity by employing photosensitizer-nanoparticle conjugates [80-86]. The targeted PDT is based on molecular recognition towards specific cell surface receptors, which can be achieved by conjugation with a specific antibody. Recently, targeted PDT has been employed in breast cancer cells using Her2 antibody-phthalocyanine-gold nanoparticle conjugates [87].

Tetrasulfonated aluminum phthalocyanine was selected in our study as a hydrophilic and anionic photosensitizer.[87]

First, the results of the confocal microRaman/fluorescence images and spectra on the noncancerous and the cancerous human breast tissues of the patient (P104, infiltrating ductal cancer, GX) without addition of photosensitizers will be presented. In the next step, we add photosensitizers at concentrations corresponding to standard PDT practical applications. Before we present the results, we will describe the types of cancer analyzed in this study. Ductal cancer develops in the epithelial cells of the ducts. During disease progression, the normal polar organization of the luminal epithelial cells is lost. As these cells differentiate and proliferate, the epithelial cells completely fill the lumen of the duct. In infiltrating ductal cancer, the transformed epithelial cells cross the basement membrane, and migrate through the basement membrane into the surrounding structures, which are predominantly but not exclusively composed of adipose tissue, fibroblasts, connective tissue, and collagen fibers. These structures can be easily observed in Figures 20A and 21A, where we present histological images. The histological images demonstrate the morphology, as hematoxylin and eosin (H&E) staining allows the visualization of various structures in tissues. Hematoxylin
stains all basophilic components blue, especially the nucleus, containing DNA and RNA, and the rough endoplasmatic reticulum, with an accumulation of ribosomes, as a result of a coordination bond between aluminum and the phosphorous atoms of the DNA and RNA. Eosin stains the cytoplasm, connective tissue and collagen fibers (eosinophilic substances) red due to ionic bonds between the anionic dye and cationic plasma proteins [88,89].

The histological images demonstrate the morphology of the tissue but cannot provide any information about the biochemistry of the observed structures. However, biochemical information can be provided by Raman imaging. The histological and Raman images and the typical average Raman spectra of the breast tissue from the margin of the tumor mass and the cancerous breast tissue from the tumor mass are presented in Figures 20 and 21.
Fig. 20. Patient P104, the breast tissue from the margin of the tumor mass: H&E-stained histological image (a), microscopy image (1000 x 1000 µm, 2000 x 2000 pixels, spatial resolution 0.5 x 0.5 µm) composed of 121 single video images (b), Raman image (550 x 350 µm, 250 x 150 points per line/lines per image, resolution 2.2 x 2.3 µm) (c), microscopy image (550 x 350 µm, 250 x 150 points per line/lines per image, resolution 2.2 x 2.3 µm), images for the filters for spectral regions: 1490 – 1580 cm⁻¹, 2850 – 2950 cm⁻¹, and 2900 – 3010 cm⁻¹ (d), average spectra used for the basis analysis method and single spectra corresponding to different areas of Raman image (colors of the spectra corresponding to colors of the Raman image presented in part (c)) (e), microscopy image (550 x 350 µm, 250 x 150 points per line/lines per image, resolution 2.2 x 2.3 µm) and single spectra of various sites of the sample, colors of the spectra correspond to the colors of the crosses in the microscopy image; integration times 10 sec, 2 accumulations (f). Reprinted from: Halina Abramczyk,
Fig. 21. Patient P104, the breast tissue from the tumor mass: H&E-stained histological image (a), microscopy image (2000 x 2000 μm, 300 x 300 pixels, spatial resolution 0.66 x 0.66 μm) composed of 400 single video images (b), Raman image (80 x 80 μm, 60 x 60 points per line/lines per image, resolution 1.3 x 1.3 μm) (c), microscopy image (2000 x 2000 μm, 300 x 300 pixels, spatial resolution 0.66 x 0.66 μm), images for the filters for spectral regions: 1490 – 1580 cm⁻¹, 2850 – 2950 cm⁻¹, and 2900 – 3010 cm⁻¹ (d), average spectra used for the basis analysis method and single spectra corresponding to different areas of Raman image (colors of the spectra corresponding to colors of the Raman image presented in part (c)) (e), microscopy image (2000 x 2000 μm, 300 x 300 pixels, spatial resolution 0.66 x 0.66 μm) and single spectra of various sites of the sample, colors of the spectra correspond to the colors of the crosses in the microscopy image; integration times 10 sec, 2 accumulations (f). Reprinted
Detailed analysis of Figs. 20 and 21 shows that the Raman images (c) reveal the inhomogeneous distribution of the different compounds in the samples and resemble almost perfectly the microscopy images (b) and the conventional histological images (a) obtained from the tissue sections stained with hematoxylin and eosin (H&E). The Raman image is not prone to subjective interpretations and monitors biological tissue without any external agents, in contrast to histological assessment, that’s why the main advantage of the ‘Raman biopsy’ compared with conventional histological analysis, is that it provides not only the morphological structure but also direct biochemical information. In figures 20 and 21 one can see the vibrations characteristic of various structures such as carotenoids, lipids, and proteins. Thus, Raman imaging plays an important role in the spectroscopic characterization of human breast tissues both from the tumor mass and from the margin.

A detailed inspection of Figures 20f and 21f demonstrate that the areas from the tumor margin contain a markedly higher concentration of adipose tissue (triglycerides, fatty acids) than the cancerous tissue from the bulky tumor mass that is dominated by proteins. The Raman lipid profile characteristic of the adipose tissue (lipid (I) [13]) is dominated by peaks at 2854, 2888 and 2926 cm$^{-1}$ and is characteristic of unsaturated fatty acids and triglycerides. The peaks at 1004, 1158, and 1518 cm$^{-1}$ correspond to vibrations typical of the C-C and C=C stretching modes of carotenoids. The Raman lipid-protein profile (lipid II [13]) characteristic of the cancerous tissue is dominated by the protein component, which is predominantly but not exclusively composed of type I collagen with typical vibrations of approximately 2940 cm$^{-1}$. Collagen is the main structural protein of the various connective tissues to which the transformed epithelial cancer cells migrate and invade through the basement membrane. Additional proteins are produced by the organism in
response to cancer development in the transformed epithelial cancer cells that migrate and invade through the basement membrane to stroma. For example, approximately 30% of breast cancers exhibit an amplification of the HER2/neu gene or overexpression of its protein product [90].

Analyzing the results obtained using the Raman imaging we can monitor also the accumulation of carotenoids and fatty acids in different regions of the tissue employing various spectral filters. Figures 20d and 21d present the Raman images for the tissue from the tumor margin (Figure 20d) and the cancerous tissue from the tumor mass (Figure 21d) for the filters at approximately 1518 cm$^{-1}$ (1490-1580 cm$^{-1}$), 2854 cm$^{-1}$ (2850-2950 cm$^{-1}$), and 2940 cm$^{-1}$ (2900-3010 cm$^{-1}$), corresponding to the vibrational frequency of carotenoids, fatty acids and triglycerides, and proteins, respectively. The first observation one can make is that the Raman image of the adipose in the breast tissue at the filter 1518 cm$^{-1}$ (Figures 20d and 21d) shows that the distributions of carotenoids is almost identical to that for the filter 2854 cm$^{-1}$ for unsaturated fatty acids, and the adipose tissue act as a dynamic reservoir that accumulates carotenoids to supply them to the human organs. In contrast, the cancerous breast tissue from the tumor mass does contain a very low amount of carotenoids, as demonstrated in Figure 21d at the filter 1518 cm$^{-1}$. Moreover, the image at the protein filter at 2940 cm$^{-1}$ in Figure 21d shows the distribution of proteins in the cancerous breast tissue. The comparison of the images of the cancerous tissue demonstrates that proteins are accumulated in the regions complementary to those of the fatty acids, triglycerides and carotenoids. The results obtained for the human tissue in this paper are consistent with those obtained in the previous papers [5-15].

Figures 22 and 23 present the Raman images and typical Raman spectra of the breast tissues soaked in AlPcS$_4$ aqueous solution (c=10$^{-6}$ M) for the same areas and spectral regions as in Figures 20 and 21. A detailed inspection of Figures 22 and 23 shows once again that the
vibrations characteristic of carotenoids, fatty acids, and proteins still are easily visible in Raman spectra for the human breast tissue soaked in the photosensitizer AlPcS$_4$ aqueous solution, and we don’t lose any information about the cancer changes markers if we analyze the soaked sample.

![Fig.22](image)

**Fig.22.** Patient P104, the breast tissue from the margin of the tumor mass: H&E-stained histological image (a), microscopy image (1000 x 1000 μm, 2000 x 2000 pixels, spatial resolution 0.5 x 0.5 μm) composed of 121 single video images (b), confocal/Raman fluorescence image (c) microscopy image (1000 x 1000 μm, 2000 x 2000 pixels, spatial resolution 0.5 x 0.5 μm) and images for the filters for spectral regions: 2850 – 2950 cm$^{-1}$, 2900 – 3010 cm$^{-1}$ and 3670 – 4600 cm$^{-1}$ (d), average spectra used for the basis analysis method and single spectra corresponding to different areas of fluorescence image (colors of the spectra corresponding to colors of the fluorescence/Raman image presented in part (c)) (e), microscopy image 2000 x 2000 μm, 300 x 300 pixels, spatial resolution 0.66 x 0.66 μm) and single spectra of various sites of the sample, colors of the spectra correspond to the colors of the crosses in the microscopy image (f). Integration times: 0.3 sec for Raman spectra and 0.1 sec for fluorescence spectra. *Reprinted from: Halina Abramczyk, Beata Brozek-Pluska, Jakub Surmacki, Jacek Musial, Radzislaw Kordek, Analyst, 2014, 139(21), 5547-59. Copyright 2014 The Royal Society of Chemistry.*
Fig. 23. Patient P104, the breast tissue from the tumor mass: H&E-stained histological image (a), microscopy image (100 x 80 μm, 60 x 60 points per line, resolution 1.3 x 1.3 μm) (b) confocal fluorescence/Raman image (80 x 80 μm, 60 x 60 points per line, resolution 1.3 x 1.3 μm) (c) microscopy image (100 x 80 μm, 60 x 60 points per line, resolution 1.3 x 1.3 μm), images for the filters for spectral regions: 1490 – 1580 cm\(^{-1}\), 2850 – 2950 cm\(^{-1}\), and 2900 – 3010 cm\(^{-1}\), 3670 – 4600 cm\(^{-1}\) (d), average spectra used for the basis analysis method and single spectra corresponding to different areas of fluorescence image (colors of the spectra corresponding to colors of the Raman image presented in part (c)) (e). Integration time: 0.3 sec for Raman spectra and 0.1 sec for fluorescence spectra. Reprinted from: Halina Abramczyk, Beata Brozek-Pluska, Jakub Surmacki, Jacek Musial, Radzisław Kordek, Analyst, 2014, 139(21), 5547-59. Copyright 2014 The Royal Society of Chemistry.

In addition, we can follow the localization of photosensitizers using fluorescence images from the same areas as those obtained for the Raman images. Therefore, combining the Raman/fluorescence imaging, one can obtain information both on the localization of the photosensitizer and the biochemistry of the tissue structures where the photosensitizer is localized. We have demonstrated that the absorption of AlPcS\(_4\) is dominated by a monomeric form in aqueous solution. [17] Green light (532 nm) has been employed in this study because this may be of clinical benefit in selected cases where lesions do not require significant illumination depth, using of green light can be easier also for medical doctors [6].
In the literature has been shown that hydrophilic photosensitizers generally localize in lysosomes [17,77,91], while the lipophilic dyes are concentrated in the plasma membrane, mitochondria, endoplasmic reticulum, and nuclear membranes [41]. AlPcS₄ is a hydrophilic and anionic photosensitizer.

One can observe in Fig. 24 that the AlPcS₄ is localized predominantly in membrane structures of the adipose tissue (corresponding to the violet color in the fluorescence images in Fig. 24b) and in the epithelial cells spreading through the basement membrane of ducts into the supporting stroma (corresponding to the green color in the fluorescence images in Fig. 24b). The subcellular localization of the photosensitizer is related to interactions between hydrophilic AlPcS₄ and proteins produced in the transformed epithelial cells. The photosensitizers did not accumulate in connective tissue [67], which is the predominant component of stroma. As the hydrophilic photosensitizer, AlPcS₄ molecules do not enter into the hydrophobic adipose.

![Fig. 24. Patient P104, the breast tissue from the margin of the tumor mass: Raman images without AlPcS₄ (a) and fluorescence images of the tissue soaked in AlPcS₄ and irradiated with doses: 79 J/cm² (I), followed by the next 318 J/cm² (II) and 79 J/cm² (III) (b) and the fluorescence spectra of AlPcS₄ (c). The colors of the fluorescence spectra correspond to the colors of the fluorescence images b). Integration time: 0.1 sec. Reprinted from: Halina Abramczyk, Beata Brozek-Pluska, Jakub Surmacki, Jacek Musial, Radzisław Kordek, Analyst, 2014, 139(21), 5547-59. Copyright 2014 The Royal Society of Chemistry.](image-url)
This preferential localization is even more visible in the breast tissue from the tumor mass in Figure 25, where the photosensitizer is distributed exclusively in the infiltrating epithelial cells in the supporting stroma (bright yellow areas) and is absent in the adipose tissue (dark circle areas at the bottom). The dark area at the top represents CaF$_2$ substrate.

Fig. 25. Fluorescence images (80 x 80 μm, 60 x 60 points per line, spatial resolution 1.3 x 1.3 μm) from the spectral region 3670-4600 cm$^{-1}$ of the tissue from the tumor AlPcS$_4$ irradiated using doses: 79 J/cm$^2$, followed by the next 318 J/cm$^2$ and 79 J/cm$^2$ and the Raman spectra of AlPcS$_4$ from the bright yellow areas. Integration time: 0.1 sec. Reprinted from: Halina Abramczyk, Beata Brozek-Pluska, Jakub Surmacki, Jacek Musial, Radzislaw Kordek, Analyst, 2014, 139(21), 5547-59. Copyright 2014 The Royal Society of Chemistry.

Figures 24 and 25 show that the intensity of fluorescence decreases (photobleaching) with increasing fluence, which depends both on the increased power of the laser from 10 mW to 40 mW (Figs. 24b and 25b) and the duration of irradiation (Figures 24b and 25b). The low
intensity of fluorescence simply indicates photodegradation of the photosensitizer during PDT process. Direct observation of the fluorescence presented in Figures 24 and 25 can help medical doctors to identify of the optimal amount of photosensitizer as well as doses useful during PDT.

The comparison between both the histological and Raman images of the noncancerous and cancerous tissues demonstrates also that the normal tissue contains markedly higher levels of adipose cells, which contains a high fraction of unsaturated fatty acids. This remark may suggest that the presence of the adipose tissue may be inversely related to breast cancer development [61-70]. This observation, analyzed in the context of the present study and a number of literature studies, suggests that fatty acids and the products of their metabolism play an important role in the molecular mechanisms of carcinogenesis [91]. In this context it is extremely important to monitor not only the photodegradation of the photosensitizer, but also to control biodegradation of the main constituents of the tissue upon PDT irradiation.

Summarizing, Raman microspectroscopy and confocal Raman imaging combined with confocal fluorescence allow studying distribution of phthalocyanine (AlPcS₄) in the noncancerous and cancerous breast tissues. Moreover, Raman imaging can be treated as a new and powerful technique in the photodynamic therapy of cancer, increasing our knowledge of the mechanisms and efficiency of photosensitizers by better monitoring the localization in cancer cells and clinical assessment of the therapeutic effects of PDT. [6]

**Femtosecond spectroscopy of phthalocyanines**

Advances of femtosecond methods are crucial to the understanding of ultrafast phenomena in nature in many biological systems including human tissues.
To understand in detail the mechanisms of interaction of phthalocyanines and human tissues we have recorded transient femtosecond absorption spectra of aluminum phthalocyanine.[18]

In order to study energy dissipation and dynamical alterations in the phthalocyanine structure, a system was triggered with laser and monitored with time-resolution of 50 fs. To get an insight into mechanisms of energy dissipation we have monitored the dynamics of AlPcS₄ in the interfacial region of the human breast tissues upon excitation at 677 nm, which promotes the S₀→S₁ transition, with the main contribution from the (a₂u)→(e₉), transition in the Q-band.

Once upon excitation the molecules were probed with different time delays at different wavelengths (570 nm, 602 nm, 670 nm).

Figure 26 shows the transient absorption signals ΔA(t) of AlPcS₄ at the interface of the cancerous and noncancerous tissues and in the film at the glass support as a function of the time delay when pumped with 677 nm and probed with 664 nm. The results are presented in the full time window up to 1 ns (Fig. 26a) and in the narrow window up to 10 ps (Figure 26b).

Fig.26. Transient absorption signal ΔA(t) of AlPcS₄ in noncancerous (●), cancerous (▲) human breast tissues and in film (♦) as a function time delay in the full time window up to 1 ns (a) and 10 ps (b), pumped at 677 nm and probed at 670 nm. Reprinted from: Halina Abramczyk, Beata Brozek-Pluska, Marc Tondusson, Eric Freysz, J. Phys. Chem. C 2013, 117, 4999–5013. Copyright 2013 American Chemical Society.
Figure 26 shows that directly upon excitation at 677 nm, a negative signal at 670 nm has been recorded. The negative signal has been assigned to the instantaneous bleaching of the ground state $S_0$ due to the $S_0\rightarrow S_1$ transition in the Q band. The recovery of the bleach at 670 nm was described by three-exponential functions with the time constants of $130\pm0.10$ fs, $1.53\pm0.19$ ps, and $37.86\pm5.25$ ps for the cancerous tissue and $110\pm0.10$ fs, $1.34\pm0.16$ ps, and $40.72\pm7.86$ ps for the noncancerous tissue. The recovery of the bleach in the AlPcS$_4$ film is found to be also three-exponential but with different time constants $830\pm100$ fs, $7.31\pm1.02$ ps, and $56.03\pm6.58$ ps. Comparison of these time constants shows that the dynamics recovery of the ground state $S_0$ for the noncancerous and cancerous tissues is similar. In contrast, all the time constants are shorter than those characterizing the AlPcS$_4$ film dominated by the aggregation effects, which indicates that the dynamics of the photosensitizer is sensitive to the specific microenvironment of the biological tissue.

Figure 27 shows that the bleach of the negative signal of AlPcS$_4$ in water at 670 nm accompanied by a sudden rise at 602 nm.

![Fig.27](image-url)

**Fig.27.** Transient absorption signal $\Delta A(t)$ of AlPcS$_4$ in noncancerous (●) and cancerous (▲) human breast tissues and in film (♦) as a function time delay in the full time window up to 1 ns (a) and 10 ps (b), pumped at 677 nm and probed at 602 nm. *Reprinted from: Halina Abramczyk, Beata Brozek-Pluska, Marc Tondusson, Eric Freysz, J. Phys. Chem. C 2013, 117, 4999–5013. Copyright 2013 American Chemical Society.*
The positive signal has been assigned to the excited state absorption (ESA) of the excited state $S_1$ generated by the $S_0 \rightarrow S_1$ transition in the Q band. The results clearly demonstrate that the dynamics of AlPcS$_4$ in the noncancerous tissue is markedly faster than that in the cancerous tissue. These decays are found to be three-exponential and are fitted with time constants of 810 ± 40 fs, 5.63 ± 5.49 ps, 59.90 ± 12.85 ps for the cancerous tissue and 176 ± 20 fs, 847 ± 422 fs, 6.03 ± 1.92 ps for the noncancerous tissue. In the contrast to the biological interfaces, the film of AlPcS$_4$ exhibits the negative bleaching signal with the recovery described by three-exponential with the time constants 163 ± 25 fs, 1.56 ± 0.42 ps, and 21.59 ± 4.14 ps.

Figure 28 shows the comparison of the transient absorption signal $\Delta A(t)$ of AlPcS$_4$ as a function time delay in the noncancerous, and cancerous human breast tissues, in the film and in the aqueous solution.

**Fig.28.** Comparison of the transient absorption signal $\Delta A(t)$ of AlPcS$_4$ as a function time delay in noncancerous (●) (a), cancerous (▲) (b) human breast tissues, film (♦) (c), (pumped at 677 nm and probed at 670 nm), aqueous solution (▼) (concentration $10^{-3}$ M, pumped 677 nm, probed at 664 nm). *Reprinted from: Halina Abramczyk, Beata Brozek-Pluska, Marc Tondusson, Eric Freysz, J. Phys. Chem. C 2013, 117, 4999–5013. Copyright 2013 American Chemical Society.*

The recovery of the bleach of AlPcS$_4$ in solution at 670 nm in contrary to results for biological background was fitted using two-exponential function with the time constants of 5.09 ± 0.99 ps, and 203.16 ± 18.75 ps [92] and three-exponential function with 4.27 ± 1.68 ps,
47.21 ± 124.44 and 232.52 ± 81.00 ps. Fitting data in the early time window extending to 2 ps found that the initial recovery of the signal of AlPcS₄ in water solution at 570 nm is mono-exponential and can be fitted with a time constant of 405±164 fs in water.[92] The time constants in solution are of the same order as those obtained by Howe et al. for PcS₄ and ZnPcS₄ in DMSO probed at 720, 790, 820 nm.[92]

The results presented so far show that one can observe noticeable differences between the dynamics of the photosensitizer in solution and at interfacial of the biological tissue. The dynamics of AlPcS₄ in solution is much slower than that at the biological interfaces of the tissues and in the film, for which we find no evidence of the slow component 232.52±81.00 ps typical for solution. This observation confirms that aggregation in the solid phases results in shortening of the relaxation times. It has been show that excited-state lifetimes of phthalocyanines homodimers are short and in some cases, ligand-to-ligand charge transfer seems to be an effective deactivation pathway. Nikolaitchik et al. have shown that according to the exciton theory in face to face dimers transitions between ground state and the lower exciton state are symmetry forbidden and the lack of the fluorescence in such systems indicates that upper exciton state rapidly converts to the lower one, which undergoes subsequent nonradiative relaxation, including intersystem crossing.[93] The absence of fluorescence in cofacial dimers was related also to the decrease of the triples quantum yields and the shortening of triplet state life times, and dramatic increase of internal conversion rates by a factor of 20-300. The enhancement of internal conversion rates may be related to the fact that the exciton splitting places the lower exciton state closer to the ground state. The increased intersystem crossing rate may also be manifestation of the lowering of the energy gap with the lower exciton state closer to the corresponding triplet state. Similar observation have been made by Ma et al. for iron phthalocyanine.[94] They have shown that in the condensed phases effective intermolecular interactions and the molecular distortion resulting
in a shortening of the excited state lifetime and very high inter system crossing rate. The influence of aggregation on femtosecond excited state dynamics has been investigated also by Peyghambarian et al. [95] They have shown that the induced absorption signal develops as the absorption bleaching signal decays suggesting the exciton decay into a subgap state. The fluorescent phthalocyanine dimers have been investigated by Speirs et al. [96] They have shown that the fluorescence is typical for dimers deviated from an eclipsed cofacial structure.

Table 4 compares the time constants characterizing the dynamics of the ground state recovery of AlPcS$_4$ at biological interfaces for the noncancerous and cancerous tissues, in film, and in aqueous solution. One can see that the dynamics recovery of the ground state $S_0$ for the noncancerous and cancerous tissues is similar in contrast to the dynamics of AlPcS$_4$ in solution, which is much slower.

<table>
<thead>
<tr>
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<th>Time constants</th>
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<tr>
<td><strong>Noncancerous tissue</strong></td>
<td>110±10 fs, 1.34±0.16 ps, 40.72±7.86 ps</td>
</tr>
<tr>
<td><strong>Cancerous tissue</strong></td>
<td>130 ± 0.1 fs, 1.53 ± 0.19 ps, 37.86 ±5.25 ps</td>
</tr>
<tr>
<td><strong>Film</strong></td>
<td>830±100 fs, 7.31±1.02 ps, 56.03±6.58 ps</td>
</tr>
<tr>
<td><strong>Solution</strong></td>
<td>4.27±1.68 ps, 47.21±124.44 ps, 232.52±81.00 ps</td>
</tr>
</tbody>
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Table 4. Comparison of time constants of AlPcS$_4$ at biological interfaces for the noncancerous and cancerous tissues, film (for pump 677 nm, probe 670 nm) and solution (for pump 678 nm, probe 660 nm).[18]

Similar picture emerges from comparison between the dynamics at biological interfaces and in solution for the positive ESA signal decay at 602 nm. Figure 29 shows the comparison of the transient absorption signals $\Delta A(t)$ of AlPcS$_4$ as a function time delay.
Fig. 29. Comparison of the transient absorption signal $\Delta A(t)$ of AlPcS₄ as a function of time delay in noncancerous (●) (a), cancerous (▲) (b) human breast tissues (pumped at 677 nm and probed at 602 nm), aqueous solution (▼) (c) (concentration $10^{-3}$ M, pumped 677 nm, probed at 570 nm). Reprinted from: Halina Abramczyk, Beata Brozek-Pluska, Marc Tondusson, Eric Freysz, J. Phys. Chem. C 2013, 117, 4999–5013. Copyright 2013 American Chemical Society.

The comparison between Figure 29a, b and c shows that the decay of ESA signal in AlPcS₄ at biological interfaces is markedly faster than that in solution. The decay in solution is found to be much slower than that observed at biological interfaces of the tissues and in the film. This observation confirms once again that the aggregation in the solid phases results in shortening of the relaxation times.

The time constants found for solution were equal to $2.37 \pm 0.71$ ps, $28.07 \pm 24.18$ ps, $345.81 \pm 107.87$ ps. Fitting data in the early time window extending to 10 ps found that the initial decay of the signal at 570 nm is mono-exponential and can be fitted with a time constant of $560 \pm 94$ fs in water. Table 5 compares the time constants characterizing dynamics of the excited state $S_1$ decay of AlPcS₄ at biological interfaces for the noncancerous and cancerous tissues, in film, and in aqueous solution.
Table 5. Comparison of time constants of AlPcS₄ at biological interfaces od the noncancerous and cancerous tissues, film (for pump 677 nm, probe 602 nm) and solution (for pump 678 nm, probe 571 nm).[18]

<table>
<thead>
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<th>Time constants</th>
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<tbody>
<tr>
<td><strong>Noncancerous</strong></td>
<td></td>
</tr>
<tr>
<td>tissue</td>
<td>176±20 fs</td>
</tr>
<tr>
<td></td>
<td>847±422 fs</td>
</tr>
<tr>
<td></td>
<td>6.03±1.92 ps</td>
</tr>
<tr>
<td><strong>Cancerous</strong></td>
<td></td>
</tr>
<tr>
<td>tissue</td>
<td>810 ± 40 fs</td>
</tr>
<tr>
<td></td>
<td>5.63 ± 5.49 ps</td>
</tr>
<tr>
<td></td>
<td>59.90 ±12.85 ps</td>
</tr>
<tr>
<td><strong>Film</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>163±25 fs</td>
</tr>
<tr>
<td></td>
<td>1.56±0.42 ps</td>
</tr>
<tr>
<td></td>
<td>21.59±4.14 ps</td>
</tr>
<tr>
<td><strong>Solution</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>560 ± 94 fs</td>
</tr>
<tr>
<td></td>
<td>2.37±0.71 ps</td>
</tr>
<tr>
<td></td>
<td>28.07±24.18 ps</td>
</tr>
<tr>
<td></td>
<td>345.81±107.87 ps</td>
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</table>

One can see that that the dynamics of the excited state $S_1$ decay for the noncancerous tissue is much faster than that of the cancerous tissues. The dynamics of the noncancerous tissue is described by three time constants (176±20 fs, 847±422 fs, 6.03±1.92 ps) and does not contain slow components that are observed in the cancerous tissue (59.90 ±12.85 ps), in film (21.59±4.14 ps), and in solution (28.07±24.18 ps, 345.81±107.87ps).

The picture that emerges from the results presented in this part is the following. The dynamics of AlPcS₄ in the excited state at biological interfaces reveals three different time scales: a) very fast component of hundred femtoseconds, b) a few picoseconds, c) 20-60 ps, in contrast to the dynamics in solutions that exhibits additional component in the range of a few hundred picoseconds.

Figure 30 illustrates the main channels of energy dissipation once upon light excitation of AlPcS₄ in aqueous solution and at the interfacial regions of the biological tissue.
Fig. 30. Excited state relaxation mechanisms in solution (A), normal (noncancerous) tissue (B) and cancerous human breast tissue (C). Reprinted from: Halina Abramczyk, Beata Brozêk-Pluska, Marc Tondusson, Eric Freysz, J. Phys. Chem. C 2013, 117, 4999−5013. Copyright 2013 American Chemical Society.

In aqueous solution (Fig. 30c), after photon absorption, the excited state $S_n$ relaxes rapidly within 560 fs in the Franck-Condon region followed by vibrational relaxation in $S_1$ state with the time constant of 2.4 ps and return to the ground state $S_0$ via fluorescence with the excited state lifetime of 345 ps. The second channel of energy dissipation goes through the intersystem crossing to the triplet state $T_1$ followed by return to the ground state $S_0$ via phosphorescence with the excited triplet state $T_1$ lifetime of 28 ps. The phthalocyanine at the biological interfaces of the human breast tissue does not relax via fluorescence. In the cancerous tissue (Fig. 30b) after photon absorption the excited state $S_n$ relaxes in the Franck-Condon region followed by the intersystem crossing within 810 fs to the triplet state $T_1$
followed by return to the ground state \( S_0 \) via phosphorescence with the excited triplet state \( T_1 \) lifetime of 59.9 ps. The second channel goes through the vibrational relaxation in \( S_1 \) state with the time constant of 5.6 ps followed by the conical intersection to the ground state \( S_0 \) and vibrational relaxation in the ground state within 1.5 ps. In the noncancerous tissue (Fig. 30a) the main channel is radiationless decay via conical intersection with the time constants of 176 fs, and 6 ps corresponding to the wave packet dynamics in the Franck-Condon region of \( S_1 \) state, and vibrational relaxation of the excited state \( S_1 \), crossing the local barrier followed by the relaxation to the conical intersection, respectively. The constant of 1.3 ps along this pathway corresponds to the vibrational relaxation in the ground state \( S_0 \). The second channel active in the noncancerous tissue is the intersystem crossing to the triplet state \( T_1 \) within 847 fs and with the time constants of 41 ps, and 1.3 ps corresponding to the lifetime of \( T_1 \) lifetime and the ground vibrational relaxation state, respectively.

Sumarizing we have found that photosensitizer responsible for harvesting of the light energy in biological tissue find their ways for a recovery through some special features of the potential energy surfaces such as conical intersections, which facilitate the rate of radiationless transitions. We have found that the tetrasulphonated aluminum phthalocyanine interacts with the normal (noncancerous) breast tissue and cancerous tissue according to different pathways. The dynamics in the noncancerous tissue is dominated by the radiationless decay at a conical intersection in contrast to the dynamics in solutions, which is dominated by the fluorescence. The dynamics in the cancerous tissue is dominated by the phosphorescence from the triplet state \( T_1 \) state. We have found that the dynamics of \( \text{AlPcS}_4 \) reveals three time scales: 110–170 fs, 1–7 ps, and 20–60 ps. The shortest time constants have been assigned to vibrational wavepacket dynamics in the Franck-Condon region down to the local minimum of the excited state \( S_1 \), a few picosecond component has been assigned to vibrational relaxation
in the ground and excited electronic states, 20–60 ps components represent the decay from the triplet state $T_1$ to the ground state $S_0$.[18]

**Conclusions**

We have shown that spectroscopic multimodal approach (Raman imaging-IR-UV-VIS-Femtosecond spectroscopy) is a powerful alternative to the conventional methods. The multimodal approach is ideally suited to analyze the properties of photosensitizers (phthalocyanines) in liquid solutions and in solid state as well as in human noncancerous and cancerous tissues, which can be crucial for better understanding of photodynamic mechanisms in real crowded biological environments. Raman imaging represents a novel method that allows monitoring accumulation and distribution of photosensitizers in human tissues and cells. It can also provide information about the photodamage of phthalocyanines during PDT reaction. We have shown that analyzing human tissues soaked with phthalocyanines one can simultaneously obtain information about the chemical composition and distribution of different tissue compounds such as lipids, nucleic acids, and proteins as well as accumulation and distribution of phthalocyanines. The multimodal approach may bring revolution in cancer detection and PDT treatment. The approach will help to make appropriate decisions based on simultaneous biochemical images of cancer tissue, Raman vibrational biomarkers and fluorescence of photosensitizers that allows for guidance of tumor PDT illumination in real treatment time, estimation light doses required in PDT treatment, and is capable accurately delineate the tumor margins (optical biopsy).

**References**


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