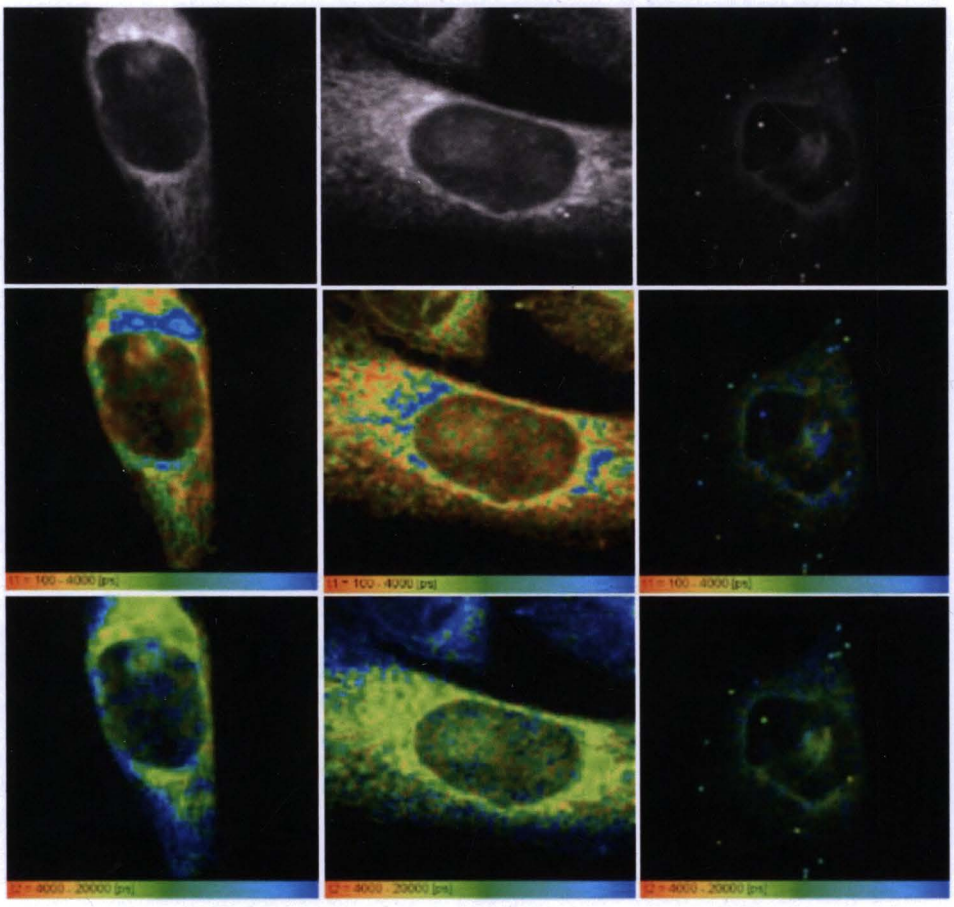
PhD Thesis

Porphycenes in photodynamic therapy of cancer cells and photodynamic inactivation of bacteria



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Porphycenes in photodynamic therapy of cancer cells and photodynamic inactivation of bacteria

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A	–	absorbance
a_1, a_2	–	signal amplitudes
ACN	–	acetonitrile
ALA	–	aminolevulinic acid
BHA	–	brain heart infusion agar
CD	–	cyclodextrin
CMC	–	critical micelle concentration
DDS	–	drug delivery system
DTBPc	–	2,7-di-*tert*-butylporphycene
$\Delta\lambda_{1/2}$	–	band width at half maximum intensity of emission
$^1\Delta_g$, $^1\Sigma_g^+$	–	singlet excited states of oxygen
DMSO	–	dimethylsulfoxide
DNA	–	deoxyribonucleic acid
Egg-PC	–	egg L-α-phosphatidylcholine
EO	–	ethylene oxide
F-127	–	Pluronic F-127
FDA	–	Food and Drug Administration
FLIM	–	fluorescence lifetime imaging microscopy
Φ_Δ	–	singlet oxygen generation quantum yield
Hp	–	hematoporphyrin
HPD	–	hematoporphyrin derivative
HPF	–	hydroxyphenyl fluorescein
HPV	–	human papillomavirus
λ	–	wavelength
LED	–	light emitting diode
λ_{em}	–	emission wavelength
MTBPc	–	2-*tert*-butylporphycene

$^1O_2$	–	singlet oxygen
PBS	–	phosphate buffer saline
Pc	–	porphycene
PC	–	phosphatidylcholine
PCT	–	photochemotherapy
PDI	–	photodynamic inactivation
PdOEP	–	palladium octaethylporphyrin
PDT	–	photodynamic therapy
PI	–	propidium iodide
Pluronic	–	Pluronic F-127
PS	–	photosensitizer
PO	–	propylene oxide
ROS	–	reactive oxygen species
S_0, S_1	–	singlet states of a chromophore
$^3\Sigma_g^-$	–	triplet state of the oxygen molecule
τ_1, τ_2	–	fluorescence decay times
THF	–	tetrahydrofuran
TTPc	–	2,7,12,17-tetra-*tert*-butylporphycene

INTRODUCTION

2.1. Photodynamic therapy (PDT)

Photodynamic therapy is an approved method of treatment for a variety of malignances including cancer. The method requires simultaneous application of two factors: light and the substance called photosensitizer to the targeted cell or tissue. Phototherapeutic action is based either on the energy transfer from the excited state of the photosensitizer directly to molecular oxygen present in the targeted cells or it is directed via intermediate radical forms to reactive oxygen species (ROS). In both cases the species created in situ is a powerful drug leading to the cell death. [1]

2.1.1 Phototherapy and photodynamic therapy – history

Therapeutic properties of light were already known by ancient Egyptians. First hints indicating this fact are dated from the V and IV centuries BC. [2] Through the ages solar light was used in the treatment of such diseases as vitiligo, psoriasis, or rickets. This kind of therapy involving only light and based on endogenous sensitizers is called phototherapy. [3]

Photodynamic therapy (PDT) covers all the methods of treatment that, apart from light, require an additional substance, called photosensitizer (PS). The term PDT evolved in the XXth century, after the range of discoveries that led to the formation of this new fascinating branch of medicine. The breakthrough that started this process was the discovery of Oscar Raab, the doctoral student of professor Von Tappeiner, who observed in 1900 that the cells of *Paramecium caudatum* were killed as the acridine orange and sunlight were introduced to the solution. Surprisingly, each of these factors, when applied separately, did not do harm to the cells. Extended studies led to the conclusion that oxygen is necessary for the process of cell death induced by light. [4] The new discipline in medicine was officially introduced to the world in 1903, along with awarding Neils Ryberg Finsen with a Noble Prize "in recognition of his contribution to the treatment of diseases, especially lupus vulgaris, with concentrated light radiation, whereby he has opened a new avenue for medical science". The use of word ‘dynamic’ in the name of PDT has been discussed through the decades. Von Tappeiner probably introduced this

misguiding term to distinguish the biological phenomenon from photographic processes discovered nearly at the same time. Further attempts to replace it with a much more proper phrase - photochemotherapy (PCT) - were unsuccessful. [5]

The biggest names of this period include already mentioned professor Von Tappeiner, who together with professor Jodlbauer gave the origin to the term of photodynamic therapy, publishing the first successful trial of bacteria inactivation by an exogenously applied photosensitizer. [6]

In the beginning of the XXth century much of attention was paid to the influence of light on the metabolism of plants. Therefore, fluorescent plant dyes were also studied. One of the researchers who joined this field with the medical applications was Walter Hausmann. [7] His studies in photodynamic effects of chlorophyll extracts on red blood cells resulted in the discovery of hematoporphyrin (Hp) as a potent photosensitizer, causing erythema, endema, and skin necrosis in mice kept in sunlight. Hp, an ion-free derivative of heme, was the compound of interest because of the structural similarity to chlorophyll.

The work of Hausmann started a new era of photodynamic research in Munich. The most popular experiment from this period was conducted by Friedrich Meyer-Betz, who injected himself with 200 mg of Hp and shined the light of the Finsen lamp on the small area of his forearm. The ulceration occurred immediately in the irradiated place. Even after days, the exposure to the sunlight caused the massive phototoxic reaction with swelling and burning sensation (Figure 1). [8]

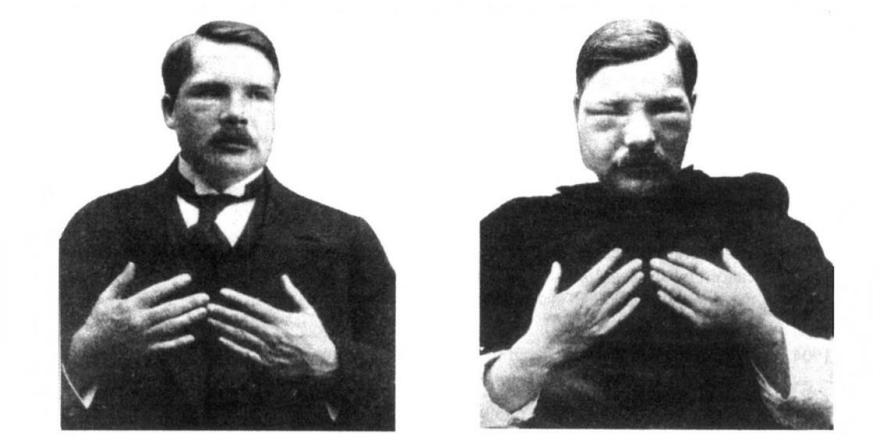


Figure 1. Friedrich Meyer-Betz before and after his experiment of injection 0.2 mg of Hp and exposing himself to light. [8]

In 1931 Hp was sold for the first time as a PDT drug in Germany. The trade name was "Photodyn". Between 1940 and 1955 a lot of studies were carried out on the patients

and tumor tissues. In this time Figge and co-workers reported the usefulness of Hp for tumor treatment and diagnostics due to its good tumor-localizing properties.

After this intense period of remarkable discoveries, the PDT went out of the spotlight for a while. Nevertheless, new photosensitizers and light sources were studied in the laboratories around the world. The 70s of the XXth century brought the development of clinical applications of PDT. The turning point was 1978, when Thomas Dougherty and his co-workers presented the results of their studies on hematoporphyrin derivative (HPD). [9] HPD was the mixture of porphyrin subunits and by-products. Dougherty proved their efficacy against the cutaneous and subcutaneous malignancies, such as basal cell carcinoma.

Until the end of the XXth century the main focus in PDT was devoted to anti-cancer treatment. New photosensitizers, new light sources and dosimetry methods were developed. The method was approved in many countries for treatment of various medical conditions. Clinical trials have been still conducted to broaden the range of applications. Until 1990 only a few articles concerning non-oncological applications of PDT were published. These were the first attempts to explore antimicrobial and antiviral potential of the method. [10][11] XXIst century brought the development especially to this branch of PDT, as the drug resistance of bacteria has become the world-wide problem.

2.1.2. Photosensitization mechanism

The basic process of PDT is photosensitization. Gilbert Laustriat, in a very clear and intuitive way defined it as "the process, which causes the system to become sensitive to light via an endogenous substance." [12] This endogenous substance is called a photosensitizer, meaning a light absorbing chemical species that initializes a photochemical reaction. In case of PDT it is a molecule which after the photoexcitation transfers the energy to the molecular oxygen. Therefore, photodynamic action requires three independent elements combined together: light, photosensitizer, and molecular oxygen. The first step is to deliver the agent to the targeted tissue. Then the place has to be illuminated with light of the wavelength covered by the range of absorption of photosensitizer.

We distinguish two main mechanisms of oxygen-dependent photosensitization, depending on the acceptor in the first stage of the energy transfer process. Apart from these

two, we can find in the literature the photoreactions of type III and IV, that refer to the oxygen-independent processes, however the definitions of those are inconsistent.

In this introduction the attention will be drawn to the processes of the first two categories, since they are most relevant to the PDT field. Even though their definitions have been widely used already for a few decades, there are still misunderstandings among scientists regarding both terms. Figure 2 shown below will support the following explanation of both phenomena.

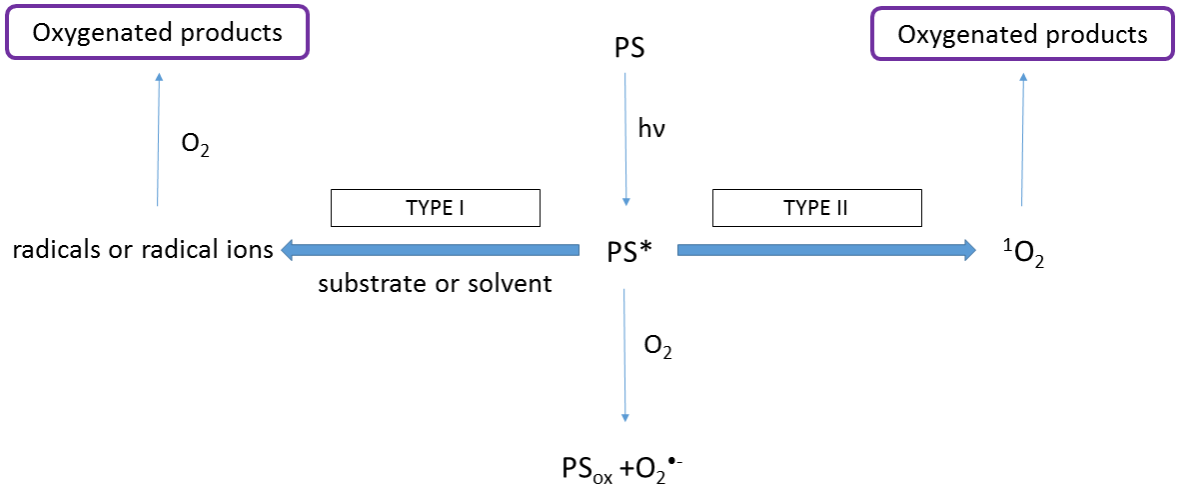


Figure 2. Scheme of photosensitization mechanisms, specifying type I and type II reactions.

These photosensitization mechanisms include degradation of biomolecules upon one-electron oxidation and singlet oxygen ($^1O_2$) reactions.

Type I mechanism is based on the photoinduced electron transfer. In this case, the triplet state of PS reacts directly with another molecule present in its neighborhood, transferring a proton or an electron to form a radical anion or radical cation. These radicals may further react with oxygen to produce reactive oxygen species. As the radical chain reaction starts, the molecular oxygen can be transformed to reactive oxygen species such as hydroxyl radical $HO^{\bullet}$ or hydrogen peroxide H_2O_2. The PSs that tend to follow this pathway include, for example, carbonyl compounds such as benzophenone.

Type II mechanism involves direct energy transfer from the triplet state of PS to triplet oxygen leading to singlet oxygen generation.

The most controversial is the ascription of superoxide radical anion ($O_2^{\bullet-}$) formation to one of the types of photoreactions. This process has been investigated for the last fifty years and still is the subject of discussion between scientists. Christopher Foote, in his article from 1991 devoted to the differentiation between both types of mechanisms, claims

that the process in which electron transfer from the sensitizer to oxygen leads to superoxide radical anion ($O_2^{\bullet -}$) and the oxidized form of PS should also be considered as type II reaction, but it is occasionally ascribed as type I. [13] M.S. Baptista et al. in their article from 2017 claim otherwise. In their review one can find that $O_2^{\bullet -}$ can be generated by the donation of the electron to molecular oxygen by PS, or more likely indirectly as the result of O_2-mediated oxidation of the radical anion and then it should be rather assigned to type I. [14]

Although the photosensitization in the clinical conditions is usually the mix of processes of I and II type, there are conditions that can favor one of those, and the ratio of both depends on such factors as the type of PS used, the concentrations of the substrate and oxygen. [15] Low concentrations of oxygen favor type I reactions, whereas type II occurs typically at higher concentrations of oxygen.

Many groups of compounds were proven to exhibit the photosensitizing effect upon visible light irradiation. The most popular among them are porphyrins, chlorins, bacteriochlorins, and phthalocyanines. [16][17] However, there is still a need to design new photosensitizers with better photophysical properties, bioavailability, and selectivity. [18] Recent studies focus on photosensitizing abilities of various nanoparticles and even on such molecular structures as fullerenes. [19][20]

2.1.3. Reactive oxygen species (ROS)

Reactive oxygen species is the term referring to chemically reactive oxygen radicals as well as non-radical derivatives of oxygen. In other words, these are all the products of one- two- or three-electron reduction of molecular oxygen (Figure 3) plus singlet oxygen. [21][22] ROS react with the components of living systems, being usually toxic and dangerous for the organism. This process is the base of photodynamic therapy and the ability to produce ROS is an important parameter of a photosensitizer.

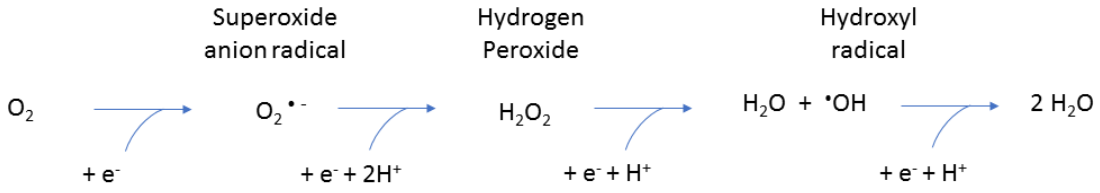


Figure 3. Stepwise reduction of molecular oxygen, leading to the formation of reactive oxygen species.

ROS and their derivatives that occur in the organism cover the range of over 20 compounds, however the most important ones for PDT are singlet oxygen ($^1O_2$), hydroxyl radical ($OH^\bullet$), superoxide anion radical ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2).

For porphycenes, which are considered type II photosensitizers, the most important factor influencing the efficiency of PDT is singlet oxygen generation yield. [23]

Singlet oxygen

The electronic structure of molecular oxygen is quite unique compared to most other molecules commonly occurring in the environment. The ground state of the oxygen molecule is a triplet state $^3\Sigma_g^-$ with two unpaired electrons in the highest occupied orbitals. These two electrons can be rearranged, creating two excited singlet forms, $^1\Delta_g$ and $^1\Sigma_g^+$.

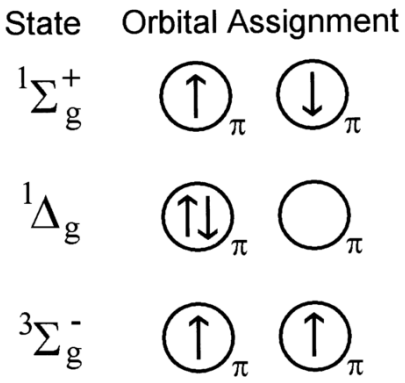


Figure 4. Possible configurations of two electrons in HOMO degenerated orbitals in the molecule of oxygen. [24]

The stability of the lower-lying singlet $^1\Delta_g$ is relatively high and its lifetime in the solution is in the range of 10^{-6} – 10^{-3} s. $^1\Sigma_g^+$ state is much less stable and its lifetime is even a million times shorter. [21] The spin and symmetry forbidden transition $^1\Delta_g \leftrightarrow {}^3\Sigma_g^-$ can be observed in absorption and emission spectra around 1270 nm. [24]

The untypical electronic structure of oxygen has a lot of very important consequences for the creation of world in the shape we know it and the possibility to create ROS forms to perform photodynamic therapy can be considered as a minor side-effect.
It is worth mentioning that the vast majority of molecules has singlet ground states, contrary to the oxygen. This limits the number of ground-state reactions that molecular oxygen can take part in.

The singlet oxygen generation quantum yield (ϕ_Δ) is a parameter carrying information about the efficiency of the process of ground state oxygen conversion to singlet oxygen in a particular solvent. The value of ϕ_Δ refers to the number of molecules of $^1O_2$ molecules generated for each photon absorbed by a photosensitizer.

In type II photosensitization mechanism $^1O_2$ is generated by energy transfer process, during the collision of excited PS with the oxygen in its triplet state, according to the equations 1 and 2.

$$PS(S_0) \xrightarrow{h\nu} PS(S_1) \xrightarrow{k_{isc}} PS(T_1) \quad (1)$$

$$PS(T_1) + {}^3O_2 \xrightarrow{k_{en}} PS(S_0) + {}^1O_2 \quad (2)$$

Where k_{en} is the rate constant of energy transfer and k_{isc} is the rate constant of intersystem crossing.

Singlet oxygen generation quantum yield is therefore limited by competitive radiative and non-radiative processes, described by equations 3 and 4.

$$PS(T_1) \xrightarrow{k_r} PS(S_0) + h\nu'' \quad (3)$$

$$PS(T_1) \xrightarrow{k_{nr}} PS(S_0) \quad (4)$$

Where k_r , k_{nr} are the rate constants of radiative and non-radiative relaxation of the photosensitizer triplet state.

Taking into account equations 1 – 4 and considering also processes that occur due to the presence of molecular oxygen, the equation describing the quantum yield of singlet oxygen generation in solution can be presented as eq. 5.

$$\Phi_\Delta = \Phi_T \phi_{en} = \Phi_T \left(\frac{k_{en}[O_2]}{k_r + k_{nr} + k_q[O_2]}\right) \quad (5)$$

Where:

Φ_Δ is the quantum yield of singlet oxygen generation
Φ_T is the quantum yield of triplet formation
ϕ_{en} is the efficiency of energy transfer
k_q is the rate constant representing all the processes of triplet state quenching by molecular oxygen. [25]

Most of the values of ϕ_Δ found in the literature refer to photosensitizers dissolved in organic solvents, whereas the values for water solutions are much more informative with respect to biological environment of the organisms. The difficulty with determination of ϕ_Δ in water is caused mainly by a very short lifetime of this species in aqueous environment,

which is around 3.5 μs. [26] For comparison, the lifetime of singlet oxygen in deuterated water is 67.0 μs [26] and in the organic solvents, such as carbon tetrachloride can even reach the domain of milliseconds. [27] The longest lifetimes are observed in the solvents considered as chemically inert. The modern and very complex explanation of singlet oxygen lifetime dependence of the solvent was recently provided by Ogilby's group [28] Their theory is based on the assumption that the interactions between solvent and molecular oxygen influence both radiative and irradiative processes. In the isolated molecule of oxygen radiative transitions between three different electronic states are forbidden, whereas for the same molecule placed in the solvent these transitions become more probable. The presence of solvent can favor singlet oxygen deactivation to the ground triplet state. ϕ_Δ is generally determined by means of radiative processes and it is difficult to obtain objectively informative values when the majority of $^1O_2$ is deactivated on the way of irradiative processes. Ogilby's group tried to quantify the influence of particular solvents on particular transitions. They found out that in contrary to the radiative constants, the non-radiative ones do not depend on the refractive index of the solvent. The values correlate with the parameter that characterizes solvent's ability to accept the electronic excitation energy from the oxygen. They propose that the electronic excitation energy of oxygen is deposited in the vibrations of oscilating solvent molecules, and they call this theory the "energy sink" model. It explains, above all, why lifetimes of singlet oxygen are the shortest in the solvents containing O-H rather than O-D bonds. According to this model the low-frequency oscillators, such as C-F, C-C, C-D, O-D, are poorer "energy sinks" than the oscillators with higher vibrational frequencies (C-H or O-H).

Due to the short lifetime and very high reactivity of singlet oxygen, direct determination of this species is very difficult. Only two methods of direct detection are widely used. One is molecular emission at 1270 nm, which is an intrinsic property of singlet oxygen applied for both detection and characterization of this species. The difficulties come from a very low yield of this forbidden transition, such as 10^{-5} – 10^{-7}, depending on the environment. Despite the limitations, the method is often used, both for steady state and time-resolved measurements.

The second direct way of singlet oxygen detection and quantification is electron paramagnetic resonance (EPR). The method is very sensitive, but the errors due to co-existing ions and solvents are very likely to happen. Moreover, the application is limited by the high prices of instrumentation and relative complexity of the method.

Due to the mentioned obstacles a lot of probes for indirect detection of $^1O_2$ and other ROS have already been developed and still many new ones are under investigation. Although the sensors are never perfect and their use requires a lot of restrictions, still they provide a readily accessible tool for the determination of ROS in both *in vitro* and *in vivo* conditions. The probe compounds are divided into three groups: spectrophotometric, fluorescence, and chemiluminescence sensors. Spectrophotometry is the most comfortable and simple method, where detection and quantification of $^1O_2$ is based on the absorbance of the created sensor complex with singlet oxygen. The drawbacks of such approach are low sensitivity, poor photostability and water solubility.

For high sensitivity it is better to employ fluorescence probes. The most popular of this group is Singlet Oxygen Sensor Green. [29][30]

2.1.4. Desired properties of photosensitizers

An ideal photosensitizer applied in PDT should be characterized by following features:

- Synthesis from available precursors and easy reproducibility
- Optimal ADME properties (administration, distribution, metabolism, excretion)
- High singlet oxygen quantum yield
- Strong absorption in the region of optical therapeutic window with high molar absorption coefficients
- Effective accumulation in tumor tissue and low dark cytotoxicity
- Stability and solubility in the body fluids and easy delivery methods. [31][32]

2.2. Porphycenes

Porphycene is the structural isomer of porphyrin, first synthesized by Vogel et. al. in 1986. [33] It retains the overall $C_{20}N_4H_{14}$ formula and the system of conjugated π-electrons. Due to the lower symmetry of a molecule it exhibits slightly different electronic absorption pattern from porphyrin. Generally, for the porphyrinoids the spectrum consists of two branches. According to the convention the higher energetic band is called the Soret band and the lower energy band is called Q band. Both correspond to $\pi - \pi^*$ transitions. In the case of porphycenes the Q band is an order of magnitude more

intense than in higher symmetry porphyrins and shifted to longer wavelengths. This makes porphycenes particularly good subjects for photodynamic therapy studies, since the region between 600 and 850 nm is favorable for this treatment and is called therapeutic window.

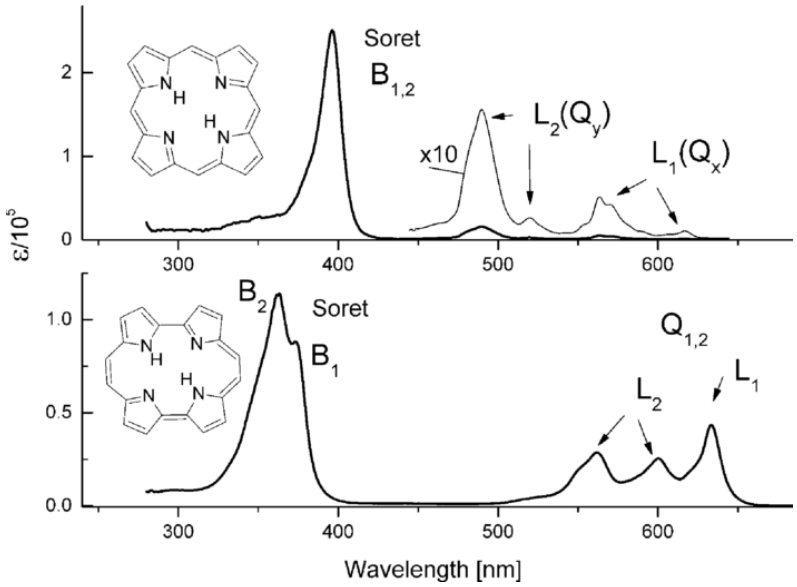


Figure 5. Room temperature electronic absorption spectra of porphyrin in toluene (top) and porphycene in n-hexane (bottom). [34]

The excited state deactivation of porphycenes also follows a somewhat different pathway than for porphyrins. Porphycenes generally have higher quantum yields of fluorescence, while their yield of triplet formation is lower than 50 %. The significant efficiencies are also reported for internal conversion process S_1–S_0. For the parent compound the respective values of the above-mentioned parameters are 0.36, 0.42 and 0.22. When the compound is substituted with phenyl groups fluorescence and intersystem crossing lose their efficiencies and the internal conversion becomes a dominating channel of S_1–S_0 relaxation. In terms of the potential use in PDT the mentioned efficiencies of triplet formation are quite moderate. Depending on substituents they can vary from 1 to 45%. [23][35] However, the high absorption coefficients and almost 100 % yield of singlet oxygen formation from the triplet state make porphycenes promising photosensitizers. [36] Well-known photophysics of these compounds and the possibility of synthesizing porphycenes with a huge variety of different substituents, attached in different positions, make them the good candidates for versatile PDT studies. [37][38][39][40] Moreover, they were proven to exhibit good tumor targeting properties [41] and they have already been successfully applied as anticancer agents. [42] Several porphycenes bearing methoxy or hydroxy groups turned out to be between 17 and 220 times more efficient against cancer

cells than a commercially available PDT drug. [43] The fact of huge reported differences in the photosensitizing effects *in vivo*, despite of only minor differences in the electronic structure of compounds is interesting and encouraging for the investigation of photobiological properties of porphycenes. There are also a few reports about antimicrobial applications of porphycene-core compounds. They focus on cationic derivatives and polylysine conjugates of porphycenes. [44][45][46][47] The missing part of the research conducted so far is the phtotobiological studies devoted to the parent, unsubstituted porphycene, which could be the base for comparison between other differently substituted derivatives.

Porphycenes can be also used in the studies of physical properties of the cells. Meso-alkylated porphycenes were proven to exhibit viscosity-controlled tautomerism, which is reflected in a large increase of the decay time of fluorescence with growing viscosity of the environment. [48] Due to this effect, porphycenes could be used as viscosity sensors for intercellular environment.

2.3. Drug delivery

The main obstacle in the delivery of most of organic compounds to the cells is their relatively high hydrophobicity. There were many ways invented to overcome this problem. The most popular ones are functionalization of the molecules with hydrophilic groups or the use of an external medium such as drug delivery system (DDS). Drug delivery is the process of administering a pharmaceutical compound to achieve a therapeutic effect in living creatures. The substance considered as a potential drug due to its performance in *in vitro* trials, when administered to the organism might either cause therapeutic effect or not. This depends on how much of the substance in its active form actually reaches the targeted tissue. Therefore it is common to use various drug delivery systems. Their function includes protecting the drug from metabolic activity of the enzymes along their way in the body and also preventing it from reacting with the tissue before reaching the target place. This leads to the higher bioavailability, longer time of circulation in the blood and lower toxicity for the patient. There is a range of often used DDSs such as liposomes, micelles, microspheres, prodrugs, cyclodextrins, and many more. According to the application they might be slowly degradable, stimuli-reactive and even targeted. [49]

In the case of porphycenes, which are highly hydrophobic, a few ways of delivering the photosensitizer to the cells have been reported. In the *in vitro* studies dimethyl sulfoxide (DMSO) was often used as a solvent. The procedure includes dissolving a hydrophobic dye in DMSO and adding small portions of such solution to the physiological buffer. [50][51][52] Another approaches rely on lipid bilayer vesicles, such as liposomes [42][53] and conjugation of the dye with bigger molecules soluble in water, such as polylysine. [46][46]

2.3.1. Liposomes

The liposomes (Figure 6) were discovered in the 60s' of the XX century and they were first reported to be used as DDSs in the clinical treatment of humans in 1976. [54] They can be described as the vesicles consisting of phospholipids, which makes them biodegradable and non-toxic DDS medium. Phospholipids occur in nature as the building blocks of cellular membranes. Liposomes are self-organized structures formed by hydrophilic and hydrophobic interactions of phospholipids with the aqueous phase. Due to the double-layer shell structure they might be used for encapsulating both hydrophobic and hydrophilic compounds. [55][56][57] They can reach diameters from around 20 nm to even microns, depending on their composition and the preparative method.

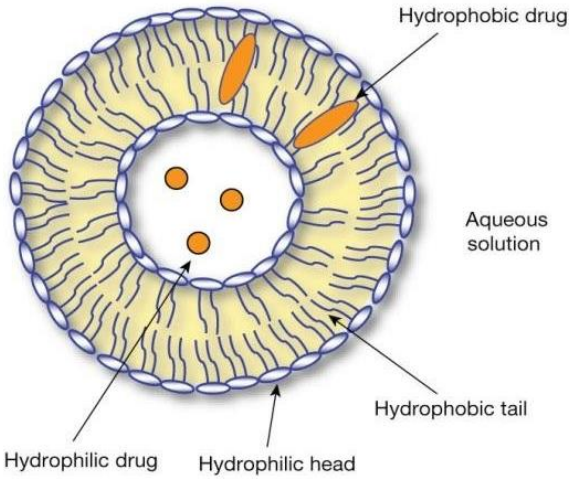


Figure 6. Structure of phosphatidylcholine liposome carrying hydrophobic and hydrophilic drugs. [54]

The phospholipid used most often for the creation of liposomes is phosphatidylcholine (PC). PC liposomes were proven to be a versatile DDS for

encapsulation of the drugs of very diverse lipophilicity. They also have good diffusion properties through such tissues as epidermis and dermis [58], which can be relevant for their use in photodynamic therapy.

2.3.2. Polymeric micelles

Polymeric micelles (Figure 7) exhibit high and versatile loading capacity and they tend to be stable under physiological conditions. They can be spontaneously formed from amphiphilic molecules in a certain range of concentrations and temperatures. [59] The lowest concentration of a surfactant allowing micelles formation is called critical micelle concentration (CMC). Below this value (determined for a particular temperature and type of solvent) the surfactant occurs in the solution in the form of monomers. Micelles with lower CMC levels are thermodynamically more stable. They can be obtained by increasing the hydrophobic fraction of amphiphiles. [60][61] Among novel drug delivery systems, very promising are those based on pluronic block copolymers. They consist of hydrophilic ethylene oxide (EO) and hydrophobic propylene oxide (PO) blocks. Arranged into the EO–PO–EO structure, such copolymers can form micelles, of which the size and shape depends on the length and the relative ratio of the components. Various pluronics have been reported to be extremely promising for drug delivery and PDT. [62][63]

Liposomes and micelles differ in the arrangement of amphiphilic molecules. Liposomes consist of lipid bilayer, so the hydrophilic drugs can be encapsulated in the central part of the vesicle, whereas hydrophobic compounds occupy the coating. Micelles are formed by the single layer of surfactant and they can carry a loading of a hydrophobic drug inside, between the lipophilic tails of polymers.

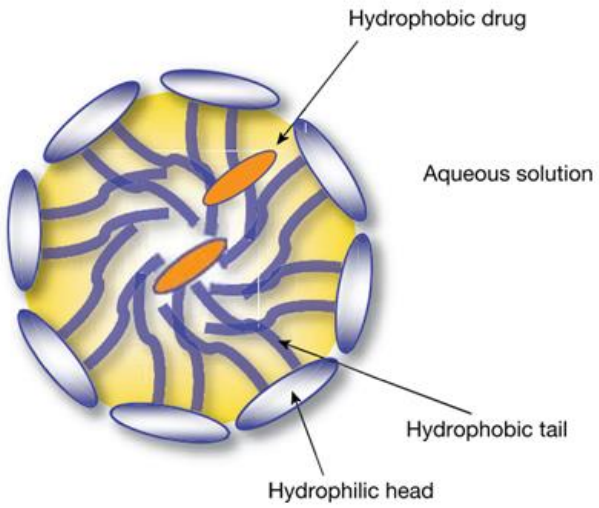


Figure 7. Structure of the micelle carrying a hydrophobic drug. [54]

2.3.3. Cyclodextrins

One of the common ways of delivery of poorly water-soluble substances is the use of cyclodextrins (CDs), which are chemically and physically stable macromolecules produced by enzymatic degradation of starch. They are water-soluble and biocompatible, which makes them perfect for medical applications. They form bucket-shaped molecules (Figure 8) with a hydrophilic outer surface and a lipophilic cavity. Hydrophobic compounds can be encapsulated inside the cavity due to the non-covalent interactions without any chemical reactions. One of the most widely used representatives of the cyclodextrin family is *β*-cyclodextrin. It is considered ideal for drug delivery due to perfect cavity size, efficient loading availability, and relatively low cost. [64]

An inclusion complex of CDs, apart from increasing water solubility of drugs, can also reduce their toxic side effects to some extent, which not always is a desired property. [65]

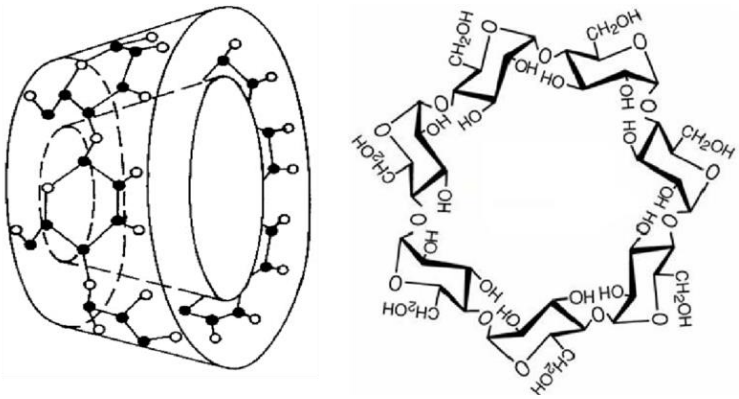


Figure 8. Structure of β-cyclodextrin. [66]

2.4. Optical therapeutic window

Tissue permeability to light is a crucial factor for the efficiency of photodynamic therapy. Photodynamic action can only occur if both light and photosensitizer are delivered to the target lesion. The tissue is however not fully transparent and the incoming light undergoes many physical processes, including refraction, reflection, absorption and scattering. In terms of the light therapy, all should be taken into account. The topic is very broad and complex. In 2009 Plaetzer et. al. published a very detailed review concerning this issue. [67] In this introduction the focus will be put on these aspects that are important for the *in vitro* studies devoted to PDT.

Refraction and reflection are the processes that can be minimized by the perpendicular illumination of tissue. They are not really relevant for *in vitro* studies, where the cells are in the solutions, but their consideration is very important in the clinical application of PDT. Another problem is scattering, which is dependent on light intensity and directionality. It causes the loss in the fluence rate, so it decreases the power of light illuminating the unit area of tissue. It is the complex issue, but generally red light is scattered less than blue. This property favors the use of photosensitizers that are excited with longer wavelengths.

The most important process that limits the light administration to the tissue is absorption. Chromophores that have to be considered in the case of living tissue are water, oxyhemoglobin (HbO_2), deoxyhemoglobin (Hb), melanin and cytochromes. Their absorption spectra define the optical window for PDT (Figure 9). [67][68]

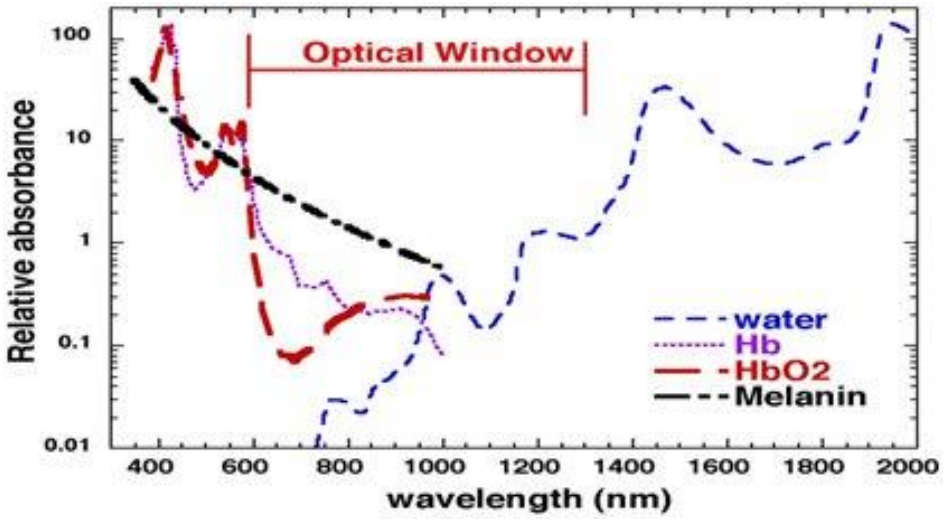


Figure 9. Absorption spectra of chromophores present in living tissue: water, oxyhemoglobin (HbO_2), deoxyhemoglobin (Hb), melanin. [69]

On the basis of the spectra shown above one can see that the range of wavelengths suitable for PDT is limited to 600 – 1200 nm. In reality this range is further narrowed down to the wavelengths lower than 850 nm, since above this value the triplet state of photosensitizer has the energy too low to efficiently produce singlet oxygen.

Excitation at 630 nm, such as the one required for porphycene derivatives, provides the depth of tissue penetration of approximately 3 – 5 mm, depending on the tissue type. [70] Figure 10 shows the penetration of light of different wavelengths through the skin.

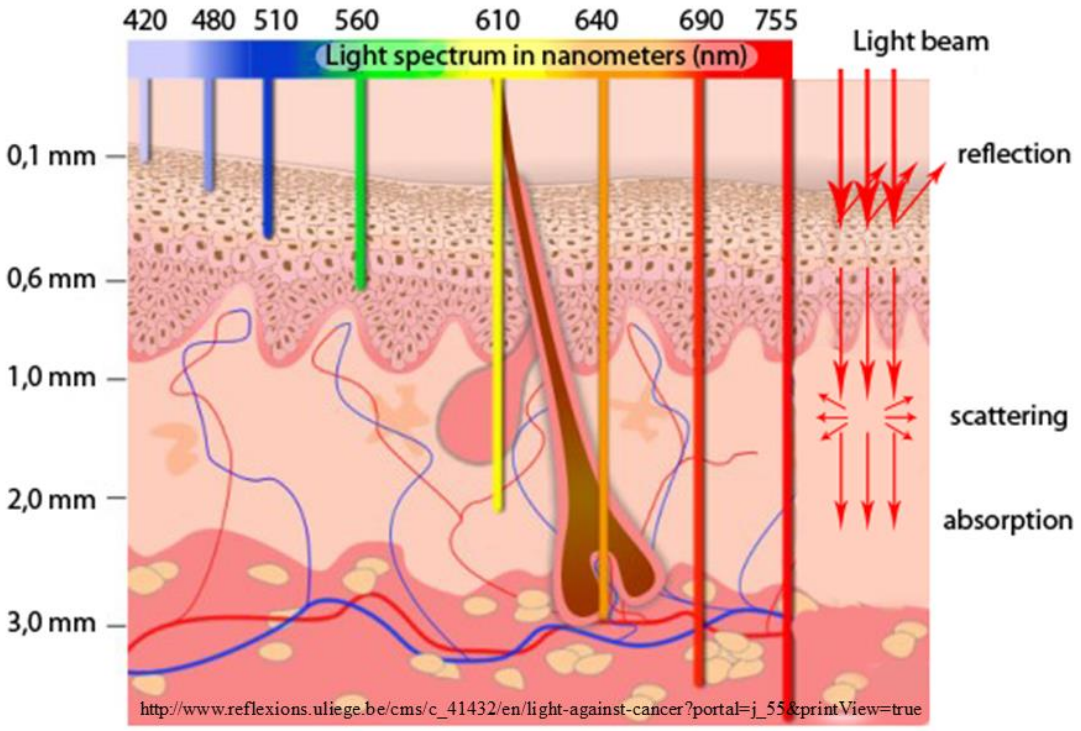


Figure 10. Propagation of light of different wavelengths through the tissue. [71]

2.5. Anticancer PDT

Among all the investigations in photodynamic therapy, the vast majority has been devoted to the anticancer application of this method. According to the results of clinical studies, this method is particularly effective in early-stage tumors. PDT can be used in people with certain types of inoperable cancer to help them live longer and improve their quality of life. PDT is proven to work and be safer than most common therapies, but despite being the first drug-device combination approved by FDA, still it is not widely used to treat cancer today. It is offered in some treatment centers, and it is being studied in many clinical trials. Its popularity grows in such applications as localized cancers and pre-cancer skin treatment. PDT has several advantages over the traditional methods of treatment. First of all it is not as invasive as surgery and does not give any long-term side effects if it is used properly. It can be also targeted very precisely and repeated many times in the same spot of the body, contrary to the case of radiation. It is generally cheaper and less time-consuming than other anticancer treatment methods. [72] Still, PDT faces also some limitations. The most difficult to overcome is the limited access of light to some regions of the body, which makes PDT useful mostly for the treatment of surface tissues and the organs that can be reached with the light source. Moreover people undergoing PDT

become very sensitive to light for some time, so special precautions must be taken after the drugs are put in or on the body. There are several limitations for PDT. Obviously, it cannot be applied for the people oversensitive to light, with such disease like porphyria. Because of its localized nature this sort of treatment is not currently effective against the metastatic lesions spread over different places of the body, however the research has been carried out to find the proper PDT conditions for inducing systemic immunity.

2.5.1. Selectivity in anticancer PDT

One of the most important issues in PDT drugs design is the selectivity of uptake by the malignant tissue. If the selectivity is good, the accumulation of the PS in cancer cells is much higher than in healthy cells and the side effects for patients are reduced. Moreover, none of approved photosensitizers accumulate in cells' nuclei. This prevents DNA damage that could be cancerogenic itself and favorable to the mutations causing development of immunity to the treatment. PS selectivity is influenced by such factors as hydrophobicity or hydrophilicity of the molecule. The scale to express its value in pharmacology is the logarithm of the octanol/water partition coefficient (logP). [31] Lipophilicity of the drug is one of the factors determining its place of action. Hydrophilic molecules generally tend to remain in the circulation until they are excreted, whereas the hydrophobic compounds leak out of the vessels and can be in this way delivered to the tumor tissue. Based on the primary place of action PSs are often divided into two categories, cellular and vascular. [73]
The efficiency of tumor treatment depends on the drug accumulation inside the malignant cells. The tumors differ from healthy tissues and the most important difference is their rapid growth. On the one hand, the quicker the tumor grows the more dangerous it is, but, on the other hand, this property can be used as an advantage in the treatment. Due to extraordinary production of vascular growing factors in malignant cells, the systems of blood vessels in tumor is chaotic and suffers the lack of functional lymphatic drainage and elevated interstitial fluid pressure. [74] This special property of tumor vasculature is used for accumulation of medicines inside the tumor and is referred to as enhanced permeability and retention effect. This phenomenon does not concern free drugs, but can be very effective in the case of so called nanomedicines, which covers micelles and liposomes encapsulated drugs, as well as proteins and macromolecules and other particles that are large enough to avoid renal clearance. [75]

2.5.2. Approved photosensitizers

Although the concept of PDT is already known for a century, the list of the clinically applied photosensitizers is still very short. Allison and Siabata in their clinical review from 2010 mention 18 approved photosensitizers. [76] Following what happened to them till now one can find out that just a few substances are available commercially for anticancer use. Some photosensitizers, such as Lutexaphyrin (Antrin®) had been studied in clinical trials with promising results, however at some point they disappeared from the spotlight. [77] Some of them are registered clinically used drugs for non-cancer applications. The representative of such group is Verteporfin (Visudyne®), which has given good results in the treatment of pancreatic cancer in phases I and II of clinical trials [78], but so far is widely used only in ophthalmological treatment of cornea neovascularization. Other example is Hexvix® (a derivative of ALA) that has been used clinically, not in the treatment, but in the diagnostics of bladder cancer. [79] A narrow group of photosensitizers have been approved locally in anti-cancer treatment. One of this group Photolon® (Talaporfin) is officially approved in Russia and Belarus, but only under academic research in USA, Poland, and Bulgaria. Another, Foscan® (Temoporfin), has been applied in the European Union, Norway, and Iceland for palliative treatment of head and neck cancer, but has not reached the global range. Special attention should be put to four medicines whose clinical use was approved in Europe as well as in United States for treatment of cancer and pre-cancer lesions. Porfimer sodium (Photofrin® or Photobarr) is the most widely used and studied photosensitizer. It is activated by red light. It has been approved in the USA to treat patients with cancer of the esophagus, Barrett's esophagus, and in European Union to treat non-small cell lung cancer. PDT can help those who cannot have other types of treatment, such as surgery or radiation therapy. Aminolevulinic acid (ALA or Levulan®) is a drug that is put right on the skin, used to treat actinic keratosis, a skin condition that can become cancer, and is used only on the face or scalp. A special blue light source, rather than laser, is used to activate this drug. Methyl ester of ALA (Metvix) is one of several other forms of ALA, but with better bioavailability. It is approved for treatment of some types of actinic keratosis of the face and scalp. Methyl ester of ALA is activated with red light. The very recently commercialized TOOKAD® Soluble with the active substance padeliporfin brings a lot of hope for the patients with prostate cancer, since long phase of clinical trials, brought very promising results in this area of treatment. [80]

2.6. Cell death mechanisms in PDT

Cell death induced by PDT drugs is a complicated process and can basically follow three different pathways: apoptosis, necrosis and autophagy. The pathway depends on various factors, such as the type of the cells treated, dose and type of the photosensitizer, as well as its cellular localization. It is important to remember that the cell death followed in *in vitro* conditions may not resemble the process occurring in the living organism. *In vivo* treatment has to face unequal light distribution and inhomogeneous photosensitizer distribution, which leads to various cell death responses and mostly causes occurring of the mix of the cell death mechanisms. The initial site of PDT-related damage may determine which cell death pathway is first activated. Further choice of the pathway depends on the cellular response to PDT. It is possible that the cell tries to rescue itself by activating the autophagy process. Apoptosis can only occur if the cell is damaged to such extent that the repair mechanisms cannot be applied any more. Necrosis is induced when the proteins taking part in both autophagy and apoptosis process are destroyed and the cellular integrity breaks down. [81]

2.6.1. Apoptosis

Apoptosis, also called programmed cell death, is a normally occurring physiological process used by the organism to remove useless, improper or infected cells. In adult tissues cell death balances cell division so that the size of the tissue is kept constant. In the cells with impaired ability of apoptosis, such as cancer cells, proliferation exceeds apoptosis and the tissue is growing in an uncontrolled way, leading to the formation of tumor. The treatment of cancer is focused on inducing the apoptosis with some external agent. When the apoptotic signal arrives in the cell, it starts the cascade of highly ordered, energy-dependent biochemical processes. Morphological changes include cell shrinkage, condensation of nuclear chromatin, and formation of apoptotic bodies. A characteristic feature of this process is blebbing of the membrane. The organelles are then tightly packed inside of the blebs, together with cytoplasm. Apoptotic bodies created in such way might be then phagocytosed by macrophages and degraded with phagolysosomes. [82]
Regulation of apoptosis is done by means of two different groups of proteins: pro- and antiapoptotic. Together with caspases they are called the markers of apoptosis and they are always present in apoptotic cells. Two different, but convergent apoptotic pathways

have been described: the intrinsic or mitochondrial pathway and the extrinsic, called also death receptor signaling pathway. Both can be the target of PDT. [83]

2.6.2. Necrosis

Necrosis is a rapid, energy-independent process induced by the physical or chemical damage. It results in the quick degradation of larger fractions of cells. Necrosis is characterized by a pyknotic* nucleus, cytoplasmic swelling, and progressive disintegration of cytoplasmic membranes, which lead to the lysis of the cell and release of the intracellular material. Necrosis occurs in disordered fashion and does not exhibit the features of programmed cell death. However, new reports show that in certain circumstances the necrosis can be driven by some intracellular signaling mechanisms. [84][84] The pathway of necrosis induced by PDT is dependent on the location of photosensitizer in the cell: e.g., drugs located in plasma membranes cause cell death by the loss of membrane integrity. If they attach to lysosomes, they may disrupt the lysosomal membrane and trigger the release of lysosomal proteases that may also lead to necrosis. In PDT treatment it is important to keep control over necrosis, since the excessive process can turn into the inflammation and might cause kidney failure and death of the patient. On the other hand, the vascular PSs are expected to cause necrotic effect in both vasculature and tumor tissue. The release of intracellular products, toxic chemicals, and excess calcium blocks the vasculature cutting the tumor food and oxygen supply. The other consequence of necrosis introduction is the immune response, including release of inflammatory mediators such as cytokines, growth factors and proteins. This activates neutrophils and other white blood cells in the treated spot. [84] Stimulation of the immunological system is recently considered to be one of the most significant factors leading to the cure of cancer diseases with PDT. Extensive research has been conducted in this area. [85] The results of these studies are supposed to improve the efficiency of the method and help upgrading PDT from a localized to systemic treatment.

* Pyknosis – nucleus shrinking in dying cells, based on irreversible condensation of chromatin. The process occurs both in necrotic and apoptotic cells.

2.6.3. Autophagy

Autophagy is the third type of cell destruction. Basically, in this process the cell is eaten by itself. In the PDT treatment of tumors it can induce dual response of the treated tissue and it is still controversial whether simulation of autophagy might help in tumor treatment. On the one hand, it is the process of cell death, which might well work for cancer cells. On the other hand, it might be considered as a defense mechanism that favors survival of the treated cells and is a base of cancer immunity to PDT treatment.

2.7. HeLa cells

HeLa is a mammalian cell line, originating from human cervical cancer taken in 1951 from Henrietta Lacks. It is the oldest and most commonly used cell line utilized for the laboratory research. Properties that make it so abundant in science include immortality, quick proliferation, and enormous durability as compared to regular human cells. These features derive from the genetic pattern of the cell line, which is different from Henrietta Lacks genome. The source of the dissimilarity is the influence of Human papillomavirus (HPV), which tends to integrate with the DNA of cervical cancer cells, affecting the cells karyotype. Normal human cells have 46 chromosomes, whereas HeLa has 76 to 80 mutated chromosomes. They can replicate infinitely due to the overactive telomerase enzyme that they produce. It prevents them from shortening telomers during every division. For the regular cells, which have a limited number of divisions, this process leads to the aging and apoptosis, because the telomers get shorter every time when the cell divides. [86]

2.8. Antimicrobial PDT

The concept of PDT application against bacteria has been known for decades, but it gathered a substantial interest in the XXIst century. Antimicrobial photodynamic therapy (APDT), also called photodynamic inactivation (PDI), is one of the promising tools for fighting infections, which can be considered as an alternative to the common ways of treatment in the era of growing resistance to antibiotics. Recently, PDI has often been proposed as the promising method of treatment for some kinds of localized infections and extensive studies in this area have been carried out. [87][88] The best results in terms of

applications have been recently obtained in the field of dentistry and concern the treatment of periodontitis and disinfection of root canals. [89][90][91][92]

One of the most threatening problems of nowadays are nosocomial infections or the 'healthcare associated infections'. They are caused by prolonged hospital stay and often entail serious health issues leading to death. The most popular species causing nosocomial infections include *Streptococcus* species, *Acinetobacter* species, enterococci, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Legionella* and *Enterobacteriaceae* family members, namely, *Proteus mirablis*, *Klebsiella pneumonia*, *Escherichia coli*, *Serratia marcescens.* Main interest is directed to drug-resistant species, which are particularly dangerous, such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, *Pseudomonas aeruginosa* and *Klebsiella pneumonia.* [93]

Staphylococcus aureus

A pathogen considered as the most responsible for nosocomial infections is *S. aureus.* It is Gram-positive cocci, non-spore forming, facultative anaerobe. It is the widespread species playing commensal role, colonizing mostly nasal passages. Under particular conditions, such as decreased immunity during hospital treatment, it can cause serious life-threatening infections of superficial and also deep tissues.

Staphylococcus epidermidis

Staphylococcus epidermidis inhabits the healthy human skin and mucosal microflora. As a commensal bacterium it has a low pathogenic potential. However, in the last decades it was noted as the most common cause of nosocomial infections related with indwelling medical devices. A feature that helps this microorganism to develop its pathogenic ability is easiness of biofilm formation. This is a serious problem, since the biofilms have substantially higher resistance to antibiotics than the separated colonies. [94]

Enterococcus faecalis

The second cause of nosocomial infections worldwide are enterococci, Gram-positive, enteric facultative anaerobic microbes. They inhabit normally gastrointestinal tracks of humans, occur in gut microbiota and play the role in spoilage process of fruit juices and meat products. They are responsible for serious nosocomial conditions such as urinary tract infections, endocarditis, bacteremia, intra-abdominal abscesses. [95]

2.8.1. Methods and criteria for evaluation of antimicrobial agents activity

Serial dilution method is one of the approaches to evaluate antimicrobial activity of a substance. It enables to distinctly identify colonies and count the number of colonies in the plate to calculate CFU (colony forming units) per milliliter of the sample. The suspension of bacteria with antimicrobial agent is subsequently diluted 10^4, 10^5, 10^6 times and so on, and further spread on the plate. Spread plate culture technique is commonly used in the laboratory to quantify the number of organisms in the solution. By using a sterile spreader, the solution is evenly spread on the Petri dish covered with agar medium. This allows to count the colonies of bacteria on the dish after incubation. After counting them and calculating the CFU/ml, the obtained values for the suspension with the antimicrobial agent can be compared with the reference suspension without the agent to evaluate its antimicrobial potential.

The most common procedure for evaluation of the PDT agents efficiency applies the initial density of cells of 10^8 or 10^7. This makes it possible to measure 6 logs reduction, meaning the eradication of 99.9999% of microbes in the sample. This number can be interpreted as the total eradication and it is attributed to the highly active antimicrobial photosensitizers. It is worth mentioning that the American Society of Microbiology set the minimal level of activity required for any antimicrobial agent as 3 logs of CFU. Without achieving this value the substance cannot be accepted as "antimicrobial". There are exclusions from this rule. For example, bacteria growing in biofilms are considered more resistant to PDI treatment and the required level of eradication in such case is just 2 logs. [96]

AIMS OF STUDIES

Photodynamic therapy (PDT) and photodynamic inactivation (PDI) are promising methods of treatment of various diseases, from the microbial infections to different types of cancer. Both methods require the application of the photosensitizer with the desired properties, such as high molar absorption coefficients in the optical therapeutic window range and high quantum yield of ROS generation. The photosensitizer has to be also administered in a way that allows reaching its target point of action, such as cancer cells or bacteria.

The aim of the present studies was the evaluation of the PDT and PDI applicability of selected porphycene-core compounds, which included:

- Evaluation of photophysical properties of the compound, such as molar absorption coefficients, quantum yields of fluorescence, and fluorescence lifetimes in different solvents.
- Selection of the delivery system that can be used for *in vitro* applications of the porphycenes in PDT of the cancer cells and PDI of bacteria. During the experimental choice of the medium, it was crucial to take into consideration the high hydrophobicity of the porphycene compounds and the requirement of biocompatibility of the final formulation. On this basis, three potential types of carriers were subjected to the tests: cyclodextrins, phosphatidylcholine liposomes, and pluronic micelles.
- Estimation of quantum yields of singlet oxygen generation of the studied compounds in organic solvents and in the formulation used for their administration to the cells.
- Evaluation of the efficiency of the proposed compounds in photodynamic inactivation of Gram-positive bacteria, including simple analysis of structure – activity relationship.
- Evaluation of efficiency of the proposed compounds in photodynamic therapy of cancer, performed on HeLa cell line *in vitro*. The studies were extended with the aim to broaden the understanding of the kinetics of chromophores penetration inside HeLa cells and gaining the knowledge about the mechanism of the cell death induced by PDT with porphycene.

MATERIALS AND METHODS

4.1 Methods

Electronic absorption spectra were measured on a Shimadzu UV2700 spectrophotometer. Stationary fluorescence was recorded on an Edinburgh FS 090 CDT spectrofluorometer and a Fluorolog-3 spectrofluorometer (model FL3-22, Horiba Jobin Yvon).

The equipment used for DLS experiments included SpectraPhysics Stabilite 2017 Argon ion laser light source (λ=514.5 nm, delivering up to 2W, vertical linear polarization), photon-counting photomultiplier set on a BI-200SM goniometer (Brookhaven Instruments Corp.) and a PC-controlled 522-channel BI-9000AT cross-correlator.

Microscopy studies were conducted on:

- Nikon Eclipse E200 with the technique of phase-contrast, equipped additionally with a Prior, Lumen L200D lamp and a set of DAPI-FICT-TRICT filters.
- Nikon A1R Confocal Microscope System, using 405 nm, 488 nm, 561 nm, and 635 nm laser lines.
- Leica SPE II equipped with a Leica 63x 1.4NA oil immersion objective (HXC PL APO 63x/1.40 OIL CS) and a prism spectral detection system, using 405 nm, 488 nm, 561 nm, and 635 nm laser lines.

All experiments involving irradiation of the samples were conducted by means of the set presented in Figure 11. The set consisted of a home-made amplifier and a LED Array (Roithner Laser Technik, λ_{max} = 624 nm, P = 120 mW). The emission profile of the diode is presented in Figure 12. For all the quantitative experiments full power of the array was used, the diameter of the light spot was 7.0 cm, and the Petri dish of the diameter 3.5 cm was placed in the middle, so that 80% of the entire beam energy reached the sample, resulting in a dose of light equal to 0.6 J/(cm^2 min).

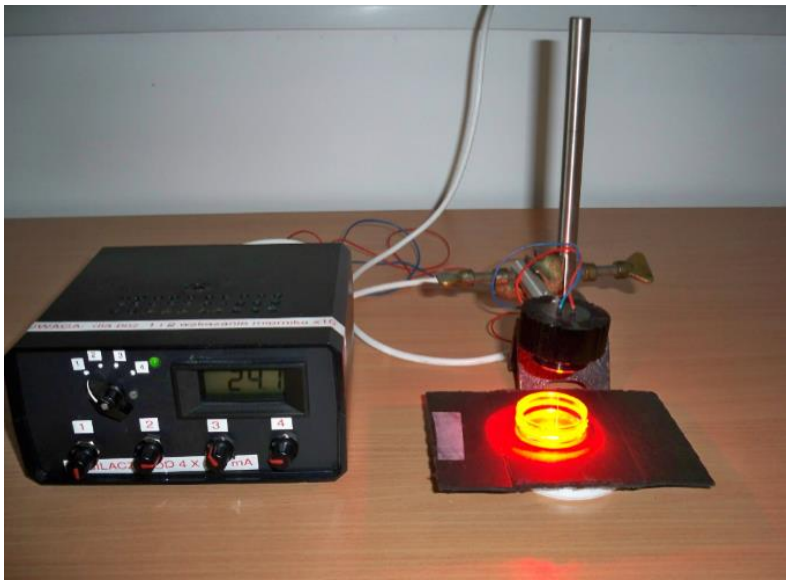


Figure 11. Home-built equipment for irradiation of the biological samples, with LED Array (Roithner Laser Technik, λ_{max} = 624 nm, P = 120 mW).

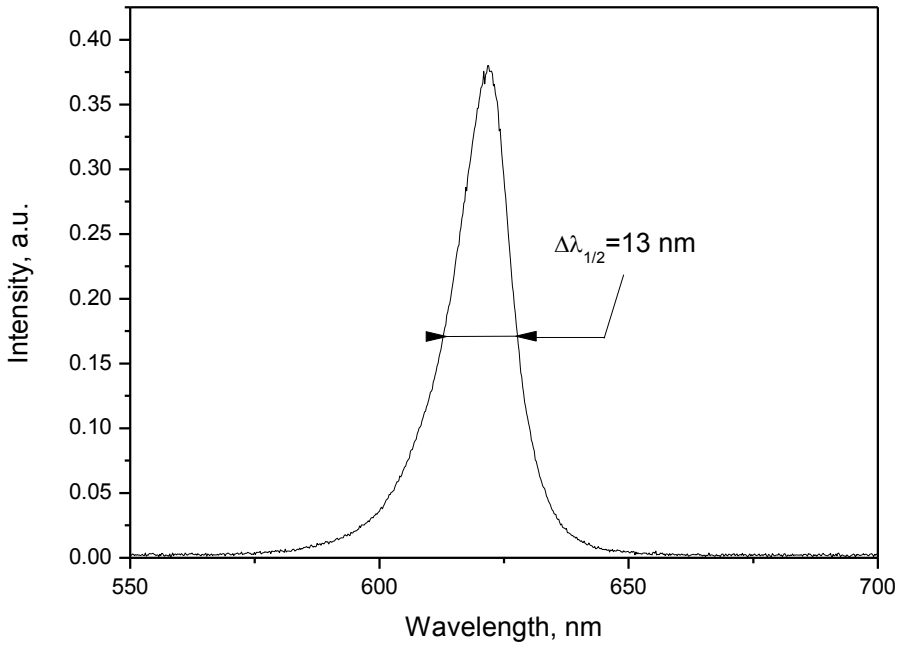


Figure 12. Emission profile of the diode array.

Fluorescence lifetime imaging microscopy (FLIM)

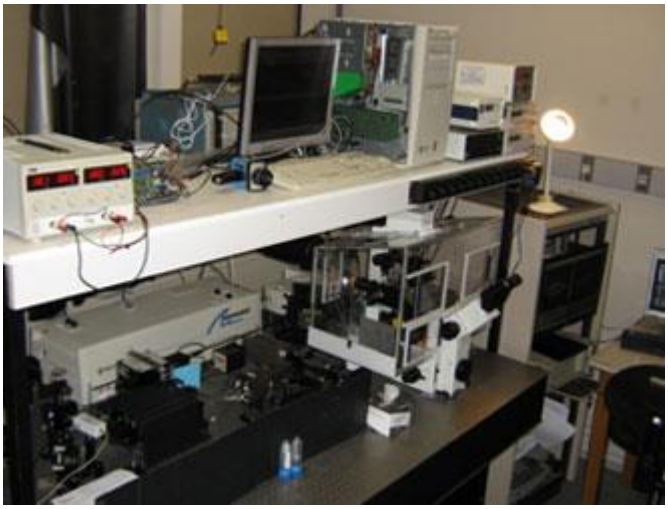


Figure 13. The setup for FLIM experiments. University of Strasbourg.

FLIM measurements were conducted in the Laboratory of Biophotonics and Pharmacology of the University of Strasbourg, directed by Ives Mely. The experimental set-up was as described in ref. [97] Time-correlated single-photon counting FLIM measurements were performed on a home-made two-photon excitation scanning microscope based on an Olympus IX70 inverted microscope with an Olympus 60× 1.2NA water immersion objective operating in the descanned fluorescence collection mode. [98] Two-photon excitation at 800 nm was provided by a Spectra-Physics laser. Photons were collected using a short pass filter with a cut-off wavelength of 680 nm (F75-680, AHF) and a band-pass filter BP 630/75m (Chroma). The fluorescence was directed to a fiber-coupled avalanche photodiode (SPCM-AQR-14-FC, Perkin Elmer), which was connected to a time-correlated single photon counting module (SPC830, Becker & Hickl). Typically, the samples were continuously scanned for about 120 s to achieve appropriate photon statistics in order to investigate the fluorescence decays. Data were analysed using the SPCImage V5.0 software (Becker & Hickl).

Singlet oxygen generation studies

Measurements of quantum efficiencies (Φ_Δ) of singlet oxygen generation ($^1O_2$, emission at λ_{max} = 1275 nm) have been performed with a home-made highly sensitive experimental set-up based on BENTHAM DTMc300 Double Monochromator and equipped with a TE Cooled photomultiplier (Hamamatsu H10330C-75, 950–1700 nm registration range). A LED with light output of 300 mW was used as photoexcitation source. The singlet oxygen quantum yield was determined by the relative method. [99] The efficiencies Φ_Δ were determined with respect to the well-known standard phenalenone Φ_Δ= 0.99 in toluene at ambient temperature. [100] The accuracy in the estimation of singlet oxygen emission quantum yield was 10%.

4.2 Samples

Porphycene and its derivatives were obtained and purified by the synthetic chemists team at the Institute of Physical Chemistry of the Polish Academy of Sciences, according to the procedures described in the literature. [43][101] The formulas of the compounds presented in this thesis are shown in Figure 14 along with the acronyms.

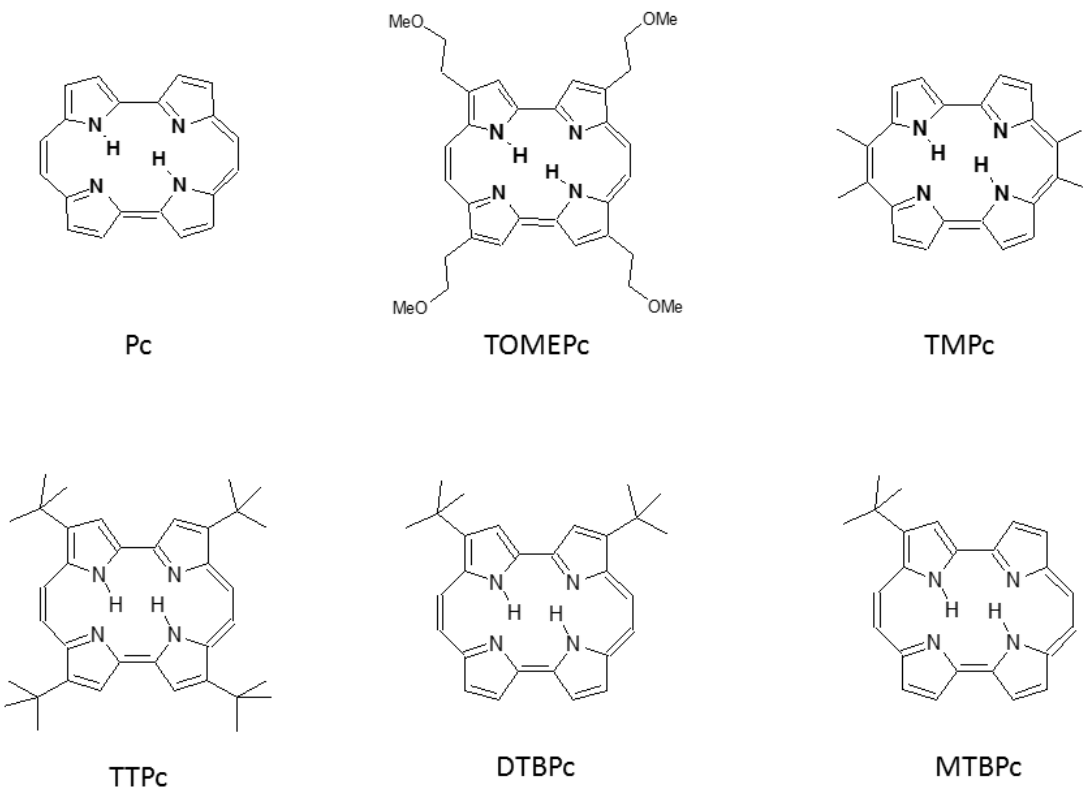


Figure 14. Studied compounds: Pc: porphycene, TOMEPc: 2,7,12,17-tetrakis(β-methoxyethyl), TMPc: 9,10,19,20-tetramethylporphycene, TTPc: 2,7,12,17-tetra-tert-butylporphycene, DTBPc: 2,7-di-tert-butylporphycene, MTBPc: 2-tert-butylporphycene.

The solvents included phosphate buffered saline (PBS) prepared from water filtered with Elix Milli-pore system and chloroform (ROTI- SOLV, UV/IR grade). Pluronic F-127 and inorganic salts for buffer preparation: sodium hydrogen phosphate, sodium chloride, potassium chloride and potassium dihydrogen phosphate were purchased from Sigma-Aldrich.

Micellar solutions

The samples containing pluronic and porphycenes were prepared using a thin film method. The compounds were co-dissolved in organic solvent so that their concentration was known. Such solutions were left to evaporate under the room conditions and then the remaining thin film was dissolved in water or PBS [102]

Liposomal solutions

The procedure of preparing liposomal samples was based on the literature method [103] slightly adapted to the requirements of the experiments presented in the thesis. It included mixing chicken egg L-α-phosphatidylcholine (egg-PC, Avanti Polar Lipids) dissolved in *tert*-butanol (Merck) with one of the porphycene compounds dissolved in chloroform. Such sample was further lyophilized to remove all traces of the organic solvent. Liposome solution was then obtained by rehydrating the dry lipid film with PBS. The vesicle emulsion obtained after hydration was sonicated and extruded repetitively through a polycarbonate film with 100 nm pores (Anatop, Whatman).

Bacteria photodestruction

Porphycene photosensitivity was evaluated for three strains from the American Type Culture Collection (ATCC): *Staphylococcus epidermidis* 14990, *Staphylococcus aureus* 29213, and *Enterococus faecalis* 29212. Bacteria were cultured on COS agar plates (Columbia Agar with 5% Sheep Blood, Becton Dickinson) overnight at 37^{o}C. The culture of bacteria was conducted in the National Institute of Medicines in Warsaw.

Bacteria were harvested from Columbia Agar plates with 5% sheep blood (Becton Dickinson) after overnight culture, washed and suspended with PBS. Suspensions of bacteria (10^7 CFU/ml, OD_{600} 0.1) with porphycene solutions were incubated at room temperature, in the dark, for 30 min. Subsequently, they were irradiated in the disposable, sterile polystyrene Petri dishes of 35 mm diameter by means of LED Array. Samples for viability studies were taken within the time of irradiation, diluted in PBS and plated on brain heart infusion (BHA) agar (Becton Dickinson) for colony counting (three times of each dilution). Tests were performed in three biologically independent replicates. Evaluation of the dark cytotoxicity, performed in parallel to each experiment, proved that the mixtures in the absence of light are neutral for the bacteria. Also it was checked that the pluronic solutions without Pc do not exhibit any antibacterial properties while irradiated.

Fluorescence markers for detecting ROS

The kinetics of ROS generation was performed with the use of fluorescence markers. Singlet Oxygen Sensor Green (ThermoFisher) was used as the marker of singlet oxygen. The concentration in working solutions was 2 – 5 μM and constant for all the samples within one experiment.

The marker used for detecting hydroxyl radical was hydroxyphenyl fluorescein (HPF from ThermoFisher). The concentration of working solutions was 10 μM. It is worth noting that HPF is not selective to hydroxyl radical, but it is sensitive also to the peroxynitile anions.

Bacteria microscopy

Microscopy studies were conducted on a Nikon A1R Confocal Microscope System, using the 635 nm laser line. Bacteria incubated with dyes were immobilized on the poly-L-lysine coated slides (Sigma Aldrich), dried in air and then covered with cover slips, through which they were observed by means of an oil immersion ×100 objective.

HeLa cell culture

HeLa cells were cultured in the specialized laboratory for human cells culture, using laminar flow cabinet Thermo Scientific MSC Advantage 1.5, microbiological safety class II; two CO_2 incubators (Nuaire NU-5810 E). HeLa cells were cultivated in monolayer cultures using Dulbecco's modified Eagle's medium with glucose in concentration of 1000 mg/l (Merck). The cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

Localization of photosensitizers within HeLa cells.

Localization of compounds within the HeLa cells were tested after 24 h incubation at 37^0C. The delivery agent used to transport of compounds to the cells was pluronic F-127 (3.5 mM) in PBS buffer. Absorbances of the solutions were as follows:

1. Pc/pluronic $A_{630} = 0.51$
2. TOMEPc/pluronic $A_{634} = 0.40$
3. TMPc/pluronic $A_{661} = 0.16$
4. TTPc/pluronic $A_{634} = 0.43$

The amount of solutions added to the cell culture dish was 100 μl per 2 ml of medium

Live/dead cell differential staining

Determination of HeLa cells viability was conducted using the method of differential staining. The method requires application of two dyes. One stains only living cells, and another stains only dead cells. In this case I used the most popular set of dyes, which means propidium iodide (PI) and calcein AM. The calcein assay is based on the conversion of the non-fluorescent calcein AM dye to the fluorescent calcein dye by intracellular esterase activity in live cells. The process of conversion cannot occur in the dead cells, so they stay calcein-negative in microscope images. Propidium iodide is membrane impermeant and therefore does not enter viable cells with intact membranes. When the membrane is broken, PI can intercalate into the nucleic acids, which causes a dramatic increase of fluorescence and is therefore used to identify dead cells.

The procedure was carried out in the time range from 30 min to 2 hours after irradiation of the cells. At this time the samples were kept in the incubation chamber. Propidium iodide solution (1mg/1ml of water) purchased from Sigma Aldrich was always used in the proportion 200 μl of the solution per 1 ml of cell culture medium. Calcein AM in DMSO (1:1 m/m) was always used in the proportion 2 μl of the solution per 1 ml of cell culture medium. Microdishes with the cultured cells and both staining solutions were incubated for 30 min and then observed under a fluorescence microscope.

Polystyrene microbeads

Corning microcarriers of the size of 125-212 μm, purchased from Sigma-Aldrich, were used for the expansion of the cell culture area in the studies of kinetic of dye penetration inside the HeLa cells. To this purpose, the content of a purchased 10 g vial of microcarriers was aseptically transferred to a sterile container. The sterile water was added to achieve a final volume of 100 mL for a final concentration of 100 mg/mL (36 cm^2/mL).

HeLa cells were cultured in DMEM with the addition of 500 μl of the stock solution of Corning microbeads per every 3.5 ml of medium with cells. To avoid adhesion of the cells to the surface of the culture dish, the falcon tube was used instead of the μ-dish. To develop a required area of the cells the incubation was conducted for 2-3 days. From time to time the solution was stirred gently to maintain the equal distribution of the cells on the surface of microbeads.

AlamarBlue cell counting

AlamarBlue® purchased from ThermoFisher Scientific was used when the evaluation of the number of HeLa cells in the samples was necessary. The active ingredient of AlamarBlue® is a non-toxic, cell permeable compound resazurin. Upon entering cells, resazurin is reduced to resorufin. This transition is accompanied by the change of color from blue to red. Resorufin is also highly fluorescent, whereas the precursor is not. Viable cells convert resazurin to resorufin, increasing the overall fluorescence and color of the media surrounding cells.

The cells stained with AlamarBlue® according to the protocol provided by the distributor were further counted using the automatic cell counter Countess II FL (Thermofisher).

Cell death mechanism assays

Both necrosis and apoptosis have their own specific biochemical and morphological pattern. Apoptosis on the level of the cells is typically characterized by phosphatidylserine exposure, cell shrinkage, nuclear condensation, and formation of pyknotic bodies of condensed chromatin. Necrotic cells exhibit nuclear swelling, chromatin flocculation, breakdown of organelle, and cytolysis by swelling. [104] The studies of the cell death mechanisms on the molecular level prove that apoptosis is driven by proteases, named caspases and regulated by Bcl-2 family of proteins. Very important organelle connected with apoptosis are mitochondria, which, during the cell death process, release the range of proapoptotic molecules. The most important of such molecules is cytochrome *c*. [105] Most of the techniques of determination of apoptosis is based either on cellular or molecular level characteristic events. In the case of defining the necrosis, a serious difficulty occurs. The problem is that in the absence of phagocytosis apoptotic cells become secondary necrotic cells with many morphological features of primary necrosis. [106]

Annexin V–FITC / propidium iodide staining

Annexin is a calcium-dependent protein which is very commonly used as the marker of apoptosis in the cells. Due to its phospholipid-binding properties it is able to attach to the phospatidyl serine expressed on the surface of the apoptotic cell. At the same time it does not bind to the non-apoptotic cell membranes, since they tend to keep the phosphatidyl serine on the inner surface of the membrane, where annexin has no access as long as the

membrane is not broken. Annexin itself is not fluorescent, so to obtain the functioning apoptosis marker it has to be conjugated with a chromophore, such as FITC. A typical method is based on co-staining with such conjugate and propidium iodide, and gives the results easy to interpret, such as shown in Figure 15.

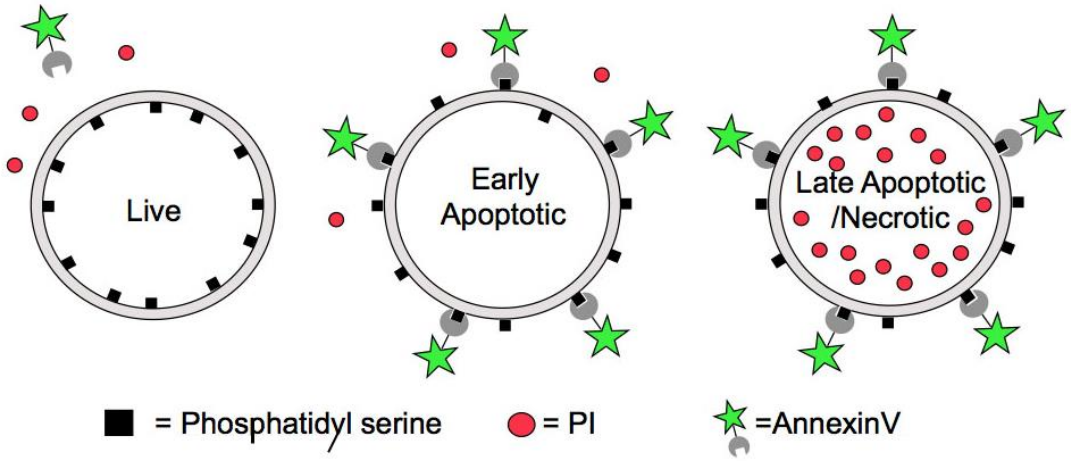


Figure 15. Schematic presentation of annexin V bounding to phospatidyl serine expressed on the surface of the cellular membrane. [107]

In this thesis Annexin V-FITC conjugate was obtained from BioLegend. The protocol used for apoptosis detection included initial washing of the cells with PBS and further with the Annexin V binding buffer (BioLegend). The cells were then stained with the Annexin V-FITC conjugate diluted 1:10 in Annexin V binding buffer and left at room temperature for 15 min. Next, propidium iodide was added in the concentration of 200 µl of PI per 1 ml of the solution. After that, the cells were washed with Annexin binding buffer and observed in the same buffer under the microscope.

Nucleus fragmentation studies

In the programmed cell death, the damaged cellular content is cleared out without producing any leftovers and without inducing the immunological response. The process requires degradation of the cellular content within the cell, without disintegration of the outer membrane. In the organism such cells are further immediately internalized by the neighboring cells or phagocytes. [108]

Hoechsts are the group of organic dyes, that can attach to the DNA helix of the cells. They are emissive, with the blue fluorescence. Due to such properties they are used for staining the nuclei of the cells in fluorescence imaging. Two most popular ones are Hoechst 33258 and Hoechst 33342. The latter (Hoechst 33342 purchased from ThermoFisher

Scientific) has been used in this thesis for staining the nuclei of HeLa cells after their photosensitization. Since it is the most lipophilic dye from Hoechst family, it can penetrate into both living and dead cells, which was crucial for the selection of this dye. The aim was to study the fragmentation of the nuclei to establish the mechanism of cell death.

Hoechst 33342 stock solution was prepared by dissolving the vial content (100 mg) in 10 ml of Millipore filtered water to create a 10 mg/ml solution. Such stock solution was further diluted with PBS in the proportion of 1:2000, prior to the experiment. For the cells staining, the medium was removed from the cell culture vessel and the amount of staining solution sufficient to cover the cells was added. After this procedure the cells were imaged by a microscope.

Caspase-3 detection studies

Caspase-3 is cysteine endoprotease responsible for the regulation of apoptosis signaling network. It is an effective caspase which is executing apoptosis and it is activated in all kinds of apoptotic cells, no matter if an intrinsic or extrinsic apoptotic pathway is followed. [105]

NucView 405 substrate (1mM in DMSO) was purchased from Biotium and the preparation of the samples was done following the producer instructions. First, 2 μM NucView solution was prepared by adding 4 μl of 1 mM stock solution to 2 ml of PBS buffer. Next, the cell culture medium was replaced with the prepared mixture and the cells were incubated at room temperature for 30 minutes. Such samples were ready to be imaged under the microscope.

RESULTS AND DISCUSSION

5.1 Photophysical properties of the studied compounds

The first stage of research for this thesis included the photophysical studies of chosen porphycenes and selection of the medium that was further used in biological tests. The initial studies were performed on porphycenes dissolved in organic solvents to avoid the formation of aggregates. The series of spectra of porphycenes shown in Figure 16 were recorded using *n*-hexane as a solvent.

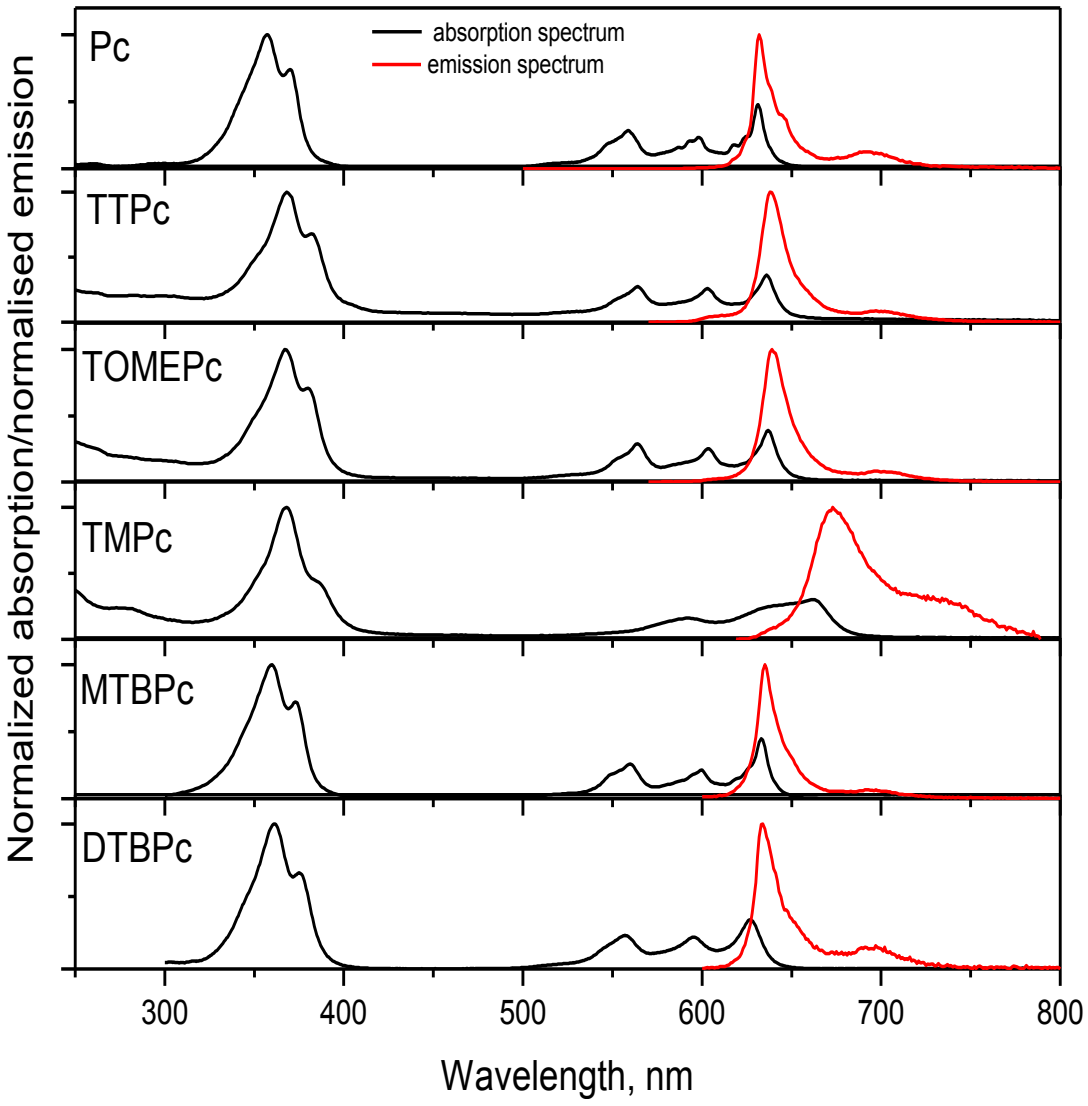


Figure 16. Absorption and emission spectra of porphycenes in n-hexane.

Table 1 presents the photophysical properties of the studied porphycenes. All of the compounds exhibit high absorption coefficients in organic solvents, over 10^5 $M^{-1}cm^{-1}$ in the Soret region. The significant feature for PDT and PDI use is the location of the Q band, with the absorption coefficients exceeding $3.5 \cdot 10^4$ $M^{-1}cm^{-1}$, and absorption range reaching over 600 nm, which fits in the optical therapeutic window. Fluorescence quantum yields of these compounds are between 0.3 and 0.5. The exception are the compounds substituted at the meso positions, represented here by TMPc and TPPc. They exhibit much lower fluorescence quantum yields, of the order of 10^{-4}. These two porphycenes were selected for these studies for different reasons than the rest of compounds. Their lifetimes of the fluorescence are much shorter than the ones of the remaining compounds shown in Table 1, but they exhibit strong dependence on the viscosity of the surrounding medium, which was employed in the research. The porphycenes substituted in the pyrrole rings exhibit fluorescence with the decay times ranging from 8 to 12 ns.

Table 1. Photophysical properties of the studied porphycenes. In the case of literature values, the references are given.

		Absorption			Fluorescence		
	Solvent	λ_{max}, nm (ε, $M^{-1}cm^{-1}$)		Ref.	Φ_F	τ_F	Ref.
		Soret band	Q_1-band				
Pc	n-Hexane				0.36	10.7	[48]
	CH_3CN	356 (115 717)	626 (39 909)	[39]	0.42	12.0	[48]
	CH_2Cl_2	358 (139 200)	630 (51 900)	[43]			
TOMEPc	Toluene				0.47		[35]
	MeOH				0.38		[35]
	CH_2Cl_2	370 (140 000)	634 (47 000)	[43]			
TTPc	CH_3CN	368 (130 054)	631 (40 053)	[39]	0.36	9.3	[39]
	n-Hexane				0.31	8.4	[39]
MTBPc	n-Hexane	359 (71 000)	633 (14 000)		0.30		
	CH_3CN	358 (120 000)	627 (43 000)		0.35		
TMPc	n-Hexane				$2.9 \cdot 10^{-4}$		[48]
	CH_3CN	365 (111 750)	654 (35 776)	[39]	$3.2 \cdot 10^{-4}$		[48]
	THF					0.003/0.017	[109]
	PVB					0.5/2.3	[48]
DTBPc	n-Hexane				0.35		
	CH_3CN	361 (120 000)	627 (40 000)		0.32		
TPPc	n-Hexane				$5.4 \cdot 10^{-4}$		[48]
	CH_3CN				$8.1 \cdot 10^{-4}$	0.006/0.030	[48]
	THF					0.005/0.031	[109]
	PVB					1.2/3.2	[48]

Singlet oxygen generation studies

The crucial property of the compounds used in photodynamic therapy is their ability to generate reactive oxygen species. Porphycenes as type II sensitizers are supposed to generate mostly singlet oxygen. The subject of this chapter was the evaluation of the quantum yield of this process. The experiments were conducted under two different sets of

conditions. First, the compounds were dissolved in toluene, the solvent providing relatively long life-time of singlet oxygen (2.9×10^{-5} s [27]). The results are shown in Table 2. In the second attempt, porphycenes were introduced in their micellar formulation, but the PBS buffer was replaced by deuterated water. Such conditions allowed to obtain the signal of $^1O_2$ phosphorescence with the used equipment. The quantum yields of $^1O_2$ generation were measured by the comparison of singlet oxygen emission intensity at 1276 nm (the maximum of the emission band) with the reference sample emission. In the case of quantum yields measured in toluene solutions, to be sure of the results, two different references were used, phenalenone (Φ_Δ = 0.99 [110]) and palladium octaethylporphyrin, abbreviated as PdOEP (Φ_Δ = 1.0 [111]). Since the correlation between both standards was very satisfactory, only phenalenone reference was further used for the measurements in deuterated water.

Table 2. Quantum yields of singlet oxygen generation of the porphycenes in toluene and in the pluronic (3.5 mM) solution in deuterated water.

compound	Φ_Δ (toluene)	Φ_Δ (deuterated water)
Pc	0.30 ± 0.01	0.28 ± 0.03
TTPc	0.38 ± 0.02	0.21 ± 0.02
TOMEPc	0.32 ± 0.02	0.23 ± 0.02
DTBPc	0.36 ± 0.01	0.22 ± 0.02
MTBPc	0.32 ± 0.01	0.23 ± 0.02

The measured yields of singlet oxygen generation were comparable among all the studied compounds and covered the range from 0.30 to 0.38. The values obtained for the solutions of porphycenes in deuterated water were slightly lower with respect to the ones in the toluene and covered the range from 0.21 to 0.28. Such results match the literature data for porphycene-core compounds. Braslavsky et. al. report Φ_Δ = 0.37 for TOMEPc in deaerated toluene. [23]

5.2. Selection of pluronic F-127 as the carrier medium for porphycenes

Porphycenes are highly hydrophobic compounds, therefore they cannot be directly dissolved in water or water buffers. Transfer of these organic compounds to the water medium without altering the basic photophysical properties was required for further biological experiments on the mammalian cells and bacteria. Initially, three approaches of solving this problem were tried. One of the ideas was using water solutions of cyclodextrins as the carriers of photosensitizers, the second was micellar solution and the third, liposomes.

5.2.1. Cyclodextrins

The research described in this chapter was supposed to answer the main question whether complexation of porphycene by cyclodextrins can provide the concentration of a photosensitizer high enough for biological experiments. In the first stage of experiments parent porphycene was added to water solutions of six different cyclodextrins in the amount exceeding its solubility in such mixture. After 7 days of shaking the samples at the temperature of 37°C, there was still unsolubilized porphycene remaining at the bottom. Because of low concentrations of porphycene in solutions, it was not possible to compare solubility of Pc among solutions of different CDs using absorbance measurements. The emission spectra (Figure 17) detected under the same conditions for the mentioned solutions revealed the highest intensity of fluorescence for trimethyl-β-cyclodextrin. This indicates stronger complexation of porphycene in the solution of this particular CD in comparison with other ones.

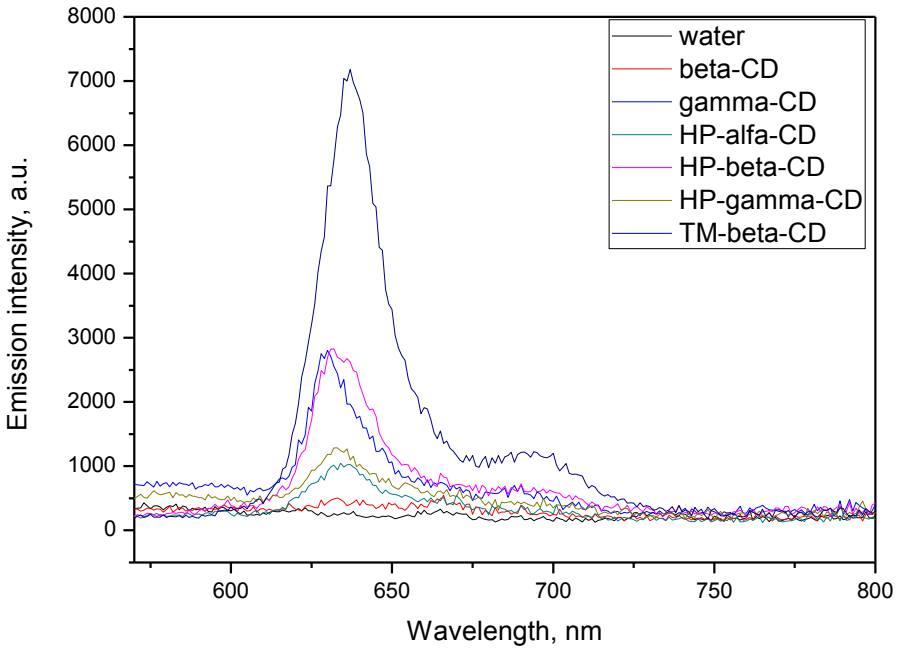


Figure 17. Emission spectra of Pc in aqueous solutions of different cyclodextrins. In each sample 0.13 mg of Pc (the amount that exceeds solubility) was added to the aqueous cyclodextrin solution (10^{-3} M).

As it was proven that trimethyl-β-cyclodextrin provides the most desired properties for biological tests, further characterization was carried out. As is visible from Figure 18 the concentration of porphycene in the samples is not high enough to be determined by means of a spectrophotometer, which indicates it must be lower than 10^{-7} M. Such concentration was much too low for biological experiments. Also, fluorescence studies of such porphycenes-cyclodextrin systems would be difficult, since replacement of water with PBS results in a dramatic decrease of fluorescence signal, due to the quenching by the phosphate anions. (Figure 19).

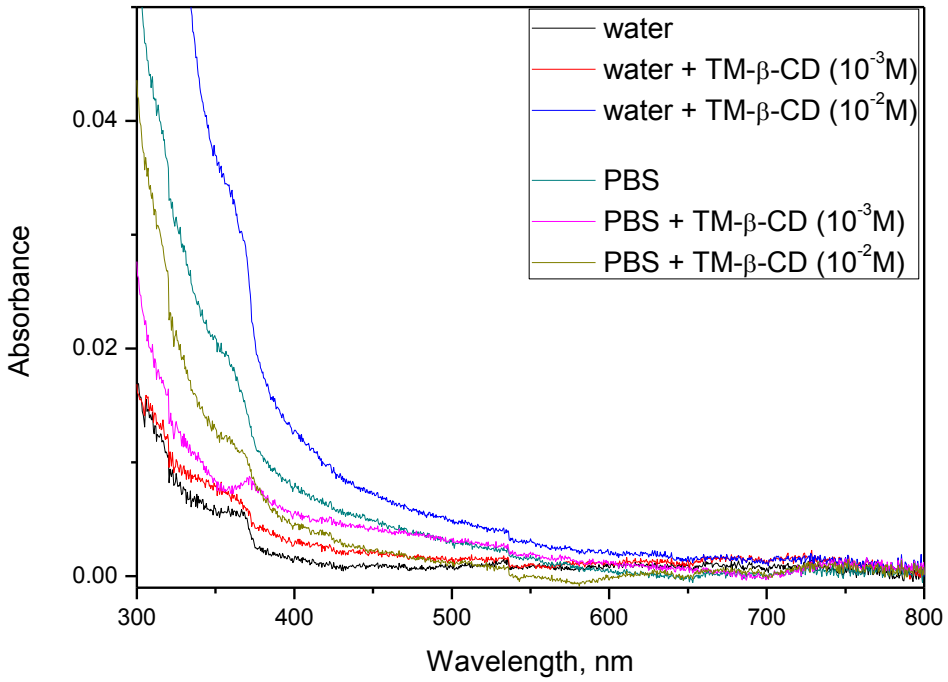


Figure 18. Absorption spectra of Pc in water and buffer solutions of TM-β-CD (different concentrations).

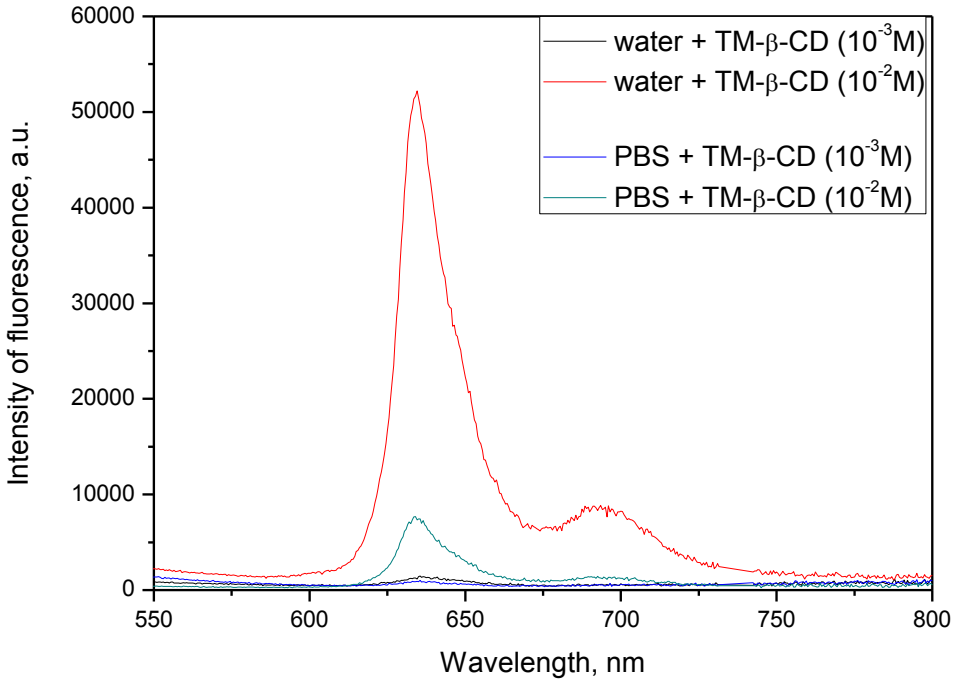


Figure 19. Emission spectra of Pc in water and buffer solutions of TM-β-CD (different concentrations).

5.2.2. Micelles

For the micellar formulation of the tested photosensitizers, the amphiphilic polymers from the group of pluronics were employed. Several products available on the market were suitable for the dyes inoculation, however Pluronic F-127 (herefrom called pluronic) appeared the most proper for further experiments, because of the best solubility in water and transparency of such solution. The second advantage of this polymer was a convenient powder form, allowing the best control over the concentration in independent experiments. The related pluronics are either liquids (Pluronic L-121) or form a viscous paste (Pluronic P-123) at room temperature. [112]

The first stage of experiments included finding the appropriate pluronic:porphycene concentration ratio for which the required photophysical characteristics would be preserved. Figure 20 presents the absorption spectra of the parent porphycene in water solutions of pluronic at various chromophore:pluronic molar ratios, with the concentration of pluronic high enough to ensure that it exists predominantly in the form of micelles. The

spectrum practically identical with that of monomeric porphycene, obtained in ACN solution, is observed when the [pluronic]/[porphycene] molar ratio exceeds 2000. When the relative concentration of porphycene becomes higher, the spectrum changes: broad absorption appears in addition to the three-band pattern, characteristic for the monomeric porphycene solutions. We assign the broad absorption to oligomers/aggregates of porphycene. Similar behavior was reported for 2,6,12,17- tetra-*n*-propylporphycene embedded in liposomes. [113]

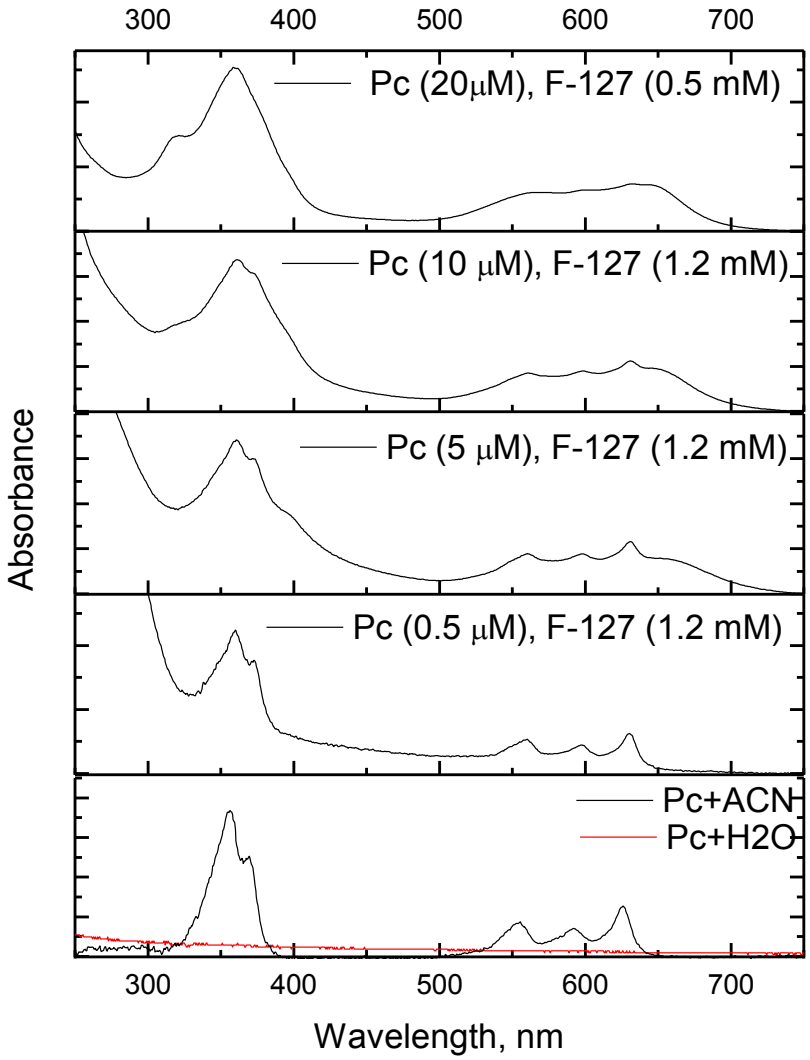


Figure 20. Absorption spectra of aqueous solutions containing Pc and pluronic in different proportions. Top to the bottom: 20 μM/0.5 mM; 10 μM/1.2 mM; 5 μM/1.2 mM; 0.5 μM/1.2 mM; solution of Pc in acetonitrile (black line), water with the addition of insoluble powder of Pc (red line).

Estimation of critical micelle concentration (CMC)

Estimation of CMC was carried out to make sure that the whole pluronic in the solution occurs in the form of micelles, which was supposed to provide optimal solubility of porphycenes in the sample. The value is crucial for evaluation of the range of pluronic concentrations that could be used in further biological experiments. Obtaining CMC was carried out using TOMEPc, due to the lowest aggregation in the pluronic solutions. For the same purpose the concentrations of chromophore used in the experiments were very low, in the range of micromoles. Figure 21 shows changes in absorption and fluorescence intensity for the water solution of TTPc with increasing concentration of pluronic. The sudden change of the slope in such relation determines CMC. The obtained averaged value is CMC=(5.2±0.4)×10^{-6} M. This corresponds well with the value obtained in other studies by means of pyrene probe for a similar solution of pluronic in PBS. [114]

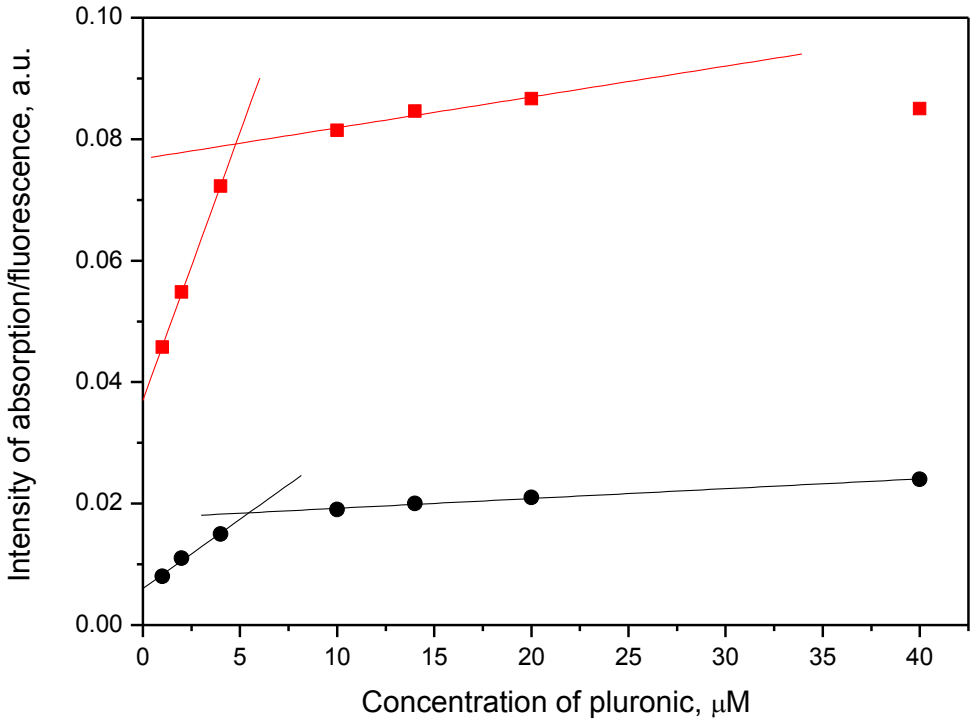


Figure 21. Dependence of fluorescence intensity (red squares) and absorbance (black circles) on pluronic concentration for the solutions of TOMEPc. The points of intersection of the regression curves determine the critical micelle concentration.

Time-resolved fluorescence experiments were conducted in order to check if the chromophores inside the pluronic micelles are distributed in the uniform way or whether they form aggregates. As presented in Table 3, fluorescence decay times of the studied porphycenes measured in micellar solution correspond well with the values obtained for the same compounds in organic solvents. The monoexponential decay pattern is a proof of the occurrence of the monomeric form of the chromophore in the micelles. The emission decays of THF/water mixtures were also recorded. They are definitely not monoexponential. In addition to the main, longer component, occurring also in pure THF, an additional, a shorter decay component is observed after addition of water. Its amplitude is about eight times smaller than that of the main component when the emission is monitored at shorter wavelengths. For the longer wavelength the contribution of short-lived component increases twice. Such behavior can be explained by the presence of the aggregated forms. The expected fluorescence of aggregates would be weaker and red-shifted with respect to the monomer emission, as is observed in Figure 20.

Table 3. Photophysical properties of Pc and TTPc in different solvents.

Compound	Solvent	Chromophore concentration (μM)	Pluronic concentration (mM)	λ_{em} (nm)	τ_1 (ns)	τ_2 (ns)	a_1/a_2	χ^2
Pc	*n*-hexane				10.7 *			
	THF				11.7 *			
	ACN				12.0 *			
	THF/H_2O 5:1	8.7	-	633	10.3	-	-	1.614
					10.6	4.0	8.6	1.305
				687	10.0	-	-	1.922
					10.5	4.3	4.8	1.187
	THF/H_2O 2:1	8.7	-	633	10.5	-	-	1.758
					10.8	4.1	8.4	1.387
				687	10.2	-	-	2.304
					10.8	4.4	4.4	1.305
	THF/H_2O 1:1	8.7	-	632	10.7	-	-	1.659
					11.0	4.1	4.1	1.315
				688	10.4	-	-	2.255
					11.0	4.1	8.1	1.242
	THF/H_2O 1:2	8.7		631	10.8	-	-	1.696
					11.2	4.7	7.4	1.306
				687	10.4	-	-	2.405
					11.0	4.3	3.9	1.235
	$Pluronic/H_2O$	0.11	50	633	12.8	-	-	1.750
				688	12.5	-	-	1.135
		1	50	633	12.0	-	-	1.416
				688	11.7	-	-	1.250
		10	50	633	11.5	-	-	1.489
				688	10.9	-	-	1.162
		20.8	0.5	633	11.2	-	-	1.369
				688	10.1	-	-	1.084
TTPc	THF	0.3	-	640	8.7	-	-	1.017
				715	8.3	-	-	1.230
	$Pluronic/H_2O$	0.84	1.3	650	9.2	-	-	1.077
				715	9.2	-	-	0.819
		0.08	1.3	650	9.3	-	-	1.060
				715	9.2	-	-	0.962
		0.04	1.3	650	9.3	-	-	0.964
				715	9.3	-	-	0.990

** literature values* [48]

Estimation of micelle size

DLS experiments were carried out using 2.5 mM filtered (pore size 0.2 μM) pluronic solutions in water at 298 K. In order to get a reliable estimate of the micelle size, relatively high concentration of the polymer was required to obtain sufficient amount of scattered light. The estimated diameter of the micelles was 29 ± 2 nm, which makes pluronic a suitable medium for encapsulation of such compounds as porphycenes, whose dimensions are of the order of $1 \times 1 \times 1$ nm^3.

Microscopy imaging of micelles

Incorporation of TTPc into the pluronic micelles was proved by means of complex microscopy studies. For this purpose, micelles had to be immobilized on the surface of glass. The procedure was carried out in the flow cells, using two different approaches. In both cases biotin-avidin conjugation was employed, so the pluronic particles had to be substituted with biotin moieties. The aim was to image single micelles containing the chromophore. The first approach applied biotinylated BSA to connect the micelles with the surface. As it is visible in the microscope image (Figure 22 A), the micelle-like structures are formed, which is visible as separated luminescent spots. However, non-regular distribution of the spots and the area of blurred fluorescence in the background suggest that the most of biotinylated pluronic sticks to the BSA instead of assembling in micelles. This result was not satisfactory; therefore another approach was taken. It required the silanization of the internal surface of the flow cell and the application of polyethylene glycol (PEG) residues instead of BSA. The resulting spherical luminescent objects in the image are single pluronic micelles containing monomeric porphycene (Figure 22 B). In the microscope images, they have the dimension limited by the microscope resolution, about half the wavelength of the used laser line.

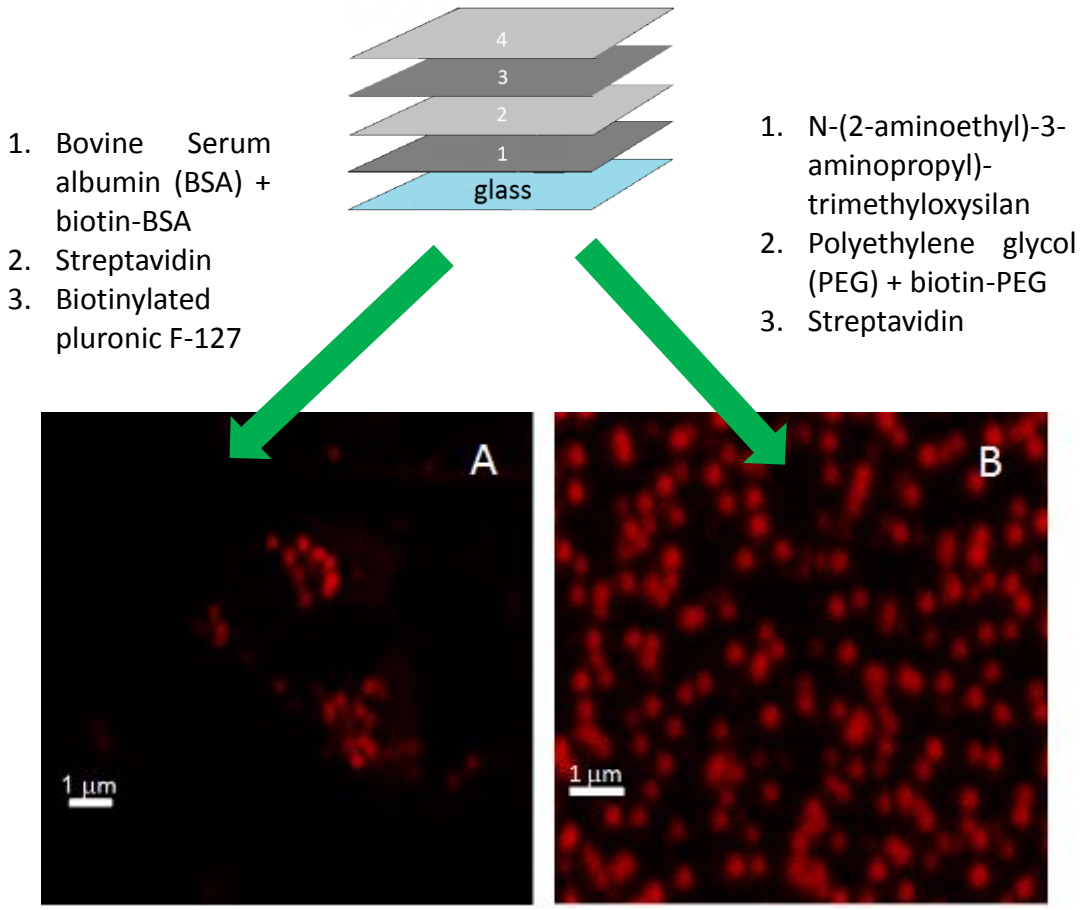


Figure 22. Fluorescence confocal microscope images of Pluronic F-127 micelles containing TTPc immobilized on the glass covered with: A) biotinylated bovine serum albumin, B) biotinylated polyethylene glycol.

Obtaining the image of separated micelles is a significant achievement, which provides the background for more advanced microscopic studies. Investigations of the chromophore emission on the level of single micelles can shed light on the nature of chromophore-micelle interactions. Particularly informative would be the analysis of fluorescence time-traces from single micelles, which would allow for estimation of the number of chromophore particles occluded in one micelle.

Viscosity inside the micelles

The viscosity inside the pluronic micelles was probed using another porphycene-core compound, 9,10,19,20-tetra-n-propylporphycene (TPPc; structure shown in Figure 23).

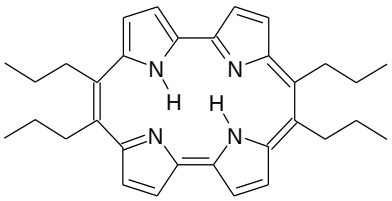


Figure 23. The structure of 9,10,19,20-tetra-n-propylporphycene (TPPc)

The decay time of its fluorescence is extremely sensitive to the changes in the viscosity of the microenvironment. [48] The evaluation of the viscosity inside the pluronic micelles is therefore possible on the basis of comparison of the fluorescence decays measured in solvents of different viscosity.

Table 4. Photophysical properties of TPPc in different solvents.

	Solvent	Chromophore conc. [μM]	Pluronic Conc. mM]	λ_{em} [nm]	τ_1 [νσ]	τ_2 [νσ]	a_1/a_2 [a]	χ^2
TPPc	Cyclohexane*				0.008	0.036	0.60	
	THF*				0.005	0.031	0.16	
	MeCN*				0.006	0.030	0.63	
	Liquid paraffin*				0.5	1.1	1.0	
	PMMA*				2.9	6.1	1.2	
	Pluronic/H_2O	0.34	2.5	667	0.7	4.3	329	1.253
		0.17	2.5	667	0.8	5.5	127	1.227

** literature values* [48]

The fluorescence lifetimes measured for pluronic aqueous solution of TPPc reveal that the interior of micelles is relatively viscous. The main lifetime of fluorescence decay measured in these studies was 0.7 ns, whereas the literature values [48] for organic solvents such as tetrahydrofuran (0.46 cP, 298 K) or acetonitrile (0.35 cP, 298 K) is around 0.005 ns and around 0.5 ns for liquid paraffin (0.86 cP, 298 K). The relationship between viscosity and fluorescence decay time of the probe is not linear, however in Figure 24 a simplified scheme of the lifetime-viscosity dependence for the mentioned substances is presented.

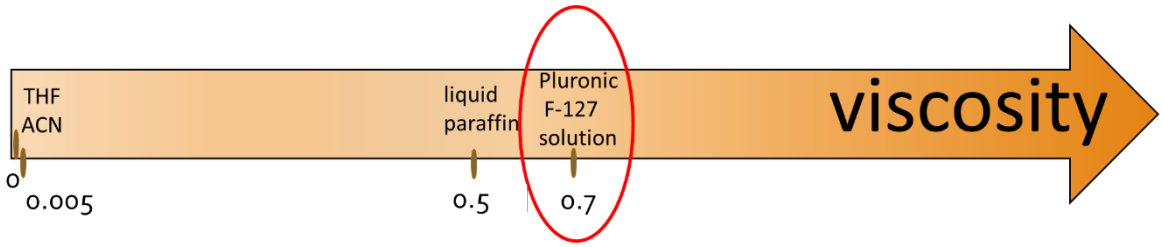


Figure 24. Schematic presentation of the medium viscosity dependence of the fluorescence lifetime of TPPc.

5.2.3. Liposomes

The third approach to obtain biocompatible water solutions of the studied photosensitizers was based on α-phosphatidylcholine liposomes. At the first stage of studies this medium looked very promising, because by embedding porphycenes in the liposomes it was possible to obtain the absorption (Figure 25) and fluorescence (Figure 26) spectra of monomeric compounds, at the same time keeping the biological-friendly aqueous formulation of the samples.

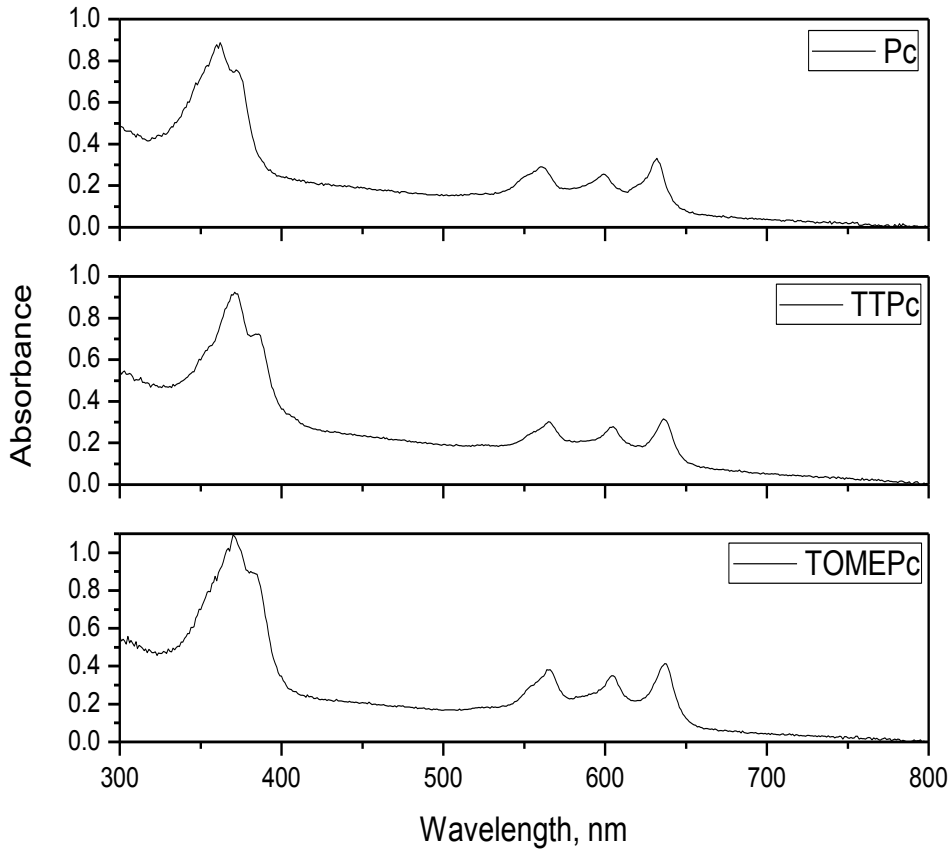


Figure 25. Absorption spectra of porphycenes embedded in eggPC liposomes.

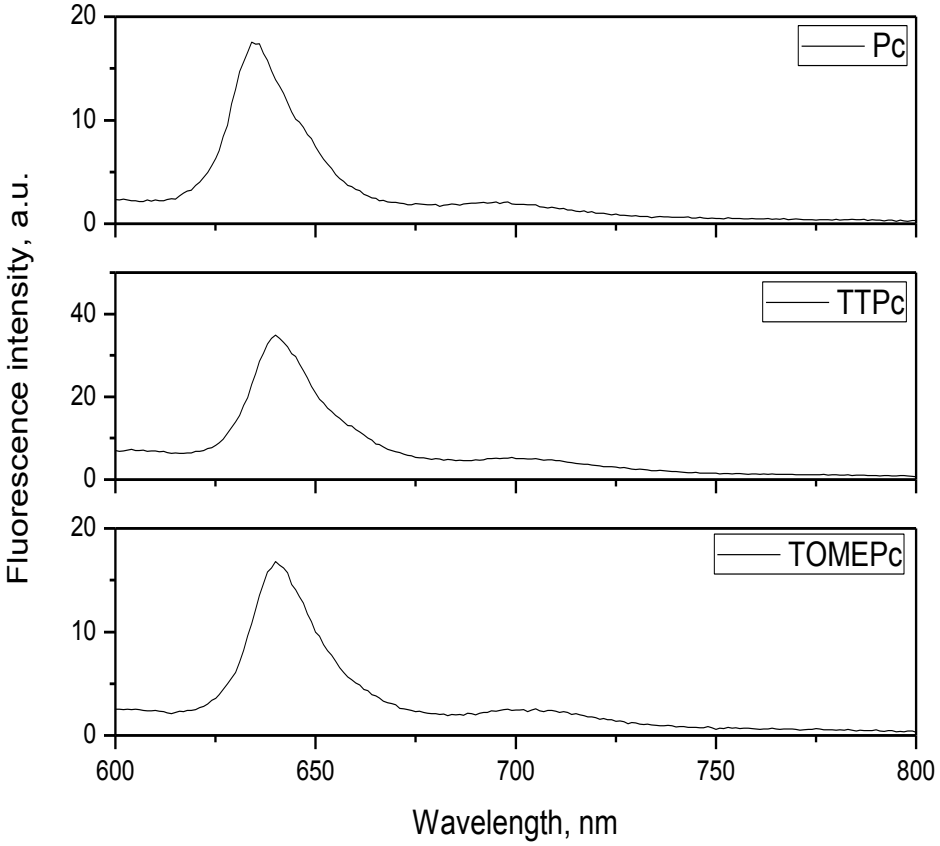


Figure 26. Emission spectra of porphycenes embedded in eggPC liposomes.

The range of concentrations of porphycenes in such solutions (estimated on the basis of the absorption spectra) was similar to the ones accessible for pluronic solutions. Despite this fact, the significant level of scattering was impairing the photophysical measurements. It is worth noting that the reproducibility of the liposomal samples prepared always in the same way, was limited. This observation, together with much lower stability of the liposomes than in the case of micellar solutions, has led to the decision of using pluronic as the main medium for the biological studies of porphycenes as photosensitizers. This choice was confirmed by further quantitative studies of HeLa cells viability after PDT treatment with Pc. Figure 27 shows that increasing concentration of Pc up to 0.7 μM gives lowering of the number of viable cancer cells to less than 20% of their initial amount (at the dose of light 18 J/cm^2), whereas the same concentration of a dye administered in pluronic micellar solution erases the cancer cells totally. The higher efficiency of PDT on HeLa cells in the case of pluronic formulation was the ultimate proof that this formulation is the best one, out of the tested possibilities, for the biological studies. It is worth

mentioning that the pluronic micelles were recently studied *in vivo* as DDSs for PDT applications. [115] They were proven to improve the melanoma treatment results in the mice, probably by enhanced ROS generation and increased tumor selectivity.

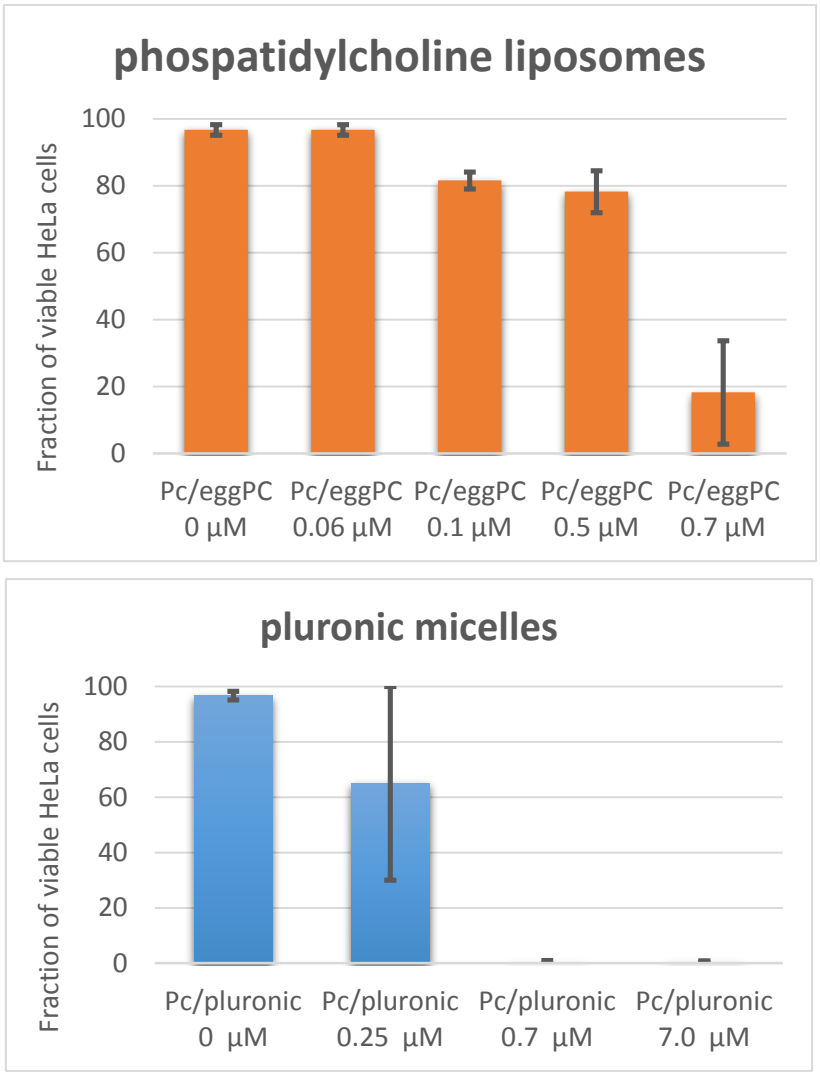


Figure 27. Viability of HeLa cells in liposomal and micellar solutions after irradiation with the dose of light 18 J/cm^2, λ= 624 nm.

5.2.4. Summary

Porphycenes are highly hydrophobic, water-insoluble compounds. To reveal their potential in PDT they need to be transferred to the biological environment. This requires water-soluble formulation. In this thesis, three different formulations were tested to decide which one is optimal for further mammalian cell and bacteria treatment. The tested carrier

media were different cyclodextrins, pluronic micelles, and phosphatidylcholine liposomes. Using cyclodextrins resulted in obtaining the solutions with very low effective concentrations of Pc, in the range of 10^{-7} M, whereas liposomes and pluronic micelles allowed for hundred times higher concentrations of the photosensitizer. Both liposomes and micelles formulations retained well the photophysical properties of monomeric porphycenes; however, the latter performed better during *in vitro* PDT trials on HeLa cancer cells. The kind of micelles selected for the biological studies consist of polymer pluronic F-127. They are stable in the conditions of experiments with CMC of $(5.2\pm0.4)\times10^{-6}$ M and the hydrodynamic diameter of 29 ± 2 nm. To prevent aggregation of porphycenes it is safe to use the excess of polymer with the molar ratio pluronic:porphycene higher than 2000:1. The most convenient range of concentrations is 1 – 10 μM of porphycene per 2.5 – 3.5 mM of pluronic. Such solutions, after filtration, can usually be stored for more than a week without the signs of aggregation or decomposition.

5.3. Bacteria photoinactivation studies

The research on phototoxicity of porphycenes against bacteria were divided into a few levels. The initial part of studies included testing three different compounds with the same Gram-positive bacteria strain, *E. faecalis*. Figure 28 shows the response of this strain to the chosen photosensitizers. Both Pc and TOMEPc induce a significant lowering of the number of bacteria in the samples upon red light irradiation. In contrast, no such effect has been detected for TTPc. The first idea to explain such discrepancy was the difference in the cell wall permeability of the dyes. Therefore, these results were encouraging for the further investigation of the dye penetration into bacteria, by means of confocal microscopy.

For further bactericidal studies Pc was selected, due to such advantages as the removal of a largest fraction of bacteria, good stability in the pluronic solution over time, and the best overlap of the spectrum with the emission profile of the diode array (Figure 12). The compound giving the most stable solution in the pluronic medium was TTPc, but, as mentioned before, it did not show any signs of cytotoxicity following irradiation.

It is worth mentioning that the antibacterial activity of the compounds was tested also with the irradiation with a different light source (λ_{max}=590 nm, P=30 mW). Both Pc and TOMEPc exhibited phototoxic effects in such conditions. Taking into account all these factors, parent porphycene was the photosensitizer selected for the quantitative studies, described in the chapter *Photodestruction of bacteria.*

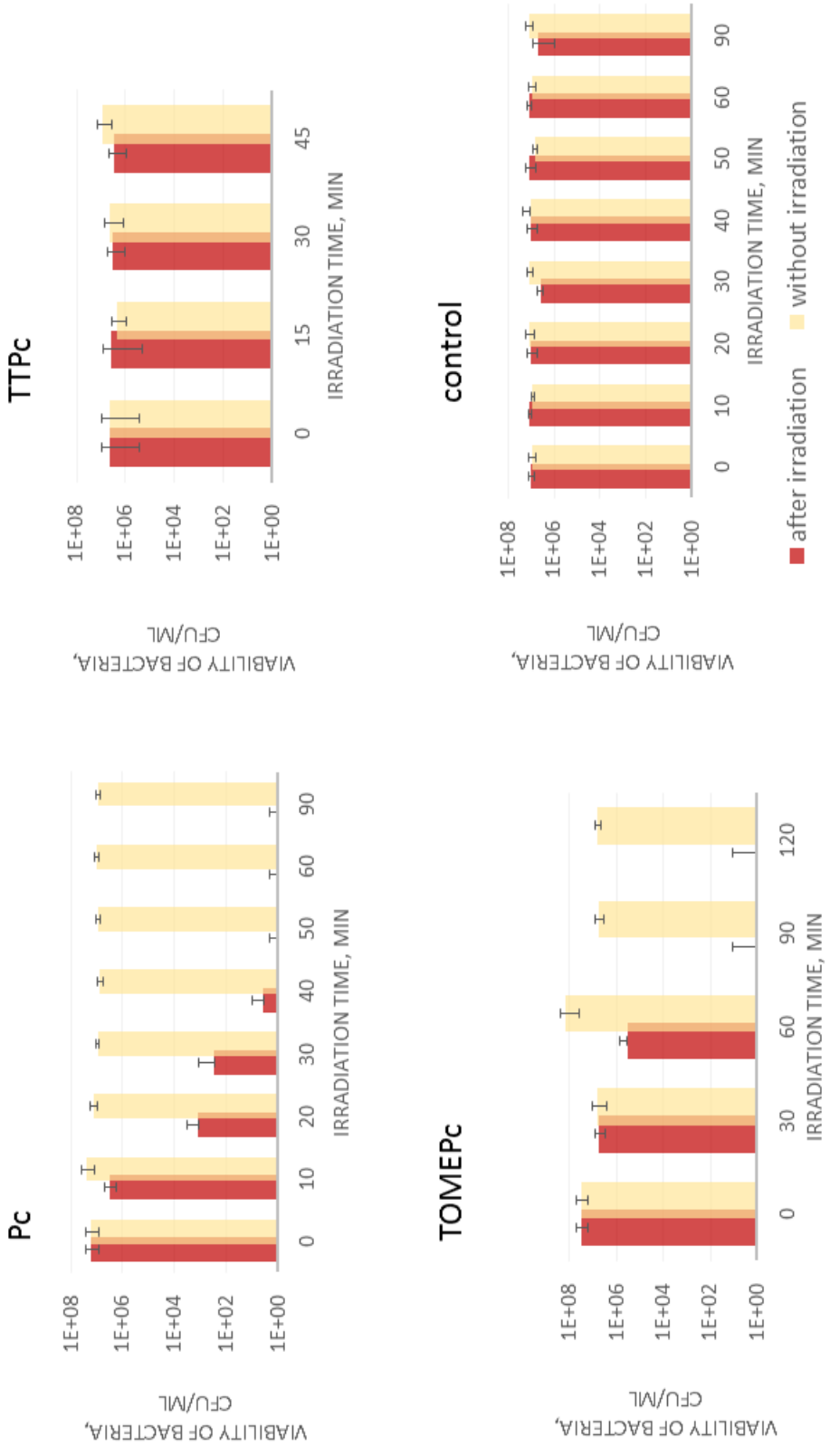


Figure 28. Dependence of the viability of E. faecalis bacteria on the time of irradiation of pluronic solution samples containing Pc (7 μM), TTPc (10 μM), TOMEPc (5 μM) Light dose: 0.6 J/ (cm² min).

5.3.1. Microscopy studies of penetration of chromophores into bacteria

The studies of parent Pc penetration inside bacterial cells were conducted on three strains: *E. faecalis, S. Epidermidis*, and *S. Aureus.* Confocal fluoresce microscopy confirmed that the chromophore enters the bacteria effectively. Figure 29 shows the overlaid images of dye fluorescence and the transmitted light view, revealing the positions of the bacteria. The majority of bacterial cells in all three samples were stained with the dye.

Initial studies of the three compounds on *E. faecalis* bacteria showed that also TOMEPc exhibits the ability to stain the bacteria, whereas TTPc does not penetrate into the cells (Figure 30). TTPc is at the same time the only compound inactive in these studies, which leads to the conclusion that the penetration through the cell wall is critical for photodestruction of bacteria. It is worth noting that the lack of fluorescence of the dye from the bacterial cells might be caused by the quenching of fluorescence or aggregation of chromophore molecules, which can also influence their photosensitizing ability. However, considering similarities in the structure of the studied compounds, such case seems less probable.

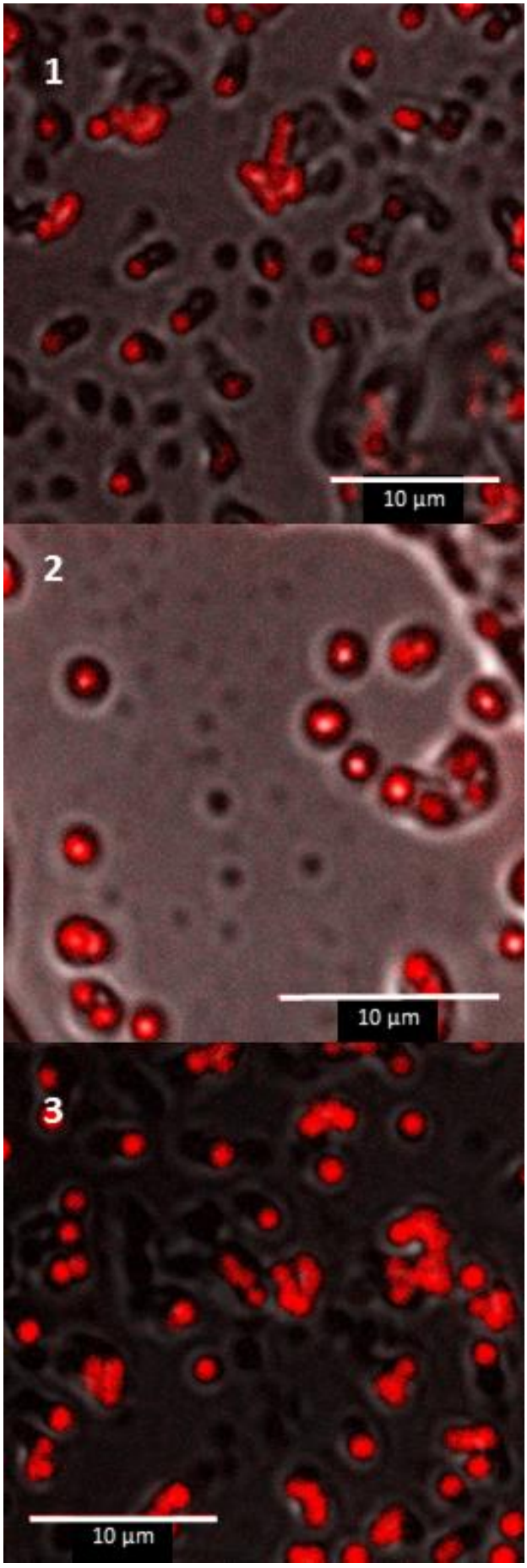


Figure 29 Confocal microscope images of 1) E. faecalis, 2) S. epidermidis, 3) S. aureus. Red channel corresponds to the fluorescence of Pc excited with 635 nm laser line. Grey channel corresponds to the transmitted light.

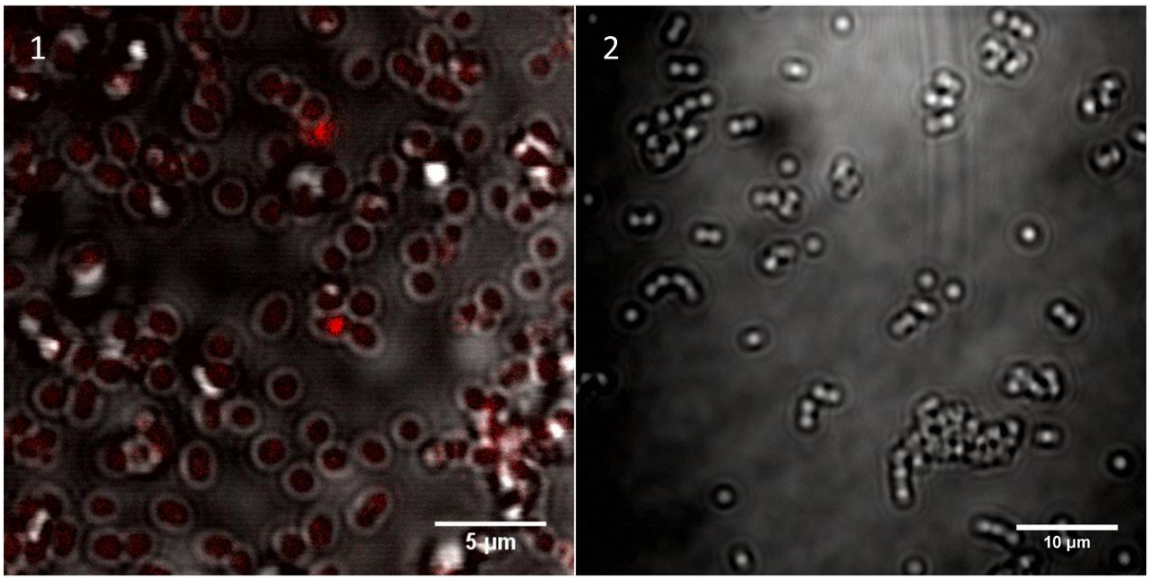


Figure 30 Confocal microscope images of E. faecalis, incubated with the solution of 1) TOMEPc and 2) TTPc. Red channel corresponds to the fluorescence of the dye excited with 635 nm laser line. Grey channel corresponds to the transmitted light.

5.3.2. Photodestruction of bacteria

This part of the research focused on how the different strains of Gram-positive bacteria will react to the treatment with the same porphycene photosensitizer under exactly the same conditions of the experiment. The selected strains included *E. faecalis, S. epidermidis* and *S. aureus.* As it was explained before, parent porphycene was selected as the photosensitizer with the best properties. All tested strains of bacteria exhibited susceptibility to co-treatment with Pc and light and all of them were completely eradicated during the time of experiment. The most sensitive species was *Staphylococcus epidermidis*, which was entirely eradicated with the dose of light equal to 6 J/cm2, i.e., after 10 minutes irradiation. Both *Enterococcus faecalis* and *Staphylococcus aureus* were illuminated for 50 minutes (30 J/cm^2) in order to decrease the number of colony-forming units to zero. It is worth mentioning that the reduction of the number of bacteria in our experiments exceeded six orders of magnitude, which is considered as the total eradication, whereas the lower limit set by The American Society of Microbiology for the compound to be accepted as antimicrobial agent is only three orders of magnitude. [16] The histograms showing the reduction of bacteria as a function of irradiation time are presented in Figure 31 for the tested bacterial strains. This figure also includes the results obtained for control samples containing bacteria and pluronic mixture without porphycene.

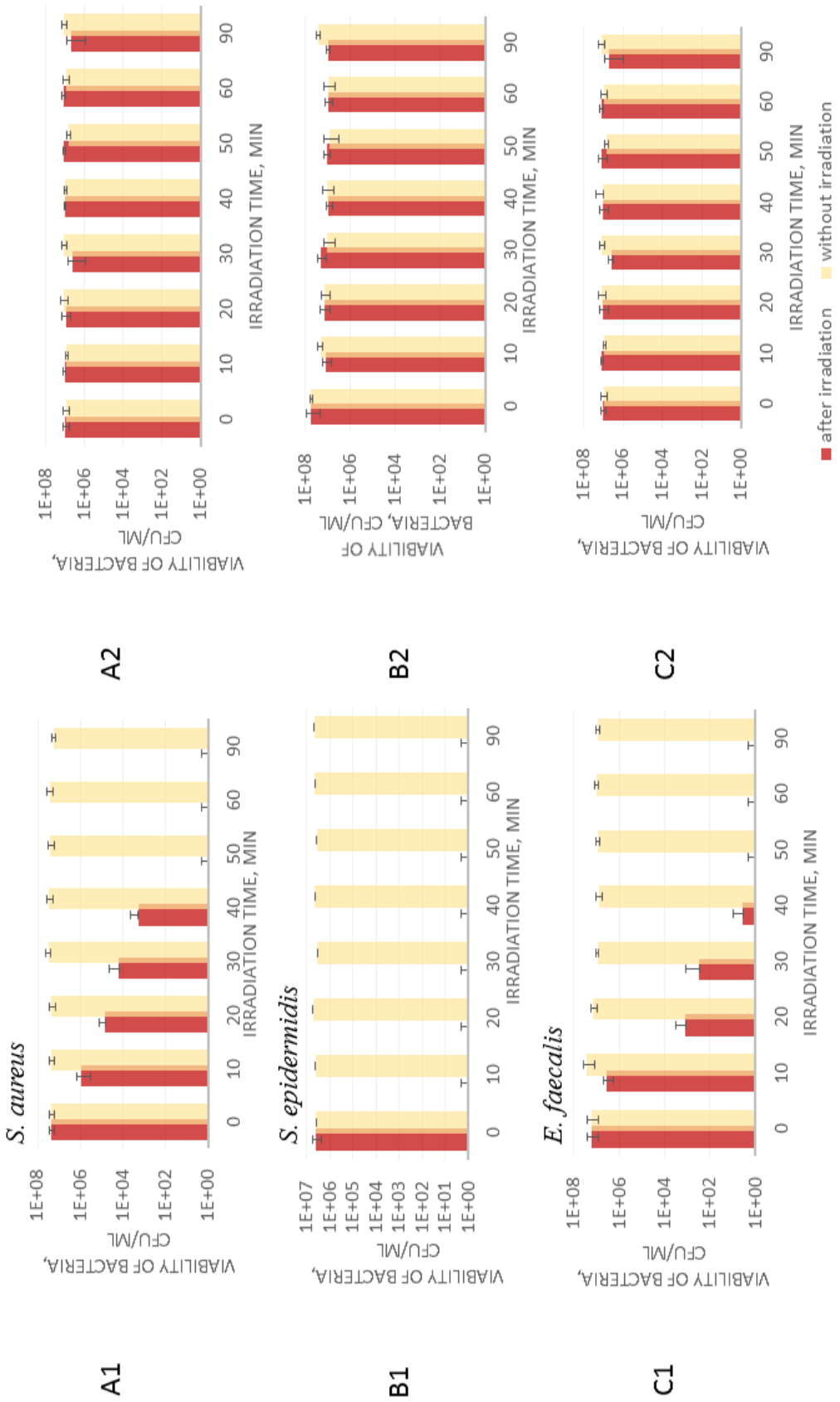


Figure 31. Dependence of the viability of bacteria on the time of irradiation of pluronic (3.5 mM) solution samples containing: A1, S. aureus and Pc (7 µM); A2, S. aureus without Pc; B1, S. epidermidis and Pc (7 µM); B2, S. epidermidis without Pc; C1, E. faecalis and Pc (7 µM); C2, E. faecalis without Pc. The results are shown as the mean value collected from 9 samples (three biologically independent replicates, three dilutions) with the error corresponding to standard deviation. Light dose: 0.6 J/(cm2 min).

It is important to highlight that the results obtained in this study can be considered as the success if compared with the previous reports concerning porphyrinoids-derived PSs. [47][116][117] The range of concentration of PSs used in the cited studies was between 10 and 25 μM and the doses of light covered the range between 10 and 180 J/cm^2. In such conditions, the decrease of the number of bacteria was about 3log. Only Ragas et al. obtained 6log destruction of bacteria with the cationic derivative of porphycene with concentration as low as 0.5 μM and, at the same time, low doses of light, such as 30 J/cm2 for *E. faecalis* and 1 μM and 15 J/cm2 for *S. aureus*. [45] Compared to these studies, the conditions we applied to initiate photodestruction of bacteria can be considered as moderately mild. Application of the constant conditions to all experiments, such as the concentration of Pc always being 7 μM, made it possible to compare the susceptibility of various bacteria strains to our PDI treatment. Using this concentration of photosensitizer enabled lowering the dose of light required for sufficient photodestruction effect to 30 J/cm^2 for both *Staphylococcus aureus* and *Enterococcus faecalis* strains. It is worth noting that irradiation with only 6 J/cm^2 was sufficient for the total destruction of *Staphylococcus epidermidis,* which makes porphycene a very efficient PS against this particular species of bacteria.

5.3.3. Structure-activity relationship for porphycenes as PDI agents

After the observation that TTPc does not cause the PDI effect on the tested microbes while other compounds do, I decided to investigate further the topic of structure-activity relationship for the studied group of compounds. A starting point was the conclusion that the presence of four *tert*-butyl groups blocked the activity of porphycene, whereas the presence of four metoxyethyl groups did not affect it. Reasonable causes for such behavior could be the difference of hydrophobicity of the compounds with different substituents, steric problems with the delivery of the compound through the bacterial cell wall, or the self-defense mechanisms of bacteria that recognize *tert*-butyl as the stranger group and do not let it inside.

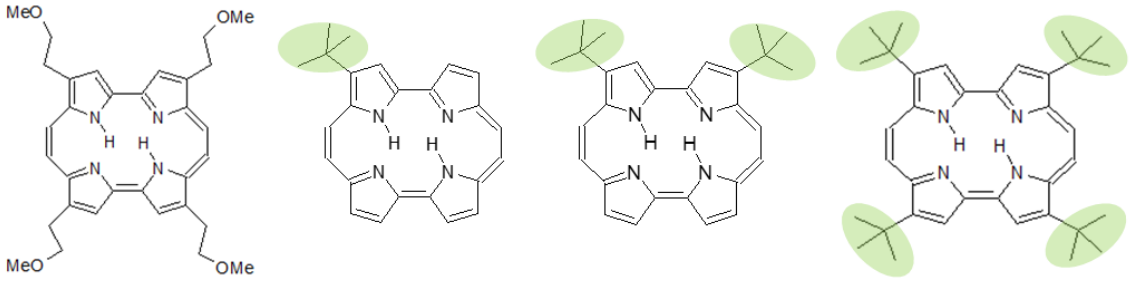


Figure 32. Porphycenes with subsequently added tert-butyl groups, studied in terms of their structure – activity relationship in PDI.

Figure 32 presents the structures of the compounds used for this experiment. The number of *tert*-butyl groups attached to the porphycene core was elevated in subsequent compounds from zero up to four. The results of the studies (shown in Figure 33) reveal that the presence of *tert*-butyl moiety does not exclude the photodynamic inactivation of bacteria. When the porphycene core is substituted with just one *tert*-butyl group, the antimicrobial effect is still high enough for lowering of the number of colony-forming units of *E. faecalis* by three orders of magnitude within two hours irradiation. Substitution of the parent compound with two or more *tert*-butyl moieties lower the antimicrobial potential so effectively that within the time of experiment the number of bacteria stays unaffected. It is not certain if the prolonged exposure to the radiation would lead to any PDI effect also for DTBPc and TTPc. From the point of view of any antimicrobial therapies it is not relevant anyway, because the time of illumination longer than two hours makes such photosensitizer practically useless. The benefit from blocked PDI and PDT properties could be the opportunity to utilize such bright mitters as porphycenes in the medical imaging of tumors, however the singlet oxygen generation has to be taken in consideration.

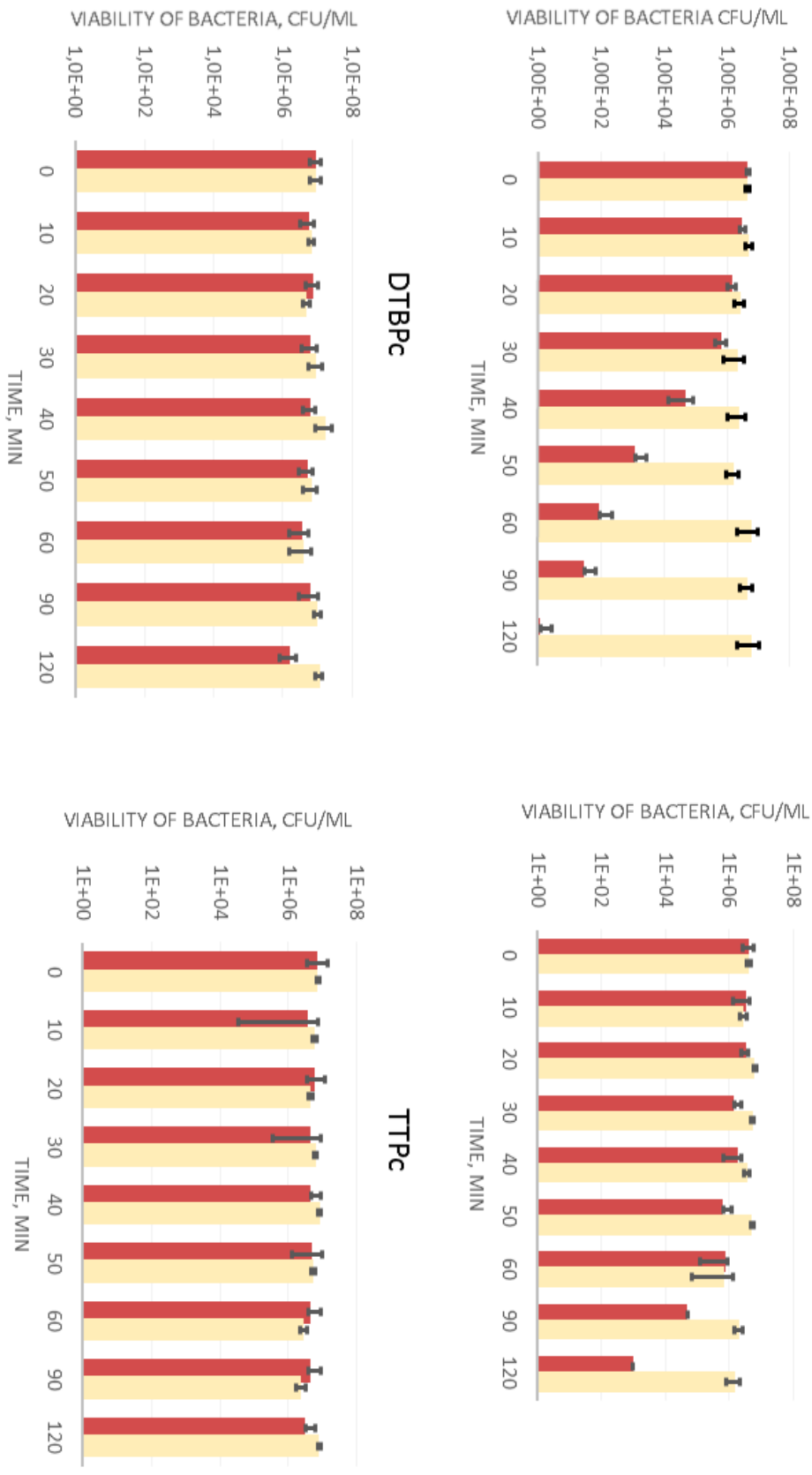


Figure 33. Dependence of *the viability of E. faecalis bacteria on the time of irradiation of pluronic solution samples containing TOMEPc, MTBPc, DTBPc, TTPc in the concentration of 7 μM. Light dose: 0.6 J/ (cm² min).*

5.3.4. Kinetics studies of singlet oxygen generation

The similar values of Φ_Δ measured for different porphycenes, together with the significant differences of their PDI activity show the big differences in the bacteria – PS interaction, depending on the structure of porphycene substituents. At the same time, it is worth noting that the biological experiments were performed under the conditions of prolonged exposure to light. Even though the Φ_Δ of the used compounds is known, the conditions within the time of experiment may be changing dramatically, influencing the effective concentration of singlet oxygen. The main quantity responsible for this impediment is the photostability of the photosensitizer, which is the measure of the number of photons that the molecule can absorb before bleaching.

The photostabilities of different photosensitizers are different and it should be taken into consideration while planning PDT and PDI experiments. In the case of the presented experiments, different photostabilities of the studied compounds could have caused the differences in the kinetics of singlet oxygen generation during the prolonged irradiation of the samples. To disprove this hypothesis, the kinetics of singlet oxygen generation was followed. The conditions of the experiment were fixed to fit as well as possible the conditions of the experiment of bacteria deactivation. The same concentrations of the photosensitizers and the same overall doses of light were used. Due to the technical issues the source of light was replaced with the laser (λ_{em} = 635 nm, P = 6 mW). The geometry of the system was also different than in bactericidal studies. The sample was irradiated in the quartz cuvette in the chamber of the spectrofluorometer, with short breaks for the spectra measurements. Singlet Oxygen Sensor Green was added to the solutions as the marker of singlet oxygen. The experiment was conducted in two variants: with stirring and without. The results are shown in Figures 34 and 35. Even a quick look at both graph leads to the conclusion that stirring is crucial for the kinetics of singlet oxygen generation in the solutions. The growth of the fluorescence of SOSG, corresponding to the amount of $^1O_2$, is linear under the stirring conditions. This observation proves that the photosensitizers do not get bleached within the experiment and therefore leads to the conclusion that the dose of light delivered to the samples does not exceed their ability to absorb, limited by their photostability. In such case the amount of the photosensitizer effectively absorbing the light is constant during the time of experiment and the rate of singlet oxygen generation is maximal, limited by the value of Φ_Δ. The opposite situation takes place when the

experiment is conducted without stirring (Figure 35). In such case the diffusion is not fast enough to provide the constant inflow of new molecules of the photosensitizer to the area of cuvette illuminated with the laser. Therefore, we note the plateau of the fluorescence signal at 529 nm after some time from starting the experiment.

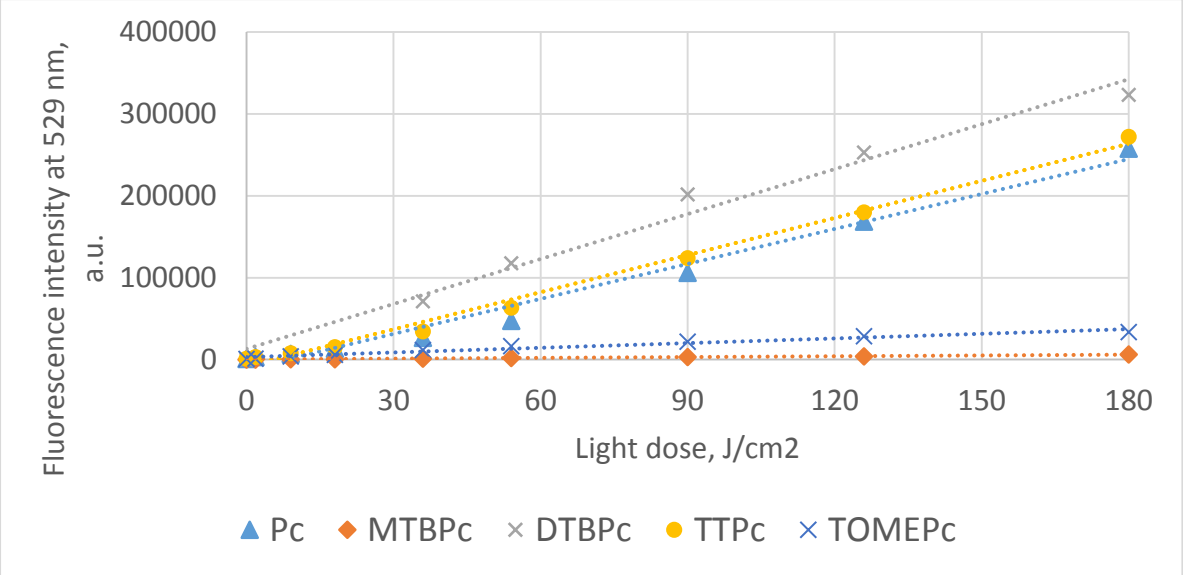


Figure 34. Chart presenting the growth of singlet oxygen amount, in the pluronic solutions of different porphycenes with increasing dose of light (λ=635 nm), probed by the fluorescence intensity of SOSG-EP (529 nm). Experiment conducted with stirring.

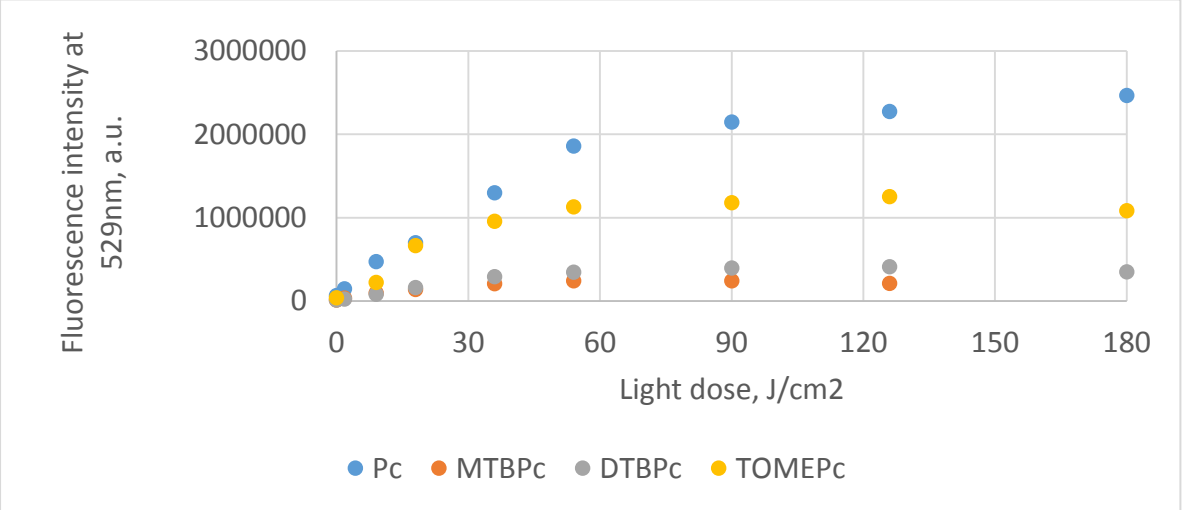


Figure 35. Chart presenting the growth of singlet oxygen amount in the pluronic solutions of different porphycenes with increasing dose of light (λ=635 nm), probed by the fluorescence intensity of SOSG-EP (529 nm). Experiment conducted without stirring.

5.3.5. Other reactive oxygen species

Application of fluorescence probes can help not only with detection of singlet oxygen, but also with the detection of other ROS. The general problem of such approach is the limited selectivity, which means that the fluorescence of the probe can be activated by more than one species. The aim of this thesis does not include following of all the ROS that are produced by the photosensitization of porphycenes. So far it was proven that the effect of photodynamic inactivation of the bacteria occurs due to generation of singlet oxygen, which is the result of type II photosensitization. With one simple experiment it is possible to check if the type I processes are also involved. [118] In this thesis such experiment was conducted with the use of hydroxyphenyl fluorescein (HPF) as the marker of hydroxyl radical. This marker is sensitive also to peroxynitrile anion. Similarly as in the case of singlet oxygen studies, the pluronic solutions of porphycenes were irradiated with the laser (λ_{em} = 635 nm, P = 6 mW) in the chamber of spectrofluorometer and stirred during the experiment. The fluorescence of HPF sensor was recorded at the time intervals during irradiation. The results are shown in Figure 36. The main conclusion is that the type I sensitization is occurring in the system together with type II processes, but the origins of such behavior would have to be further studied to say anything about the mechanisms of this phenomenon.

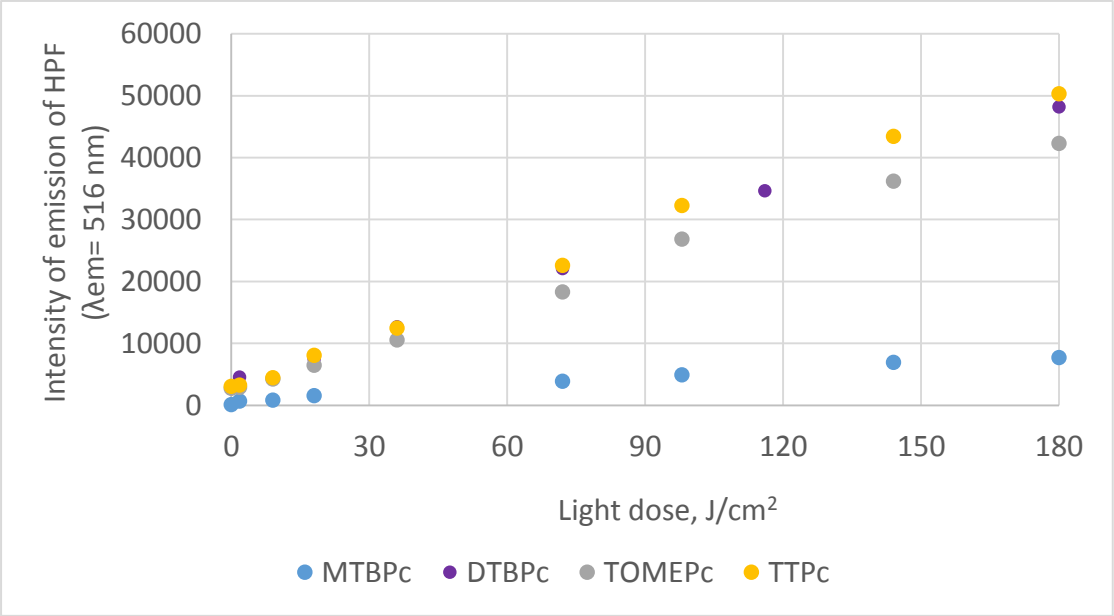


Figure 36. Chart presenting the growth of hydroxyl radical and/or peroxynitrile anion in the pluronic solutions of different porphycenes with increasing dose of light (λ=635 nm), probed by the fluorescence intensity of HPF (516 nm).

5.3.4. Summary

Photodynamic inactivation is one of the promising tools for the treatment of infections. Considering the growing resistance of the bacteria to the antibiotics, this method has a chance and a potential to become the applied way of treatment. Porphycenes whose photosensitizing abilities are studied in this thesis were applied against three different Gram-positive strains of bacteria: *E. faecalis, S. epidermidis* and *S. aureus.* The selected strains are common causes of multidrug resistant nosocomial infections, including catheter related bloodstream infections, which are very dangerous for hospitalized patients, and therefore need fast and effective treatment. The research presented in this thesis has proven that porphycenes in their micellar formulation are usually efficiently uptaken by the bacteria, however the side substituents of the porphycene core may play the crucial role in this process. When the porphycene is reaching the bacterial cells, it causes very efficient photodynamic inactivation effect under the red light irradiation. The highest activity was observed for Pc. This, altogether with the very good solubility in pluronic solution and the best overlap of the spectrum with the light source used, made this compound most suitable for the comparative bactericidal studies. In this section Pc was administered to the samples of three different bacteria strains *E. faecalis, S. Epidermidis* and *S. Aureus.* The application of constant concentration of Pc in the whole series of the experiments provided a possibility to compare the susceptibility to the treatment in various strains. 7 μM concentration of Pc in pluronic solution enabled the application of such low dose of light as 30 J/cm2 for both *Staphylococcus aureus* and *Enterococcus faecalis* strains to obtain complete eradication. Moreover, the irradiation of only 6 J/cm^2 was sufficient for the total destruction of *Staphylococcus epidermidis*. Such result makes Pc a very efficient PS against this particular species of bacteria. It is worth noting that the conditions of these experiments can be considered as moderately mild, comparing with the previous reports devoted to the porphyrinoids-derived PSs ([47][116][117]).

The structure-activity relationship studies conducted on *E. faecalis* bacteria proved that the porphycene substituents are crucial for the bactericidal potential. By introducing *tert*-butyl moieties to the molecule it is possible to completely block its antimicrobial activity. The lowering of PDI potential, however, is not a result of the photophysical

differences of the molecules. All the studied compounds were characterized by very similar quantum yields of singlet oxygen generation. The difference is the ability of the compound to get inside the bacteria cells, which is probably restricted for the compounds with *tert*-butyl groups due to the steric reasons.

5.4. Photodynamic therapy on HeLa cells

5.4.1. Initial studies of photodynamic activity of porphycenes against HeLa cells

Initial experiments conducted on the living HeLa cells were very simple and their objective was to confirm or disprove the potential of porphycenes in PDT. For this purpose the cells cultured in μ-dishes and incubated with Pc in both micellar and liposomal formulation were irradiated for 30 min with the diode array. The control sample without Pc was irradiated according to the same procedure.

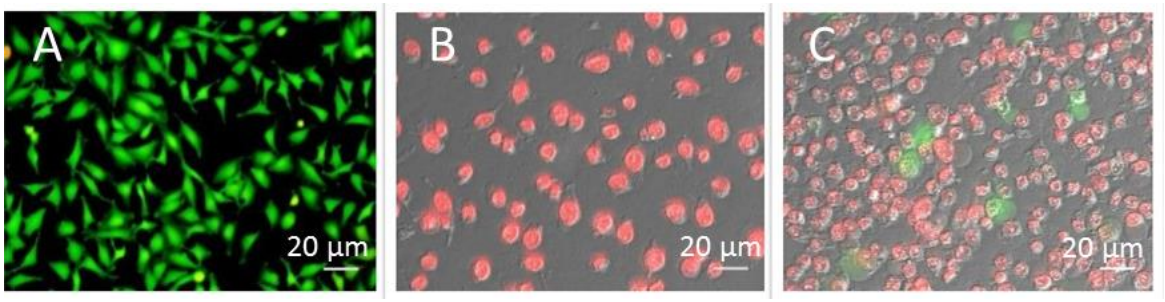


Figure 37. A – control HeLa cells, B – HeLa cells with Pluronic F-127 micellar solution of Pc (0.7 μM), C – HeLa cells with L-α-phosphatidylcholine liposomal solution of Pc (0.7 μM). Samples were illuminated for 30 minutes with the diode array (120 mW, 624 nm). Green color indicates living cells, red color indicates dead ones.

Figure 37 shows that the cells treated with Pc and irradiated, were partially (Figure 37-C) or completely killed (Figure 37-B). At the same time the control sample irradiated without photosensitizer remained unaffected, with all the cells remaining alive. This short experiment proved the ability of Pc to induce photosensitized cell death. The following trials disproved also the dark cytotoxicity of the compound. Similar results were obtained for TOMEPc and TMPc, but not for TTPc. Photodynamic effect was more pronounced in the case of the micellar formulation of administered photosensitizer, however it was observable also in the samples treated with liposomal solution.

The observed effect of photodynamic therapy occurs very rapidly and is visible even under simple transmission microscope, directly after irradiation, in the form of morphological changes in the HeLa cells (Figure 38). Adding propidium iodide indicator of dead cells to the sample straight after irradiation, reveals that the process of the cell death induced by the light is spreading over the sample in the range of minutes. That process can be therefore observed in real time. The images presented in Figure 38 are the frames of a short movie documenting the cell death after irradiation in the presence of TOMEPc. 20 minutes after switching off the light source there were no more living cells in the field of view.

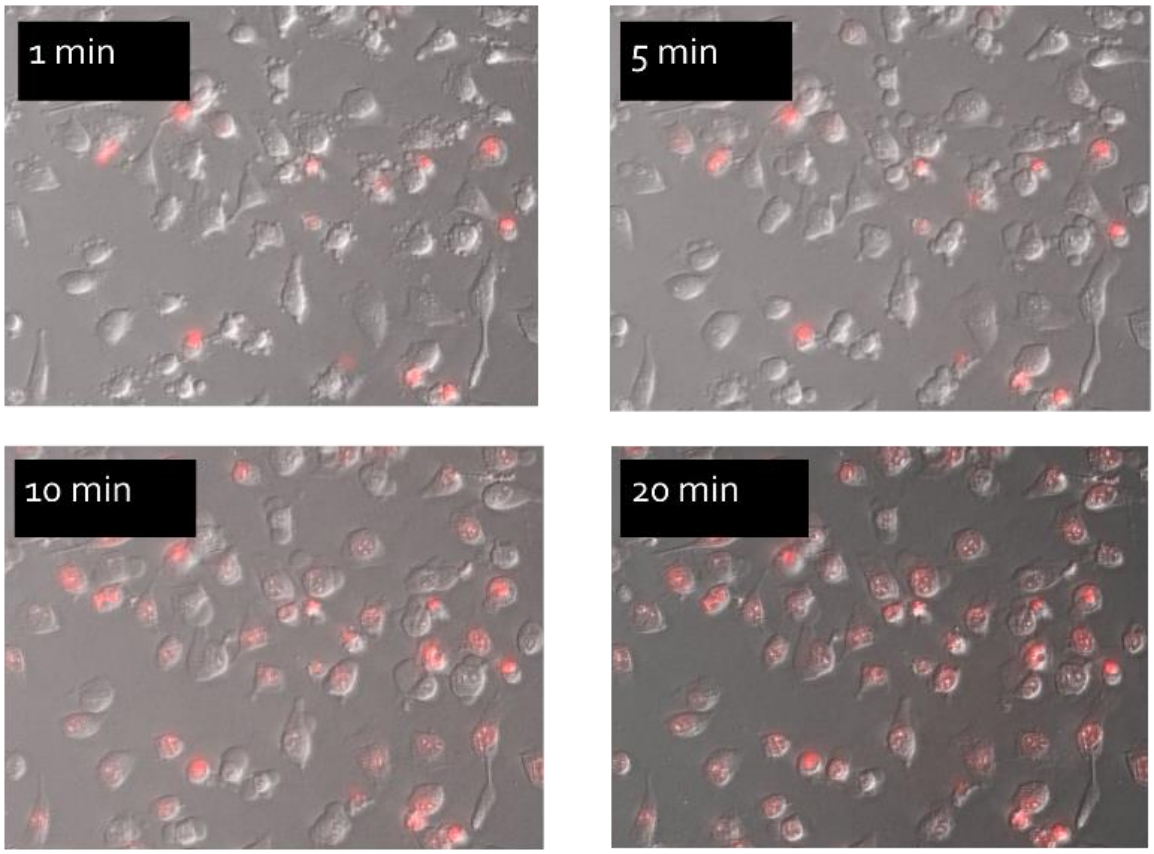


Figure 38. HeLa cells 1- 20 minutes after irradiation in the presence of TOMEPc with the light dose 18 J/cm^2. Dead cells are marked red with propidium iodide.

The common practice includes leaving cells overnight before viability tests, however in our case, since the effect was so rapid, it was much easier to observe the cell death just after irradiation, in the real time when it occurred. Storing of the irradiated samples for the longer time results in the detachment of the cells from the bottom of the dish, which makes microscopy studies impossible.

5.4.2. Localization studies

Since three out of four studied compounds exhibited photodynamic therapy effect and one did not, it was the interesting to find out what is the reason of such behavior. For this purpose, localization studies of all four compounds within HeLa cells were conducted. The obtained microscope images are shown in Figure 39.

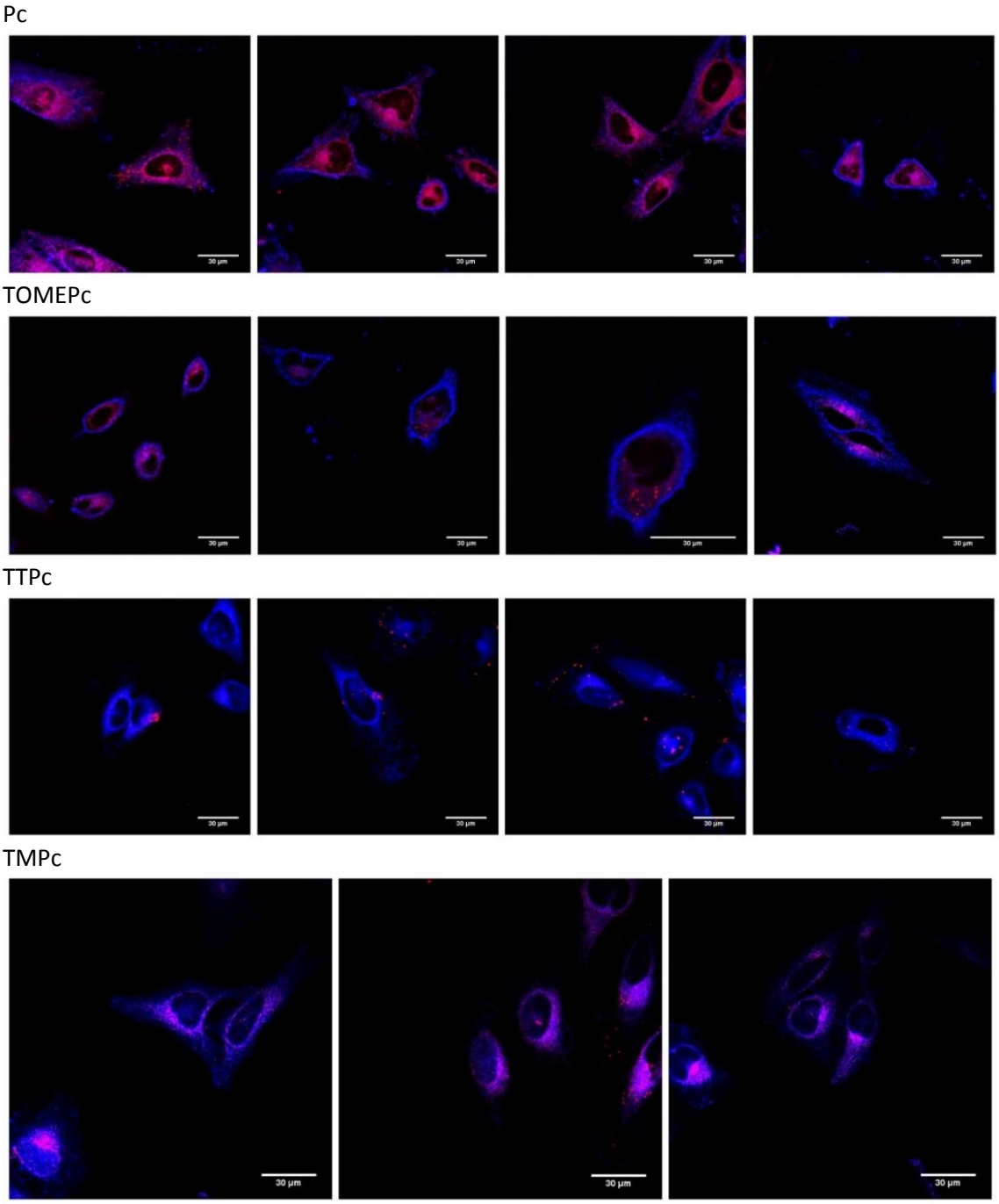


Figure 39. Confocal microscope images of HeLa cells incubated with different porphycenes (red channel) and membrane marker F2N12SM (blue channel).

The red channel corresponds to the porphycene dye and the blue one corresponds to the membrane marker F2N12SM. [119] Confocal microscope images prove that three of the compounds – Pc, TOMEPc, TMPc – follow the same pattern of dye distribution within the cell. The dyes are divided between cytoplasm and the membrane. Bright dots indicate most probably the compound embedded into the lysosomes, which is also confirmed by the fluorescence lifetimes measurements. The lysosomes are composed mostly of fats and the environment in that area is much less polar than that of the surrounding cytoplasm. In such conditions the aggregation of the hydrophobic porphycenes is prevented and their lifetimes can be much longer and therefore the brighter spots are observed (The topic of aggregation of porphycenes inside the cells will be discussed further in the next subsection).

TTPc does not effectively penetrate inside the cells. The brighter spots are visible in the microscope images of that compound, however there are no signs of membrane or cytoplasm staining. It leads us to the conclusion that the compound is recognized by the cells as the extrinsic substance, occluded in lysosomes and excreted in this way. This hypothesis is confirmed by the studies including irradiation of such system (under the standard conditions used for other compounds). No visible phototherapeutic effect is observed.

5.4.3. Fluorescence lifetime imaging

Fluorescence lifetime imaging microscopy (FLIM) was conducted for HeLa cells incubated with porphycenes before and after irradiation. The solutions of the compounds in both micellar and liposomal formulations were tested to check the lifetimes of fluorescence before the administration to the cells. The aim of such measurements was to register what happens with the dye during the PDT induced cell death. Does it stay embedded in the remaining cellular structures or maybe is it released to the cytoplasm? The changes in the microenvironment of the dye should be reflected in the decrease or increase of fluorescence decay time. The intriguing phenomenon is also the change of the viscosity of the cells during PDT induced cell death. Such topic has been raised and studied in last years. [120][121] Since it seemed that some of alkyl meso-substituted porphycenes were the perfect tool for this category of research, one of the aims of this section was to register and interpret the viscosity changes during the PDT experiments. TMPc was employed to these studies to monitor the differences in the viscosity of the cell during the cellular death process. TMPc, similar to TPPc (mentioned in Chapter 5.2.) falls into the category

of compounds whose fluorescence decay time is strongly dependent on the viscosity of its microenvironment.

The examples of the maps of fluorescence lifetimes obtained during these experiments are shown in Figures 40 and 41. Looking at all the maps with bare eye it is very difficult to judge if any important differences between them really occur. For this purpose, the statistics on 20 random points in every cell was done using the SPCImage program. The lifetimes obtained in this way are presented in Table 5. For the comparison, Table 6 shows the lifetimes of the compounds in the solutions of pluronic and liposomes before their administration to the cells.

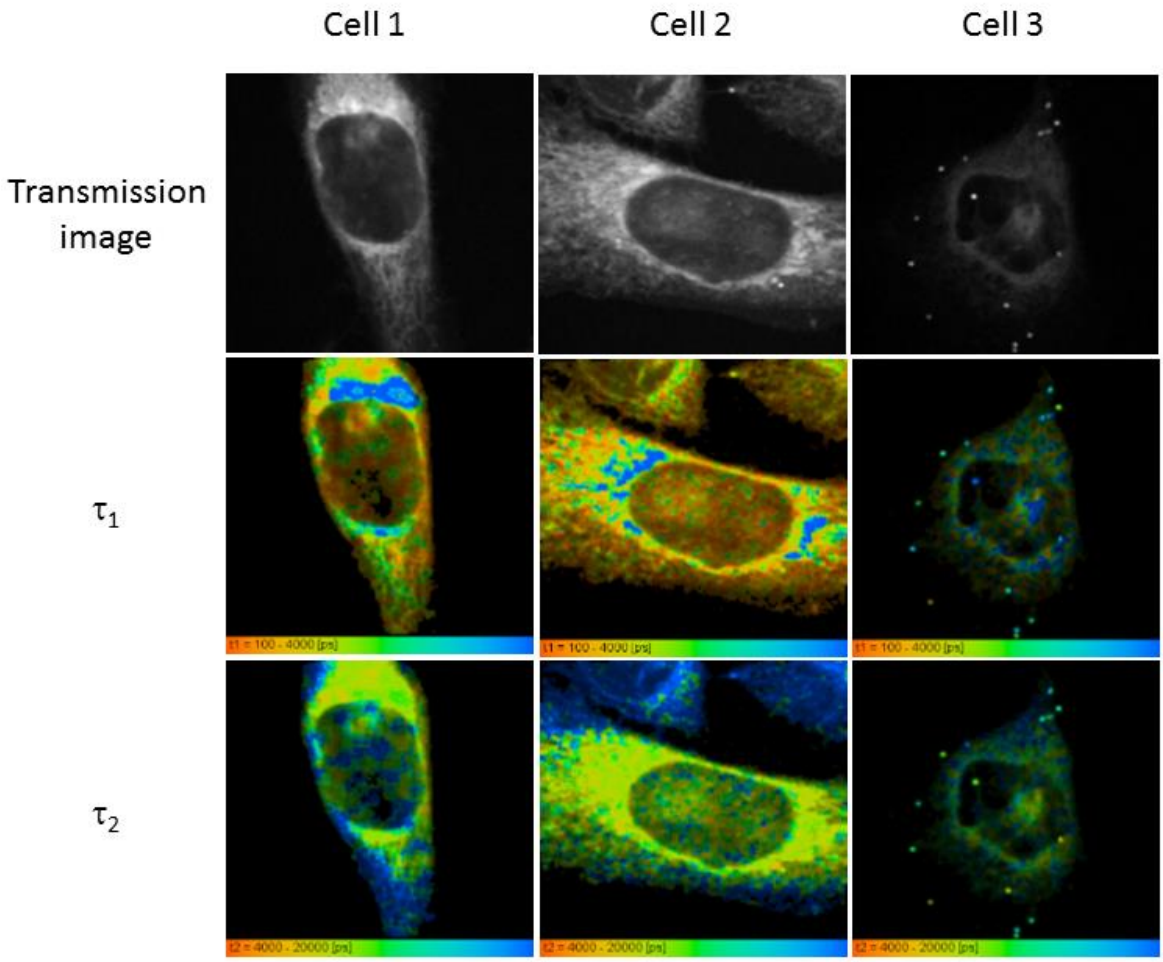


Figure 40. Map of TMPc (administered in liposomes) fluorescence lifetime distribution before irradiation. τ_1, τ_2 are the fluorescence lifetimes.

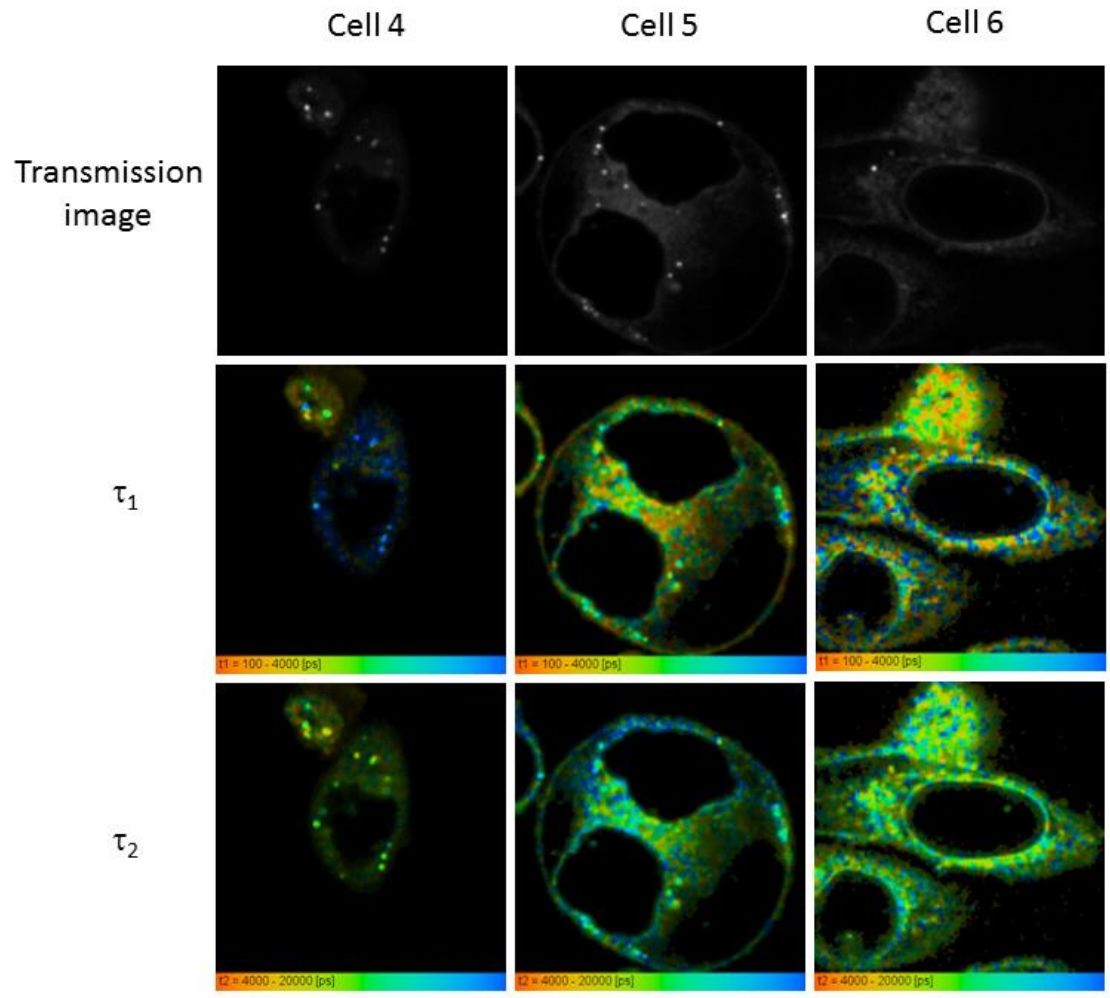


Figure 41. Map of TMPc (administered in liposomes) fluorescence lifetime distribution after irradiation. τ_1, τ_2 are the fluorescence lifetimes.

Table 5. The lifetimes of fluorescence of the porphycenes administered to the cells.

compound	pluronic				liposomes			
	before irradiation		after irradiation		before irradiation		after irradiation	
	τ_1 [ns]	τ2 [ns]	τ_1 [ns]	τ_2 [ns]	τ_1 [ns]	τ2 [ns]	τ_1 [ns]	τ2 [ns]
Pc	10 ± 1	-	9 ± 1	-	10 ± 1	-	9 ± 1	-
TOMEPc	9 ± 1	-	1 ± 0.7	10 ± 2	9 ± 1	-	9 ± 1	-
TMPc	1 ± 0.7	9 ± 2	1.5 ± 1	9 ± 2	2 ± 1	11 ± 3	3 ± 2	9 ± 3

Table 6. The lifetimes of fluorescence of the porphycenes in the micelles and liposomes solutions before the administration to the cells.

compound	medium					
	pluronic F-127			PC liposomes		
	τ_1	τ_2	A_1 %	τ_1	τ_2	A_1 %
Pc	10.67	-	100	10.64	-	100
TOMEPc	1.15	9.82	24	9.28	-	100
TMPc	0.8	2.61	74	1.26	9.45	48
TTPc	8.27	-	100	-	-	-

The obtained data are hard to interpret, but there are several main conclusions that can be drawn on their basis. First of all, it is worth to focus only on Table 5 to see that there are no significant differences between the fluorescence lifetimes of the studied compounds before and after PDT in the cells. The only exception from this observation that requires a comment with regards to TOMEPc administered to the cells in pluronic formulation. After irradiation, we can observe appearance of a short-lived component of around 1 ns, besides already existing 9 ns long-lived fluorescence. Having a look at Table 6, there comes the conclusion that such component exists also in the raw solution of TOMEPc, before introducing it to cellular environment, and does not exist in the liposomal formulation. The reasonable explanation of this fact is a minor fraction of aggregated compound occurring under the conditions of this study. The possibility of aggregation was commented previously (Chapter 5.2). Following such hypothesis it is interesting that the compound that had shown the signs of aggregation in the micellar solution, after administering this solution to the cells was not aggregated. That suggest that porphycenes in micellar formulation are uptaken by the cell by immersion, which means that they leave the micelle upon the contact with the cellular membrane and inside the cell they find even more comfortable conditions. The other argument that seems to confirm this hypothesis is the behavior of TMPc in pluronic before (Table 6) and after (Table 5) administration to the cells. Even though the lifetimes of fluorescence of this compound in the pluronic solution are 0.8 and 2.6 ns, after a transfer to the cellular environment they change to around 1 and 9 ns, which is the same as for the same compound in liposomal formulation before and after administration to the cells.

5.4.3. Uptake of porphycenes by HeLa cells

In order to evaluate usefulness of porphycenes for PDT and also for better understanding of the processes occurring in the studied systems within the cells, a series of experiments was conducted with the aim to reveal the kinetics of uptake of porphycene dyes to HeLa cells. Such studies should reveal not only the optimal time of incubation of the cells with the chromophore before irradiation, but also answer the fundamental question about the nature of the cellular uptake of porphycenes embedded inside the micelles. What was expected to be observed in these experiments was the initial growth of the concentration of the dye inside the cells followed by reaching the equilibrium with the solution manifested by approaching the plateau of internal dye concentration versus time.

Initially, the task seemed very simple. The first approach was based on using the fluorescence microscopy. The cells were kept under the microscope in the live-cell imaging chamber. The photosensitizer was added to the sample and the images of the cells were taken at time intervals within 8 hours from administration. The assumption was that the increasing intensity of fluorescence per area of the cell is a measure of growing concentration of the dye inside the cell. The results shown in Figures 43 – 45 correspond to the values of integrated fluorescence (λ_{em}= 633 nm) per the area of the cell. The data was processed using ImageJ program. Figure 42 presents the example of images used for the extraction of fluorescence intensity data.

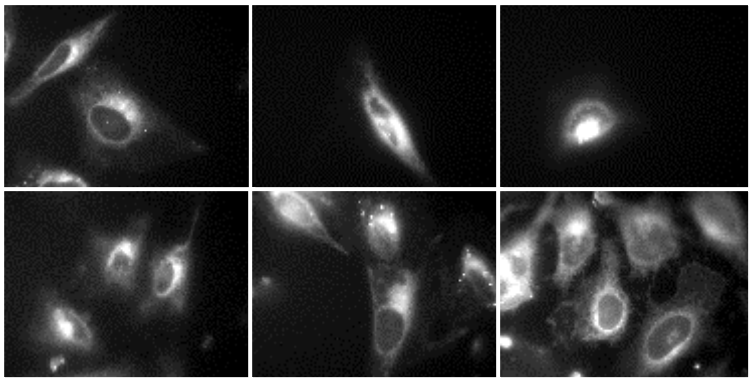


Figure 42. Examples of the HeLa cells images used for the fluorescence intensity integration.

Figures 43 - 45 show the obtained relationships of the fluorescence from the cell interior and the time of contact between the cells and the solution with chromophores.

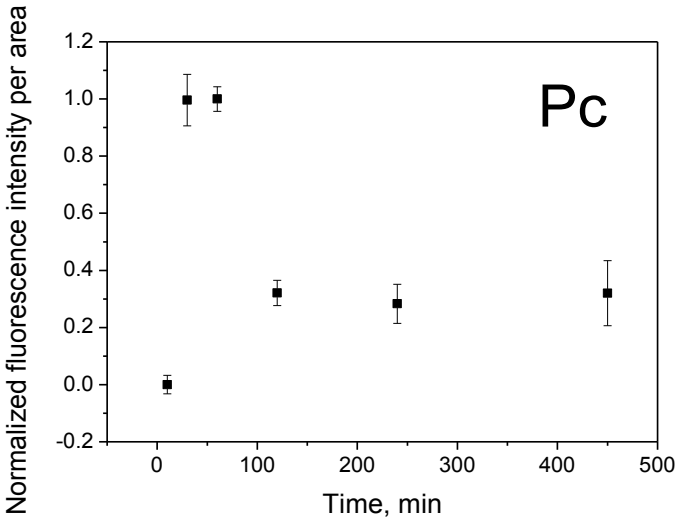


Figure 43. Dependence of fluorescence intensity on the time of contact between the cells and the micellar solution of Pc.

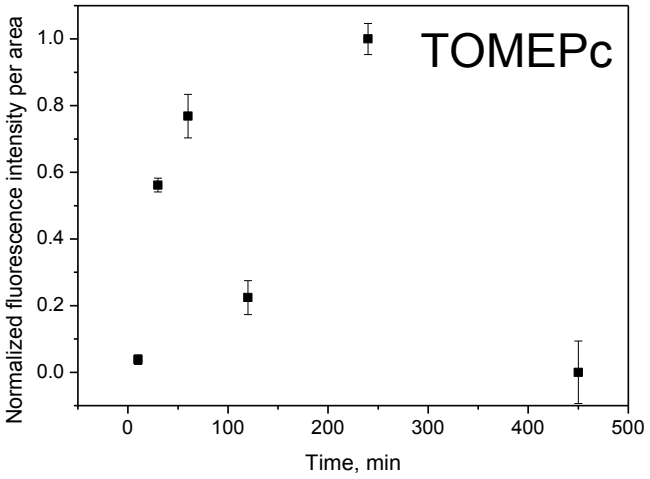


Figure 44. Dependence of fluorescence intensity on the time of contact between the cells and the micellar solution of TOMEPc.

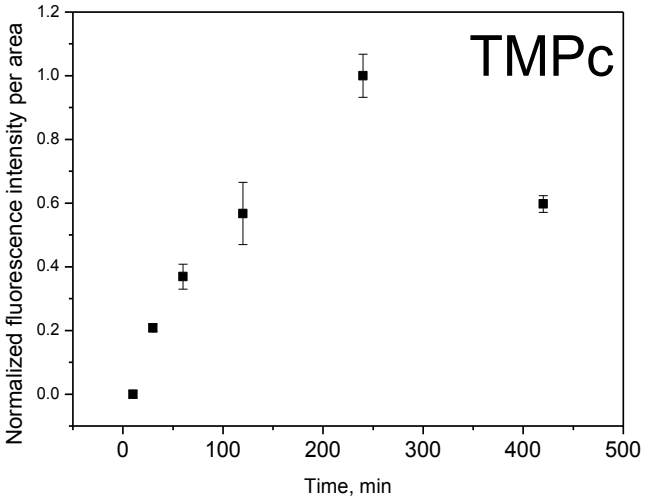


Figure 45. Dependence of fluorescence intensity on the time of contact between the cells and the micellar solution of TMPc.

The obtained traces definitely did not reveal the expected uptake kinetics. Even though it is possible to observe the plateau of concentration for Pc that is established after 100 min of incubation, two other compounds do not show any defined trend in this matter. The same experiment was conducted three times with the attention put to the short times of single cells illumination. The observed area was always changed after recording the image to reduce the effect of chromophore bleaching and the aberration resulting from PDT occurring during the experiment. Each point in Figures 43 – 45 was averaged from several different images of different cells. Apparently, it was impossible to avoid the mentioned problems and therefore the interpretation of the obtained data did not have chance to give any satisfying conclusion.

In the second approach it was easier to eliminate the cells that showed the morphological signs of cell death. However the bleaching effect was accumulated. The field of view of the microscope was kept constant for all the time of experiment. Four cells in the point of view - named 1, 2, 3, and 4 - were followed (Figure 46). Ten images were recorded in the time span of 100 minutes.

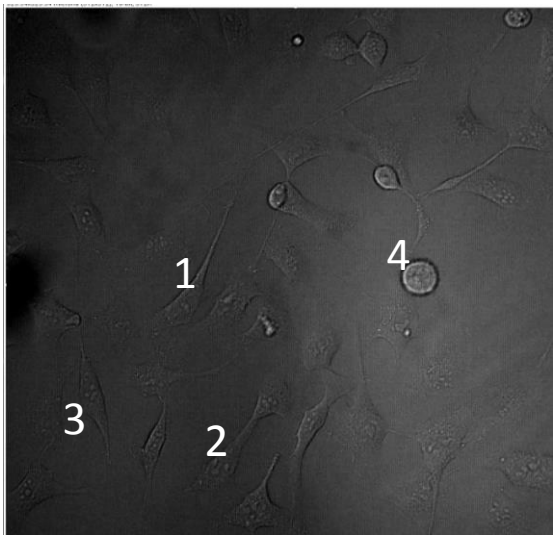


Figure 46. Transmission image of the cells 1,2,3, 4, before administration of Pc.

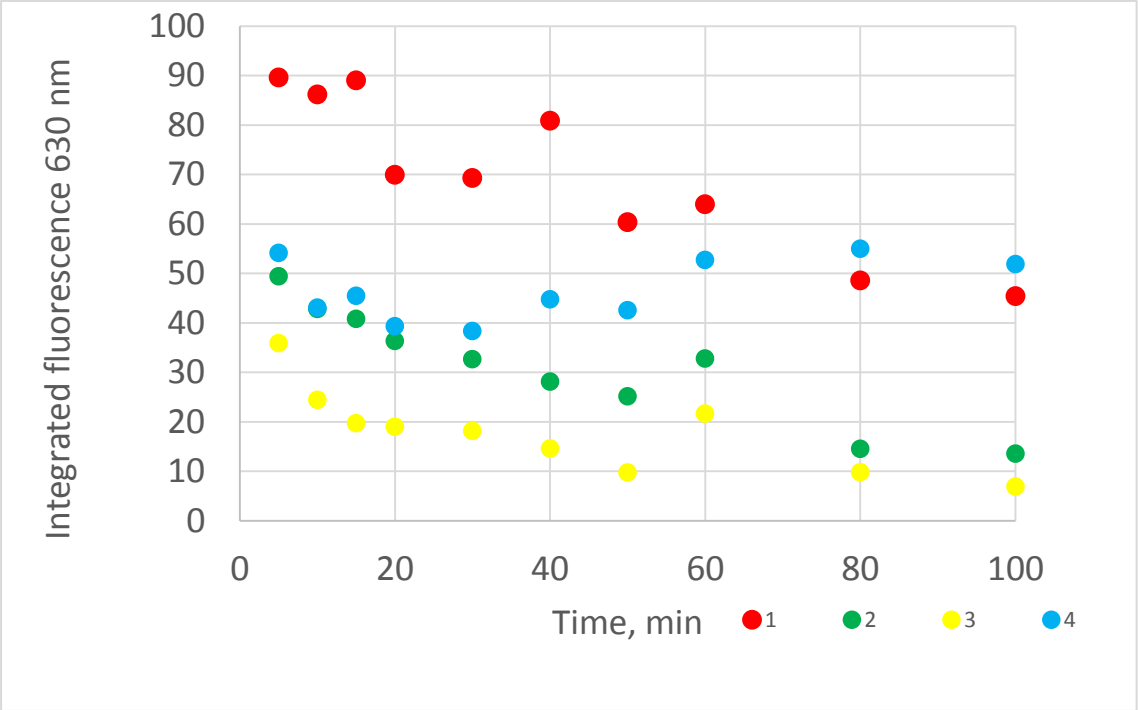


Figure 47. Evolution of integrated fluorescence (λ_{exc} = 630 nm) from single cells after the administration of Pc.

Same as previously, the integrated intensity of fluorescence from the area of the cell was calculated. This integrated fluorescence plotted against the time after administration of Pc is shown in Figure 47. The analysis of this plot provides the conclusion that prolonged observation of the same cell under the microscope results in bleaching. The emission from the cells 1, 2, 3, and 4 was generally getting lower in intensity in the course of the

experiment. The highest emission intensity was registered immediately after the administration of Pc. The power of the laser during the measurements was limited to minimum and within 100 minutes only 10 scans were conducted, which means that during the whole experiment the cells were exposed to light for no longer than 30 s. Even under such conditions, though, it was impossible to register accumulation of the dye inside the cells due to bleaching.

After the failure of fluorescence microscopy studies, a third approach was introduced. The kinetics of porphycene penetration inside HeLa cells was followed in the complex experiment by means of such a simple technique as UV-VIS absorption spectroscopy. The general idea is presented in Figure 48. Instead of following directly the changes of the dye fluorescence from the cells, the absorbance changes of the chromophore in the micellar solution placed over the cells were recorded. The difference in the measured absorbance over time was assigned as resulting from the incorporation of the dye into the cells. It is important to mention that, due to technical reasons, this experiment was conducted at room temperature.

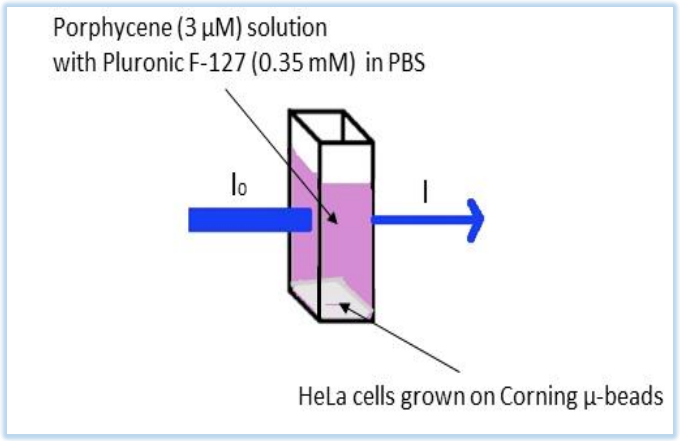


Figure 48. The scheme presenting the experiment investigating Pc penetration to HeLa cells with the use of Corning micro-beads.

The main challenge of such approach was to transfer the cell culture to the cuvette for absorption measurements. In the first trial, culturing the adherent HeLa cells on the bottom of the polystyrene cuvette did not give enough amount of the cells for following the uptake of the dye. In the second trial, polystyrene microbeads for cell cultures were used. Figure 49 presents Corning microcarriers and HeLa cells on their surface. It is visible that the cells attach efficiently to the microcarriers (Fig. 49-C). Calcein staining (Fig 49-C)

proves that contact with polystyrene surface does not affect the viability of the cells. Figure 49-D shows that only the HeLa cells absorb porphycene, whereas the microbeads remain not fluorescent.

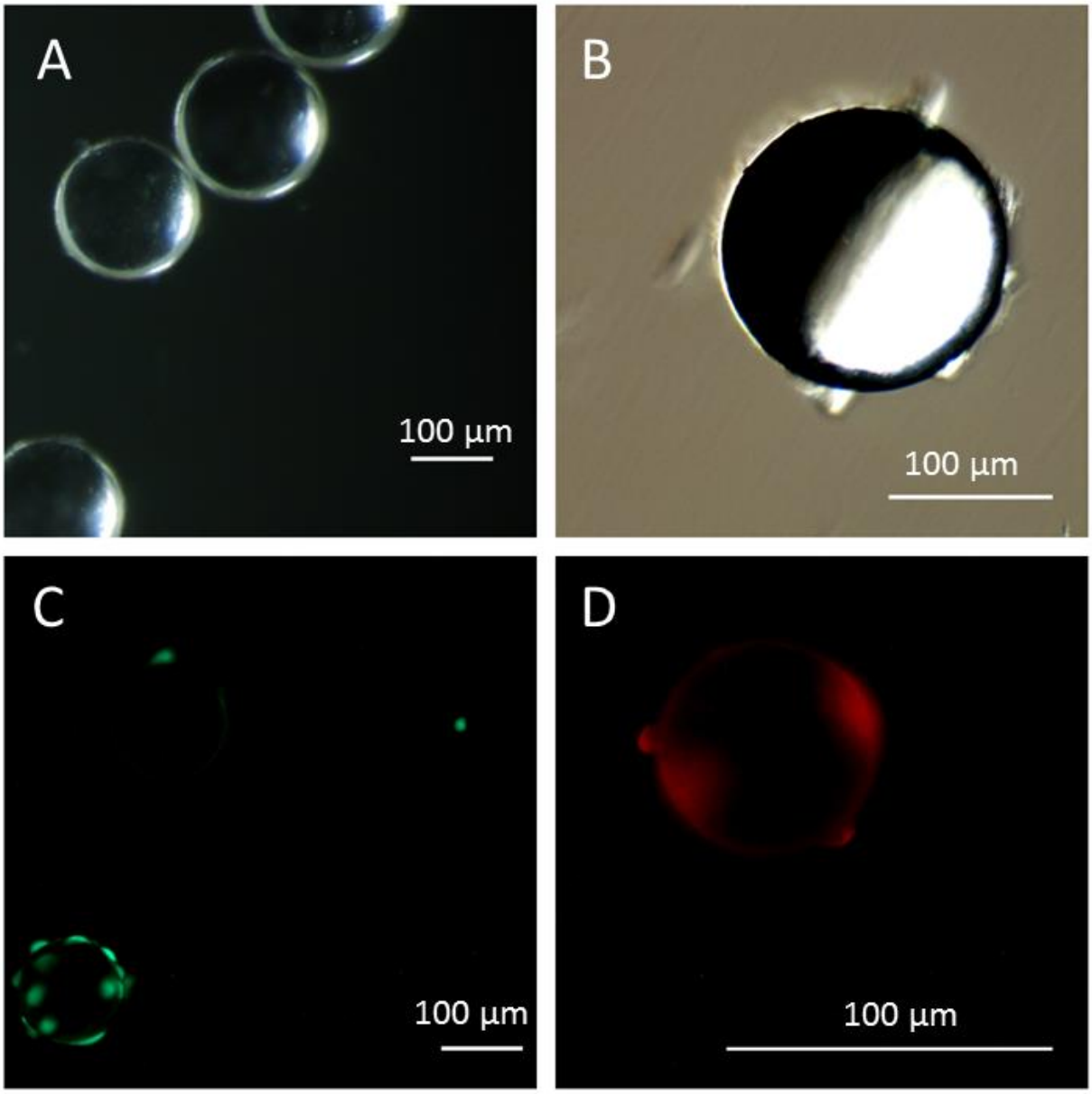


Figure 49. Corning microcarriers with HeLa cells adhered to their surface: A, B – transmission image, C – living HeLa cells stained with calceine (green channel corresponds to the fluorescence of calceine), D – HeLa cells stained with Pc (red channel corresponds to the fluorescence of Pc).

As it is visible in Figure 50, the absorbance of Pc in the micellar solution was indeed decreasing after administration of the solution to the cells.

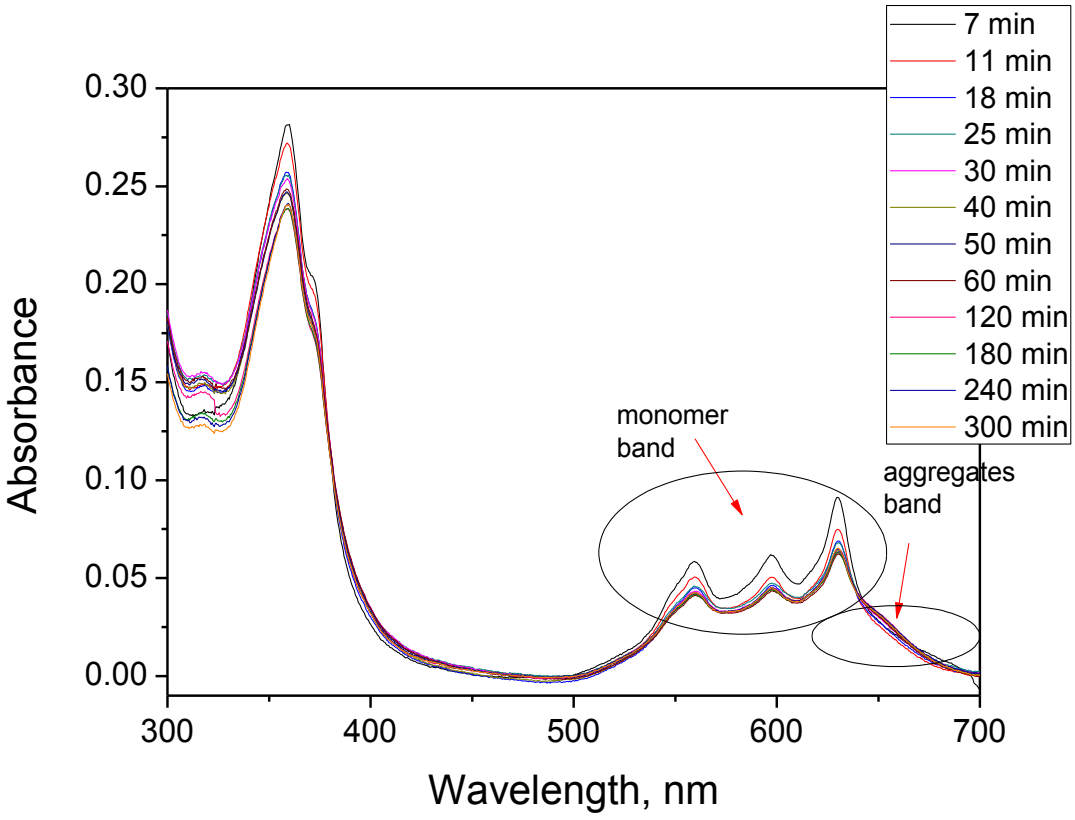


Figure 50. Absorption spectra of micellar Pc solution (initial concentration of 2μM) after its administration to the cells.

The amount of the cells in the cuvette estimated on the base of AlamarBlue® cell counting approach was around $5 \cdot 10^4$, which provides the ratio of about $7 \cdot 10^{12}$ molecules of Pc absorbed by one HeLa cell. Figure 51 shows also the kinetics of Pc uptake by HeLa cells, which reveals that the accumulation of the dye in the cells occurs mostly in the first hour after administration and then the equilibrium between the cells and the solution is established.

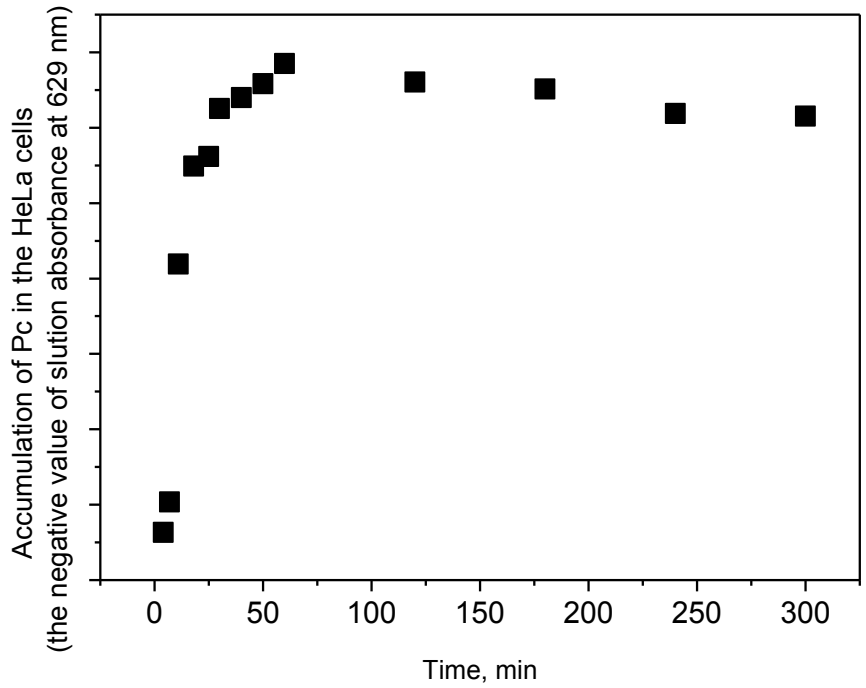


Figure 51. Scheme of the kinetics of uptake of Pc into HeLa cells taken as the negative value of absorbance decrease of the solution over the cells.

The quantitative data obtained on the basis of the experiment described above look reliable, but it is important to mention the obstacles that can occur in the used approach. It is visible in Figure 52 that a minor fraction of the chromophore undergoes aggregation within the time of experiment. It is impossible to control this kind of process and the best option is to keep the concentration of photosensitizer very low, whereas for the PDT the concentration of photosensitizer should be rather kept high. The aggregation precludes the reproduction of the experiment with the same results, since the ratio between monomers and aggregates bands differs each time and it cannot be predicted before the experiment. The proof of such behavior is provided by Figures 52 and 53, showing the results of the same experiment conducted the second time. In the case of these data quantitative estimation of the ratio of absorbed molecules is impossible, because the fraction of aggregates is high, and it is not possible to judge what is the origin of this process.

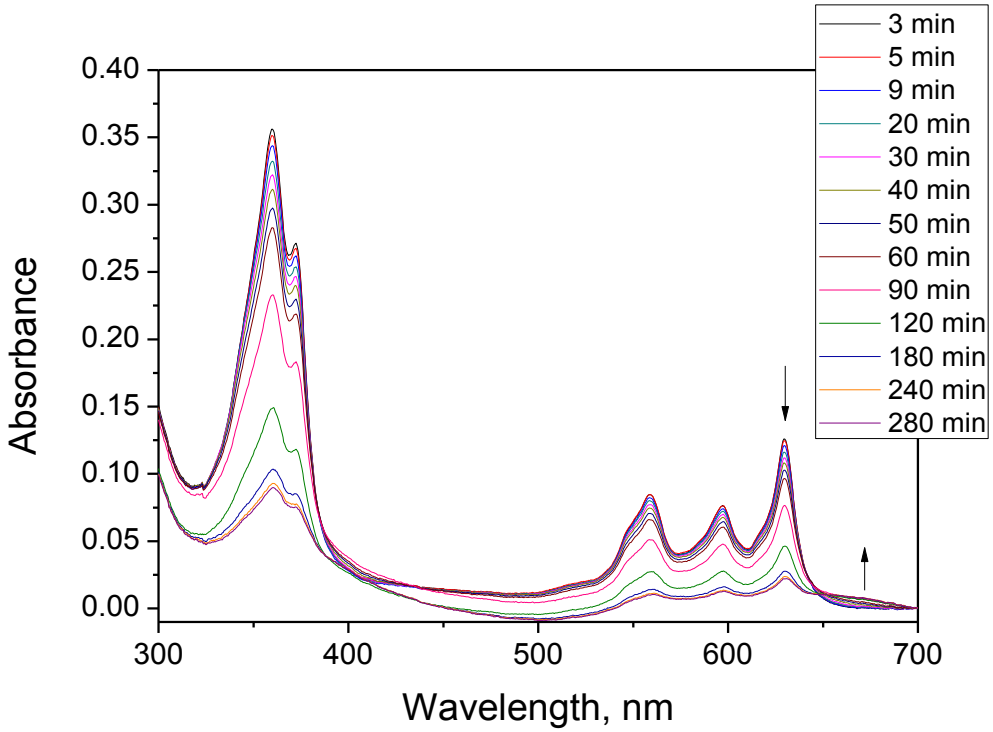


Figure 52. Absorption spectra of micellar Pc solution (initial concentration of 2.5 μM) after its administration to the cells.

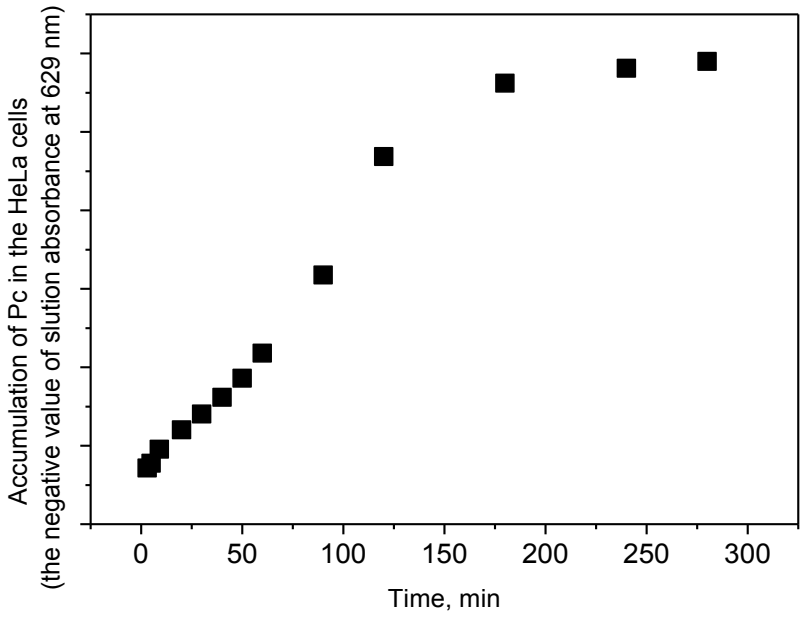


Figure 53. Scheme of the kinetics of uptake of Pc into HeLa cells taken as the negative value of absorbance decrease of the solution over the cells.

5.4.4. Mechanism of cell death

Following the success of the previous parts of studies on porphycenes as PDT agents under *in vitro* conditions on HeLa cells, further studies were carried out to prepare the ground for application of these compounds in the more complex biological studies and possible future drug development. The aim of this part of research was to determine the mechanism of cancer cells death, induced by PDT with use of porphycene PSs. It was planned to assess the relationship between such factors as dose of light, concentration of the agent, and time elapsed after irradiation and the mechanism of cell death induced by PDT. Such parameters could eventually help to estimate the conditions that favor the apoptosis in our systems, which is of great importance for developing a new drug and in the application of the substance on animals and patients.

The most typical method for distinguishing between apoptosis and necrosis is co-staining with propidium iodide and annexin V–chromophore conjugate. This method was also used in the case of described studies. Annexin V–FITC conjugate was used as the indicator of apoptosis and propidium iodide as the indicator of dead cells. Figure 53 shows the microscope image of the cells obtained after 20 minutes irradiation (12 J/cm^2) of the cells with Pc and immediately after this using the procedure of co-staining with Annexin V–FITC and PI.

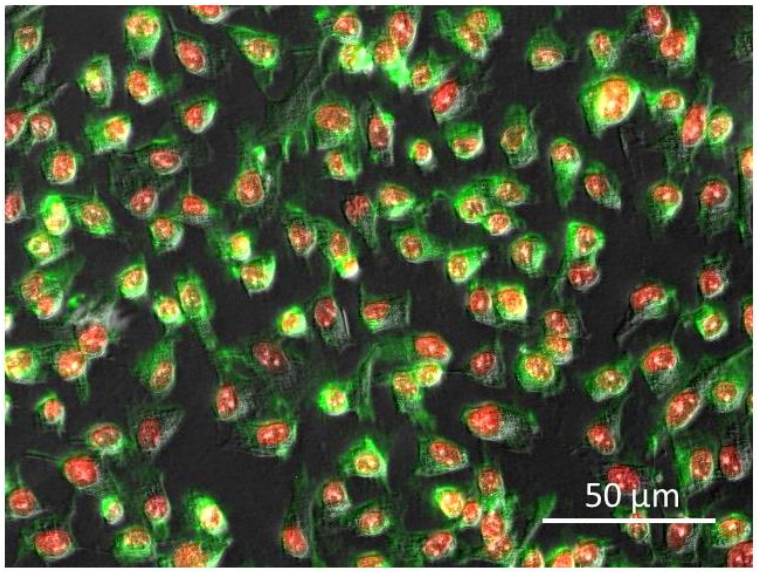


Figure 54. Image of the HeLa cells with Pc (0.4 µM) irradiated for 20 minutes. Green channel corresponds to Annexin V–FITC and red channel to propidium iodide fluorescence.

Positive result of staining with both dyes is surprising and does not bring the answer about the mechanism of cell death. Figure 55 shows the scheme that should be used in the interpretation of the microscope images in such experiment. In the case of apoptosis, the expected result is the appearance of green color, and in the case of necrosis, the red one. Staining the cells with both dyes can indicate the late stage of apoptosis, but it can as well indicate necrosis.

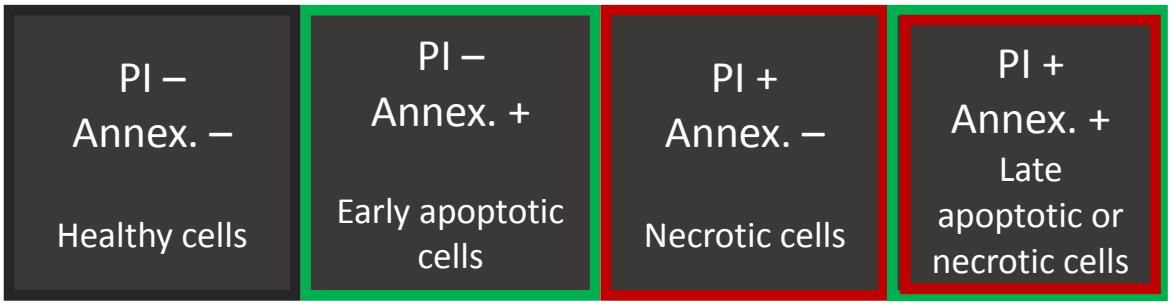


Figure 55. Scheme depicting the interpretation of PI/annexin differential staining results. (+) stands for the registered fluorescence of a dye, (-) stands for the lack of fluorescence.

In the case of apoptosis, the result can be verified by the observation of early increase of annexin positive cells before the PI-positive cells start showing up. Several experiments were conducted to catch such an image, but they failed. Lowering of Pc concentration to less than 0.4 μM or the dose of light to 6 J/cm^2 resulted in the lack of signs of cell death.

The co-staining did not determine the mechanism of cell death; therefore another technique was employed. It was based on the fact that the apoptotic nucleus should undergo fragmentation without disintegration of the cellular membrane, whereas the necrotic cells should fall into pieces without fragmentation of the nucleus. In this experiment the nucleus was stained with Hoechst 33342 and Pc was administered to the cells. After irradiation with 12 J/cm^2 dose of light and following 60 min incubation the cells were observed under the microscope. Both apoptotic and necrotic cells were recorded. The example of apoptotic cell is shown in Figure 56. Here there is no doubt about cell death mechanism. The fragmentation of nucleus occurs first and later fragmented parts are "packed" in the vesicles of cellular membrane and detached from the cell.

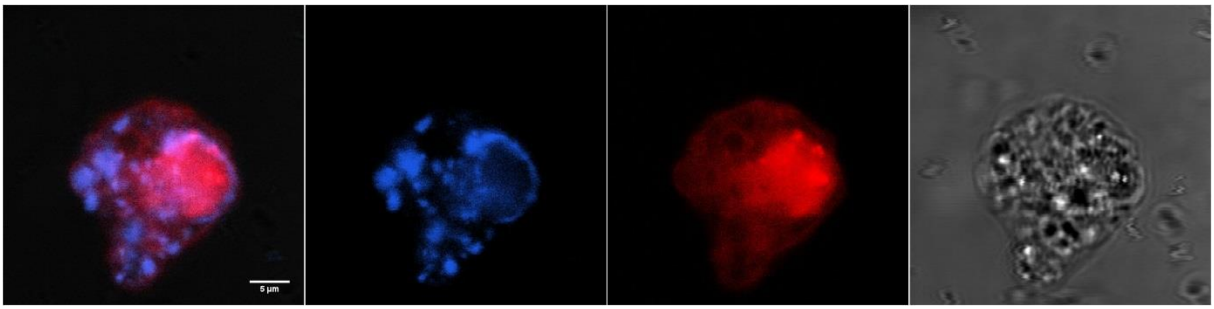


Figure 56. Apoptotic cell. Red – Pc (0.4 μM), blue – Hoechst 33342, grey – phase contrast. Irradiation power 12 J/cm^2.

Figure 57 shows the HeLa cells before and after irradiation with porphycene. Most of the cells in the irradiated sample undergo a different process than in the example above (Figure 56). They keep the nucleus in one piece even though the cellular membrane is already broken, and the cytoplasm is leaking out. This indicates the necrotic cell death. In the case of some cells it is difficult to assign the mechanism with certainty. In Figure 57 the author tried to assign the mechanism of death to each cell. Yellow circles indicate the cells assigned as apoptotic and white circles indicate the cells assigned as necrotic. The percentage of the apoptotic cells ascribed in this way was not reproducible between different samples of the same concentration of Pc and irradiated under the same conditions. The results obtained with applied method are hard to interpret and do not seem to be objective enough to draw ultimate conclusions.

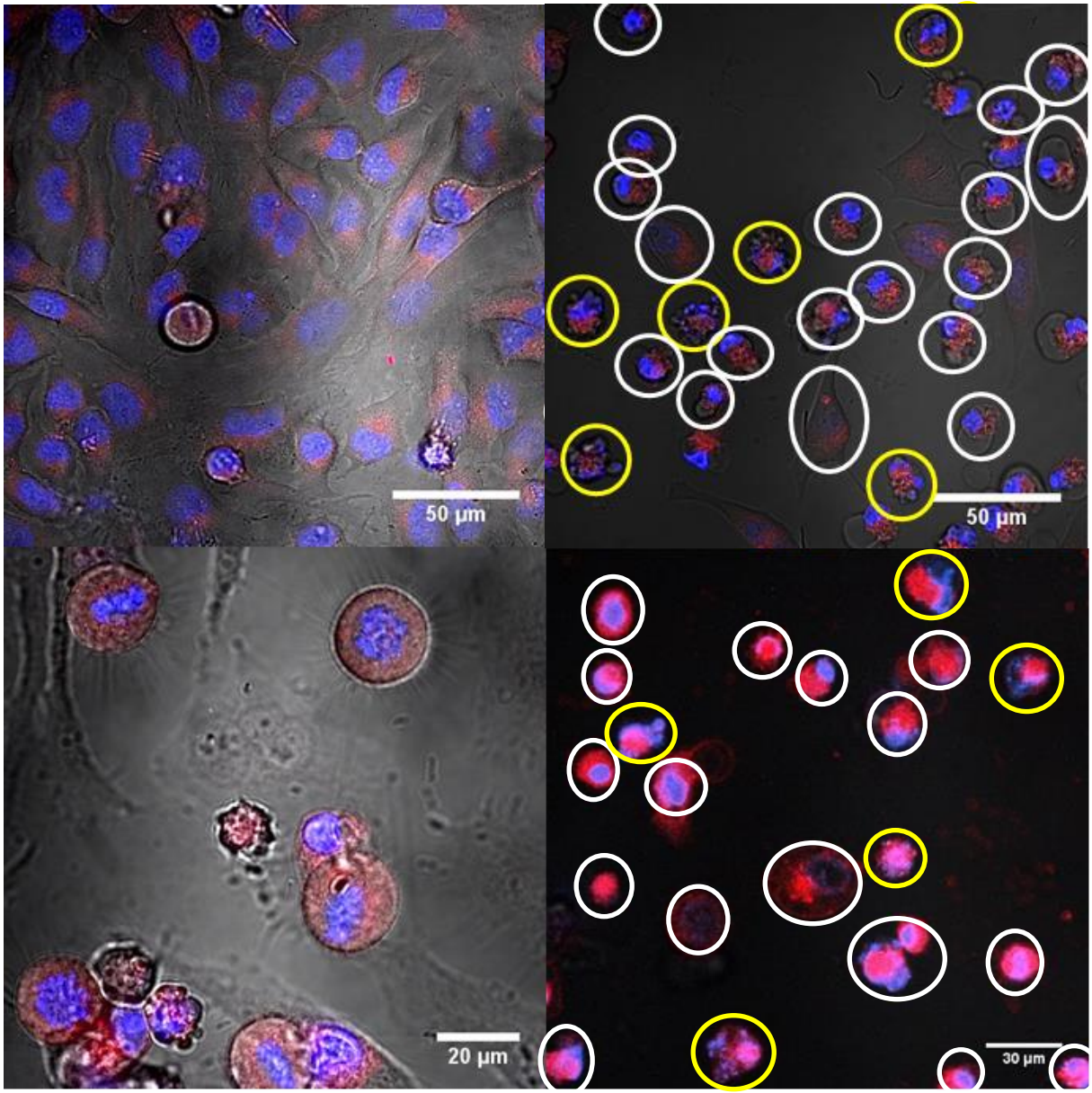


Figure 57. Left: the cells incubated with Pc (0.4 µM), without irradiation. Right: the cells incubated with Pc (0.4 µM), irradiated with the dose of light 12 J/cm^2. Red – Pc, blue – Hoechst 33342, grey – phase contrast image.

The partial success of the selection of the method for cellular death mechanism was not satisfactory. Therefore, a third approach was carried out. The same concentration of Pc and the dose of light was used. Only the indicator of apoptosis was different. NucView 405 was selected as the marker of caspase-3 activity in the living cells. Caspase-3 is one of the most important mediators of programmed cell death, connected with the death cascade initiation. Its occurrence can be therefore used to distinguish between apoptotic and non-apoptotic cells even in the early stage of the process. Figure 58 shows the reference HeLa

cells sample without porphycene and the irradiated HeLa cells with porphycene. Both samples were treated with NucView 405. The appearance of the blue fluorescence indicates the release of the caspase-3 and is characteristic for apoptotic cells. Non-apoptotic cells do not show any signs of blue fluorescence, just as in the case of the reference sample. In this experiment all the irradiated cells turned out to be apoptotic.

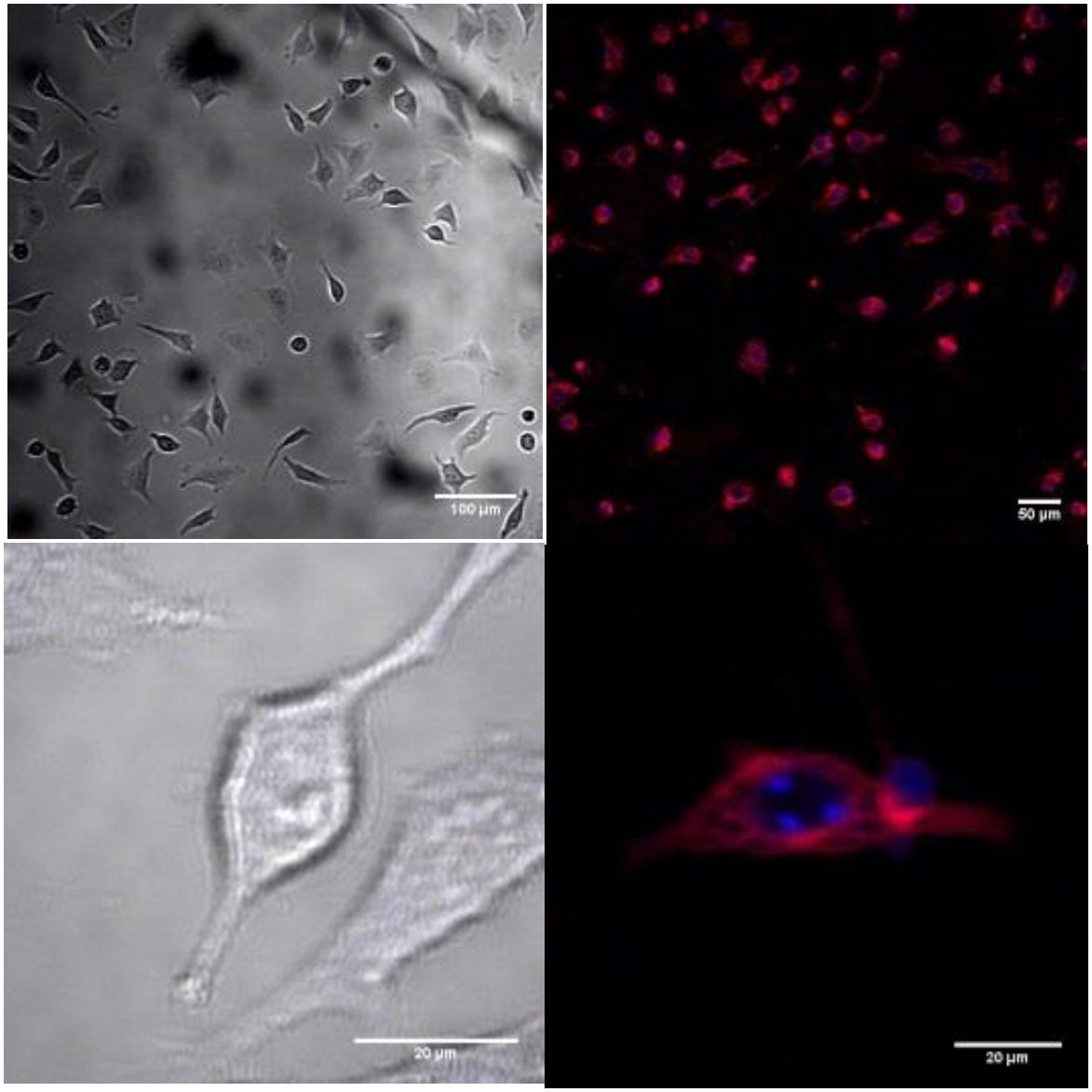


Figure 58. HeLa cells stained with NucView 405. Left, without Pc, right, irradiated with Pc (0.4 µM).

Due to such big discrepancy of the results obtained using different methods the studies over the mechanism of cell death were finished without the expected conclusions. Since in the last approach all the cells exhibited the presence of caspase-3, the most likely is that irradiation with Pc induces apoptosis in the cells, but the effect is so rapid that it is very difficult to catch the moment before the secondary necrosis starts in the sample. Also

lowering the doses of light or photosensitizer might not be helpful, since the cells do not seem to be affected immediately after irradiation, but after 24 hour incubation they are detached from the bottom of the dish and the microscopy is impossible to be performed. The good solution for further studies could be the flow cytometry of the samples treated with the low doses of light, after prolonged incubation. It would allow the observations after 24 hours for consideration of all the cells, not only the adherent ones. Such approach would broaden the statistics and allow for the smaller doses of light and photosensitizer.

5.4.5. Summary

The compounds from the family of porphycenes are efficient photosensitizers in PDT of HeLa cells performed *in vitro*. Introduction of pluronic carrier let non-ionic porphycenes penetrate to the interior of the cells effectively. Microscopic studies proved that the chromophore is usually distributed between cytoplasm and the membrane. The exclusion from this rule is TTPc, which accumulates in lysosomes and does not stain the membrane or cytoplasm at all. This location explains further the fact of untypical inactivity of this compound in PDT experiments on HeLa cells. The most probable reason is the quick excretion of TTPc from the cells, where the lysosomes are used as the carriers for this process. For the rest of the investigated compounds the PDT effect against HeLa cells was pronounced and rapid. It was possible to follow the cell death occurring immediately after irradiation of the samples in real time under the light microscope. Due to the process of cell death occurring so quickly after the irradiation it was not possible to follow the mechanism of cell death and distinguish the conditions that would favor apoptosis over the necrosis. This problem might be solved by conducting flow cytometry after 24 hours of incubation of the cells after their irradiation with gradually increased doses of photosensitizer, as the standard protocols advise. In the conditions of microscopy experiments this approach was not useful, since the cells detach from the surface of the glass shortly after their death. Despite the undertaken attempts, also the differences of viscosity in the cytoplasm of the cells during the cell death were not observed. The extensive studies of the kinetics of photosensitizer uptake inside the cells resulted in the conclusion that Pc (2 μM) penetrates into HeLa cells effectively in the about 1 – 2 hours and after that, the equilibrium with the pluronic solution is reached. The efficiency of such process in the conditions of conducted experiments was estimated as $7 \cdot 10^{12}$ molecules of the photosensitizer per cell, but, since

the measurement could be affected by aggregation of the dye, it is possible that the real value is smaller.

SUMMARY AND CONCLUSIONS

Porphycenes are promising second generation photosensitizers. Their advantage over porphyrins, their isomers representing the first generation photosensitizers, lies mostly in their light absorption properties. The crucial feature is high absorption in optical therapeutic window, which covers the wavelength range of 600 – 850 nm. Porphycene-core compounds in this thesis were studied in terms of their applicability in photodynamic therapy and photoinactivation of bacteria. It required first the evaluation of their photophysical properties, such as molar absorption coefficients, quantum yields of fluorescence, and fluorescence lifetimes in different solvents. The selected molecules were uncharged and highly hydrophobic. For this reason, the crucial part of the research was devoted to the selection and optimization of the carrier media for chromophores delivery to the bacteria and cancer cells. Three different delivery systems were investigated, including cyclodextrins, liposomes, and micelles. Finally, the micelles consisting of Pluronic F-127 polymer were selected, due to the best effective concentration of chromophores and the best stability that they provided. They also turned out to be better than phosphatidylcholine liposomes in the *in vitro* trials against HeLa cells, meaning that the same concentration of PS delivered in the pluronic micelle was providing a stronger photodynamic effect than the same concentration of PS delivered in liposomes. The pluronic formulation of porphycenes was further investigated to find the optimal parameters for PDT and PDI. The micelles are stable in the conditions of conducted experiment. The experimentally determined CMC is $(5.2\pm0.4)\times10^{-6}$ M and the hydrodynamic diameter is 29 ± 2 nm. The optimal molar pluronic:porphycene ratio advised by the author should be higher than 2000:1, which helps to prevent aggregation of porphycenes. The most convenient range of concentrations is 1 – 10 μM of porphycene per 2.5 – 3.5 mM of pluronic.

The second part of the studies was devoted to the biological application of porphycenes. Photodynamic inactivation studies were performed on three different strains of Gram-positive bacteria: *E. faecalis, S. epidermidis* and *S. aureus,* which are common causes of multidrug resistant nosocomial infections. The comparative bactericidal studies were conducted with Pc, since it exhibited the highest activity in the preliminary experiments. Comparatively low concentration of 7 μM of Pc was used for PDI studies and

it was sufficient to obtain over 6log eradication of all the used bacterial strains. It is worth noting that low doses of light in the range 6 – 30 J/cm^2 were used. The structure-activity relationship studies were also conducted, resulting in the conclusion that the *tert*-butyl substituents, contrary to apparently similar methoxyethyl moieties, lower the bactericidal activity of porphycenes and can eventually block it completely. The steric hindrance affecting bacterial cell wall penetration is the postulated reason. The photosensitizing potential measured with singlet oxygen generation quantum yield was similar for all the studied porphycenes. In toluene, Φ_Δ determined for the compounds was in the range between 0.30 to 0.38 and in deuterated water pluronic solution, which resembled the biological environment the best, the values ranged from 0.21 to 0.28. The results obtained during PDT studies of HeLa cells were matching very well the conclusions from the PDI section. Also in this case the photosensitizing effect recorded for most of the studied compounds was very pronounced, whereas TTPc seemed to be inert in *in vitro* studies. The co-localization studies in the HeLa cells confirmed that porphycenes are usually located in the cellular membrane and cytoplasm, whereas TTPc is an exception from this rule, and is accumulated in lysosomes. Lysosomes are most probably used by the cell to excrete unwanted compounds and this is the reason of the unusual inactivity of TTPc in PDT studies.

PROPOSALS OF FUTURE STUDIES

The results of the research, however promising, still leave many open questions and encourage to ask the new ones.

The bactericidal studies should be continued by investigating another porphycene derivative, with the structure shown below (Fig. 59), which is available in the library of compounds of the Institute of Physical Chemistry of the Polish Academy of Sciences.

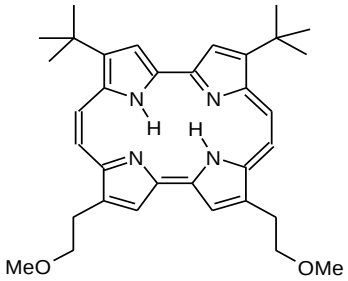


Figure 59. 2,7-di-(β-methoxyethyl)-12,17-di-tert-butylporphycene.

Simultaneous presence of *tert*-butyl and methoxyethyl substituents in one molecule makes this chromophore a good candidate for extending structure-activity PDI studies started in this thesis. It is difficult to predict if the influence of *tert*-butyl moieties, that block the ability of molecule to enter inside the bacteria cell, will prevail over the influence of methoxyethyl groups that are accepted by the bacteria.

The other thread of the studies that should be developed is investigation of photodynamic inactivation effect of porphycenes against Gram-negative bacteria. It is commonly known that the susceptibility of Gram-negative species to PDI is much lower than of Gram-positive ones, due to the lower permeability of the cell wall. This topic is one of most important challenges in antimicrobial applications of photosensitizers.

Successful attachment of porphycene-loaded pluronic micelles to the glass surface opens the pathway to the whole range of microscopic studies, including the investigations of single micelles. Observing the fluorescence time-traces from single micelles, it is possible to find out if the micelles are loaded with just one chromophore molecule, two molecules, or the whole bunch. Our preliminary experiments suggest that the average loading is 1 – 2 porphycene molecules per one pluronic micelle, however this result has to be confirmed by further studies.

Lots of opened questions were left in the field of *in vitro* anticancer studies. First of all, the similar structure-activity relationship that was conducted on bacteria should be also done on HeLa cells. Preliminary studies conducted with the use of Pc, TOMEPc, and TTPc, indicate that the relation between the structure of porphycene-core compound and its activity may be similar for human cells and for bacteria and it is based on the ability of the compound to penetrate into a cell.

The comparative studies of activity of porphycenes and commonly available drugs could be also very informative. Such preliminary experiment was already conducted in the cooperation with The Department of Immunology of Medical University of Warsaw. The activity of TOMEPc and photofrin against HeLa cells was compared. It revealed that the dose of 0.23 μg of TOMEPc induces a similar photodynamic effect (measured with the fraction of killed cells) as 10 μg of photofrin. Such big difference in the doses of both substances required for the treatment encourage to consider the economic factor that may favor porphycenes in their future way to become the PDT drugs.

The interesting series of experiments to perform can be also devoted to the comparison between the photodynamic effect of porphycenes on the cancer and healthy mammalian cells. Any signs of selectivity to the cancer cells would be an advantage, however, most probably, the photosensitization would occur in both types of cells. Anyway, it is worth remembering that the accumulation of the xenobiotics inside the cancer cells is increased with respect to the healthy cells, due to accelerated metabolism and enhanced permeability and retention effect.

BIBLIOGRAPHY

[1] D. E. J. G. J. Dolmans, D. Fukumura, and R. K. Jain, “Photodynamic therapy for cancer.,” *Nat. Rev. Cancer*, vol. 3, no. 5, pp. 380–7, May 2003.

[2] A. Cocilovo, “Colored Light Therapy: Overview of its History, Theory, Recent Developments and Clinical Applications Combined with Acupuncture T RADITIONAL Oriental medical theory,” *Copyr. Am. J. Acupunct.*, vol. 27, no. 12, pp. 71–88, 1999.

[3] M. D. Daniell and J. S. Hill, “a History of Photodynamic Therapy,” *Aust. N. Z. J. Surg.*, vol. 61, no. 5, pp. 340–348, 1991.

[4] Q. Moan, J., Peng, “An outline of the hundred year history of PDT,” *Anticancer Res.*, vol. 23, pp. 3561–3600, 2003.

[5] J. Moan and K. Berg, “Photochemotherapy of Cancer: Experimental Research,” *Photochem. Photobiol.*, vol. 55, no. 6, pp. 931–948, 1992.

[6] Jodlbauer A; von Tappeiner H, “Uber die wirkung photodynamischer (fluoreszierender) stoffe auf bakterien.,” *Munch Med Wochenschr*, vol. 51, pp. 1096–1097, 1904.

[7] E. N. Rask and W. H. Howell, “THE PHOTODYNAMIC ACTION OF HEMATOPORPHYRIN,” *Am. J. Physiol. Content*, vol. 84, no. 2, pp. 363–377, 1928.

[8] R.-M. Szeimies, J. Dräger, C. Abels, and M. Landthaler, “Chapter 1 History of photodynamic therapy in dermatology,” in *Photodynamic Therapy and Fluorescence Diagnosis in Dermatology*, vol. 2, P. Calzavara-Pinton, R.-M. Szeimies, and B. Ortel, Eds. Elsevier, 2001, pp. 3–15.

[9] T. J. Dougherty, J. E. Kaufman, A. Goldfarb, K. R. Weishaupt, D. Boyle, and A. Mittleman, “Photoradiation therapy for the treatment of malignant tumors.,” *Cancer Res.*, vol. 38, no. 8, pp. 2628–35, 1978.

[10] J. D. Macmillan and W. Andrew, “LETHAL PHOTOSENSITIZATION,” vol. 5, 1966.

[11] W. L. Morison, “Anti-viral treatment of warts,” vol. 1974, no. June 1974, pp. 97–100, 1975.

[12] G. Laustriat, “Molecular mechanisms of photosensitization,” *Biochimie*, vol. 68, no. 6, pp. 771–778, Jun. 1986.

[13] C. S. Foote, “DEFINITION OF TYPE I and TYPE II PHOTOSENSITIZED OXIDATION,” *Photochem. Photobiol.*, vol. 54, no. 5, pp. 659–659, 1991.

[14] M. S. Baptista, J. Cadet, P. Di Mascio, A. A. Ghogare, A. Greer, M. R. Hamblin, C. Lorente, S. C. Nunez, M. S. Ribeiro, A. H. Thomas, M. Vignoni, and T. M. Yoshimura, “Type I and Type II Photosensitized Oxidation Reactions: Guidelines and Mechanistic Pathways,” *Photochem. Photobiol.*, vol. 93, no. 4, pp. 912–919, 2017.

[15] A. P. Castano, T. N. Demidova, and M. R. Hamblin, “Mechanisms in photodynamic therapy: part one,” *Photodiagnosis Photodyn Ther*, vol. 1, no. 4, pp. 279–293, 2004.

[16] R. Yin and M. R. Hamblin, “Antimicrobial Photosensitizers: Drug Discovery Under the Spotlight,” *Curr. Med. Chem.*, vol. 22, no. 18, pp. 2159–2185, 2015.

[17] D. Mondal and S. Bera, “Porphyrins and phthalocyanines: promising molecules for light-triggered antibacterial nanoparticles,” *Adv. Nat. Sci. Nanosci. Nanotechnol.*, vol. 5, no. 3, p. 033002, 2014.

[18] M. Tim, “Strategies to optimize photosensitizers for photodynamic inactivation of

bacteria," *J. Photochem. Photobiol. B Biol.*, vol. 150, pp. 2–10, 2015.

[19] M. Kepczynski, M. Dzieciuch, and M. Nowakowska, "Nanostructural hybrid sensitizers for photodynamic therapy.," *Curr. Pharm. Des.*, vol. 18, no. 18, pp. 2607–2621, 2012.

[20] S. K. Sharma, L. Y. Chiang, and M. R. Hamblin, "Photodynamic therapy with fullerenes in vivo: reality or a dream?," *Nanomedicine*, vol. 6, no. 10, pp. 1813–1825, 2011.

[21] B. Halliwell, "Reactive Species and Antioxidants. Redox Biology Is.pdf," vol. 141, no. 2, pp. 312–322, 2016.

[22] G. Bartosz, *Druga twarz tlenu. Wolne rodniki w przyrodzie*, 2nd ed. Warszawa: Wydawnictwo Naukowe PWN, 2003.

[23] "Braslavsky JPPB 97.pdf." .

[24] M. C. DeRosa and R. J. Crutchley, "Photosensitized singlet oxygen and its applications," *Coord. Chem. Rev.*, vol. 233–234, pp. 351–371, 2002.

[25] R. J. Crutchley and M. C. DeRosa, "Photosensitized Singlet Oxygen and its Applications," *Coord. Chem. Rev.*, vol. 233–234, pp. 351–371, 2002.

[26] H. Wu, Q. Song, G. Ran, X. Lu, and B. Xu, "Recent developments in the detection of singlet oxygen with molecular spectroscopic methods," *TrAC - Trends Anal. Chem.*, vol. 30, no. 1, pp. 133–141, 2011.

[27] K. I. Salokhiddinov, I. M. Byteva, and G. P. Gurinovich, "Lifetime of singlet oxygen in various solvents.," vol. 23, no. 10, pp. 5642–5644, 1981.

[28] M. Bregnhøj, M. Westberg, B. F. Minaev, and P. R. Ogilby, "Singlet Oxygen Photophysics in Liquid Solvents: Converging on a Unified Picture," *Acc. Chem. Res.*, vol. 50, no. 8, pp. 1920–1927, 2017.

[29] A. Prasad, M. Sedlářová, and P. Pospíšil, "Singlet oxygen imaging using fluorescent probe Singlet Oxygen Sensor Green in photosynthetic organisms," *Sci. Rep.*, vol. 8, no. 1, pp. 1–13, 2018.

[30] S. Kim, M. Fujitsuka, and T. Majima, "Photochemistry of singlet oxygen sensor green," *J. Phys. Chem. B*, vol. 117, no. 45, pp. 13985–13992, 2013.

[31] N. Mehraban and H. S. Freeman, *Developments in PDT sensitizers for increased selectivity and singlet oxygen production*, vol. 8, no. 7. 2015.

[32] A. B. Ormond and H. S. Freeman, "Dye sensitizers for photodynamic therapy," *Materials (Basel).*, vol. 6, no. 3, pp. 817–840, 2013.

[33] E. Vogel, M. Kocher, H. Schmickler, and J. Lex, "Angew. Chem. Int. Ed. Engl.," vol. 25, no. 3, pp. 257–259, 1986.

[34] J. Waluk, "Spectroscopy and Tautomerization Studies of Porphycenes," *Chem. Rev.*, p. acs.chemrev.6b00328, 2016.

[35] S. E. Braslavsky, M. Miiller, M. Daniel, S. Pbrting, S. G. Bertolotti, S. Chakravorti, G. Kocweier, B. Knipp, and K. Schaffner, "Corrigendum Corrigendum to " Photophysical properties of porphycene derivatives [Journal of Photochemistry and Photobiology B : Biology 40 (1997) 191-1981 '," vol. 1344, no. 97, p. 1344, 1998.

[36] K. M. Kadish, K. M. Smith, and R. Guilard, Eds., *Handbook of Porphyrin Science (Volumes 6 – 10): With Applications to Chemistry, Physics, Materials Science, Engineering, Biology and Medicine*. World Scientific, 2010, 2010.

[37] I. Czerski, A. Listkowski, J. Nawrocki, N. Urbańska, H. Piwoński, A. Sokołowski, O. Pietraszkiewicz, M. Pietraszkiewicz, and J. Waluk, "The long and winding road to new

porphycenes," *J. Porphyr. Phthalocyanines*, vol. 16, no. 05n06, pp. 589–602, 2012.

[38] N. Urbańska, M. Pietraszkiewicz, and J. Waluk, "Efficient synthesis of porphycene," *J. Porphyr. Phthalocyanines*, vol. 11, no. 08, pp. 596–600, 2007.

[39] N. Urbańska, "PhD:Synteza i spektroskopia wielofunkcyjnych pochodnych porficenów," pp. 1–128, 2008.

[40] D. Sánchez-García and J. L. Sessler, "Porphycenes: synthesis and derivatives.," *Chem. Soc. Rev.*, vol. 37, no. 1, pp. 215–32, Jan. 2008.

[41] M. Leunig, E. Vogel, F. Gamarra, W. Lumper, C. Richert, D. Jocham, and A. E. Goetz, "Tumour localisation kinetics of photofrin and three synthetic porphyrinoids in an amelanotic melanoma of the hamster," *Br. J. Cancer*, vol. 68, no. 2, pp. 225–234, 1993.

[42] J. C. Stockert, M. Cañete, A. Juarranz, A. Villanueva, R. W. Horobin, J. I. Borrell, J. Teixidó, and S. Nonell, "Porphycenes: facts and prospects in photodynamic therapy of cancer.," *Curr. Med. Chem.*, vol. 14, no. 9, pp. 997–1026, Jan. 2007.

[43] C. Richert, J. M. Wessels, M. Müller, M. Kisters, T. Benninghaus, and a E. Goetz, "Photodynamic antitumor agents: beta-methoxyethyl groups give access to functionalized porphycenes and enhance cellular uptake and activity.," *J. Med. Chem.*, vol. 37, no. 17, pp. 2797–807, 1994.

[44] R. Ruiz-González, M. Agut, E. Reddi, and S. Nonell, "A Comparative Study on Two Cationic Porphycenes: Photophysical and Antimicrobial Photoinactivation Evaluation," *Int. J. Mol. Sci.*, vol. 16, no. 11, pp. 27072–27086, 2015.

[45] X. Ragàs, D. Sánchez-García, R. Ruiz-González, T. Dai, M. Agut, M. R. Hamblin, and S. Nonell, "Cationic porphycenes as potential photosensitizers for antimicrobial photodynamic therapy," *J. Med. Chem.*, vol. 53, no. 21, pp. 7796–7803, Nov. 2010.

[46] F. M. Lauro, P. Pretto, L. Covolo, G. Jori, and G. Bertoloni, "Photoinactivation of bacterial strains involved in periodontal diseases sensitized by porphycene-polylysine conjugates.," *Photochem. Photobiol. Sci.*, vol. 1, no. 7, pp. 468–470, 2002.

[47] L. Polo, A. Segalla, G. Bertoloni, G. Jori, K. Schaffner, and E. Reddi, "Polylysine – porphycene conjugates as efficient photosensitizers for the inactivation of microbial pathogens," *Photochem. Photobiol.*, vol. 59, pp. 152–158, 2000.

[48] M. Gil, J. Dobkowski, G. Wiosna-Sałyga, N. Urbańska, P. Fita, C. Radzewicz, M. Pietraszkiewicz, P. Borowicz, D. Marks, M. Glasbeek, and J. Waluk, "Unusual, solvent viscosity-controlled tautomerism and photophysics: Meso-alkylated porphycenes," *J. Am. Chem. Soc.*, vol. 132, no. 38, pp. 13472–13485, 2010.

[49] G. Tiwari, R. Tiwari, B. Sriwastawa, L. Bhati, S. Pandey, P. Pandey, and S. K. Bannerjee, "Drug delivery systems: An updated review," *Int J Pharm Investig*, vol. 2, no. 1, pp. 2–11, 2012.

[50] R. Ruiz-González, P. Acedo, D. Sánchez-García, S. Nonell, M. Cañete, J. C. Stockert, and A. Villanueva, "Efficient induction of apoptosis in HeLa cells by a novel cationic porphycene photosensitizer," *Eur. J. Med. Chem.*, vol. 63, pp. 401–414, May 2013.

[51] K. Scherer, C. Abels, W. Bäumler, G. Ackermann, and R. M. Szeimies, "Structure-activity relationships of three differently substituted 2,7,12,17-tetrakis-(β-methoxyethyl) porphycene derivatives in vitro," *Arch. Dermatol. Res.*, vol. 295, no. 12, pp. 535–541, 2004.

[52] S. Karrer, C. Abels, R. M. Szeimies, W. Bäumler, M. Dellian, U. Hohenleutner, A. E. Goetz, and M. Landthaler, "Topical application of a first porphycene dye for photodynamic therapy - Penetration studies in human perilesional skin and basal cell carcinoma," *Arch. Dermatol. Res.*, vol. 289, no. 3, pp. 132–137, 1997.

[53] C. Abels, "Targeting of the vascular system of solid tumours by photodynamic therapy

(PDT).," *Photochem. Photobiol. Sci.*, vol. 3, no. 8, pp. 765–771, 2004.

[54] D. Lembo and R. Cavalli, "Nanoparticulate delivery systems for antiviral drugs," *Antivir. Chem. Chemother.*, vol. 21, no. 2, pp. 53–70, 2010.

[55] M. Alavi, N. Karimi, and M. Safaei, "Application of various types of liposomes in drug delivery systems," *Adv. Pharm. Bull.*, vol. 7, no. 1, pp. 3–9, 2017.

[56] J. Li, X. Wang, T. Zhang, C. Wang, Z. Huang, X. Luo, and Y. Deng, "A review on phospholipids and their main applications in drug delivery systems," *Asian J. Pharm. Sci.*, vol. 10, no. 2, pp. 81–98, 2014.

[57] P. R. Kulkarni, J. D. Yadav, and K. a Vaidya, "Liposomes : a Novel Drug Delivery System," *Int. J. Curr. Pharm. Res.*, vol. 3, no. 2, pp. 10–18, 2011.

[58] C. M. Neophytou and A. I. Constantinou, "Drug Delivery Innovations for Enhancing the Anticancer Potential of Vitamin E Isoforms and Their Derivatives," *Biomed Res. Int.*, vol. 2015, no. APRIL, pp. 1–16, 2015.

[59] E. R. B. Arun Kumar, Heidi M. Mansour, Adam Friedman, "Nanomedicine in Drug Delivery," *CRC Press.*, pp. 8–12, 2013.

[60] M. C. García Vior, J. Marino, L. P. Roguin, A. Sosnik, and J. Awruch, "Photodynamic effects of Zinc(II) phthalocyanine-loaded polymeric micelles in human nasopharynx KB carcinoma cells," *Photochem. Photobiol.*, vol. 89, no. 2, pp. 492–500, 2013.

[61] Y. C. Chen, C. L. Lo, and G. H. Hsiue, "Multifunctional nanomicellar systems for delivering anticancer drugs," *J. Biomed. Mater. Res. - Part A*, vol. 102, no. 6, pp. 2024–2038, 2014.

[62] E. V. Batrakova, D. L. Kelly, S. Li, Y. Li, Z. Yang, L. Xiao, D. Y. Alakhova, S. Sherman, V. Y. Alakhov, and A. V. Kabanov, "Alteration of genomic responses to doxorubicin and prevention of MDR in breast cancer cells by a polymer excipient: Pluronic P85," *Mol. Pharm.*, vol. 3, no. 2, pp. 113–123, 2006.

[63] K. M. Park, S. Y. Lee, Y. K. Joung, J. S. Na, M. C. Lee, and K. D. Park, "Thermosensitive chitosan-Pluronic hydrogel as an injectable cell delivery carrier for cartilage regeneration," *Acta Biomater.*, vol. 5, no. 6, pp. 1956–1965, 2009.

[64] B. Gidwani and A. Vyas, "A Comprehensive Review on Cyclodextrin-Based Carriers for Delivery of Chemotherapeutic Cytotoxic Anticancer Drugs," *Biomed Res. Int.*, vol. 2015, 2015.

[65] D. Zhang, P. Lv, C. Zhou, Y. Zhao, X. Liao, and B. Yang, "Cyclodextrin-based delivery systems for cancer treatment," *Mater. Sci. Eng. C*, vol. 96, no. November 2018, pp. 872–886, 2018.

[66] R. Haynes, "Using cyclodextrin to stabilize and control colloidal micro-stickies to improve paper machine runnability," vol. 2, pp. 1268–1298, 2009.

[67] K. Plaetzer, B. Krammer, J. Berlanda, F. Berr, and T. Kiesslich, "Photophysics and photochemistry of photodynamic therapy: Fundamental aspects," *Lasers Med. Sci.*, vol. 24, no. 2, pp. 259–268, 2009.

[68] R. R. Anderson and J. A. Parrish, "The optics of human skin," *J. Invest. Dermatol.*, vol. 77, no. 1, pp. 13–19, 1981.

[69] Y. Huang, A. Chen, and M. Hamblin, "Low-level laser therapy: an emerging clinical paradigm.," *SPIE Newsroom*, no. 9, pp. 1–3, 2009.

[70] H.-W. Wang, T. C. Zhu, M. E. Putt, M. Solonenko, J. Metz, A. Dimofte, J. Miles, D. L. Fraker, E. Glatstein, S. M. Hahn, and A. G. Yodh, "Broadband reflectance measurements of light penetration, blood oxygenation, hemoglobin concentration, and drug concentration in human intraperitoneal tissues before and after photodynamic therapy," *J. Biomed. Opt.*,

vol. 10, no. 1, p. 014004, 2005.

[71] “http://www.reflexions.uliege.be/cms/c_41432/en/light-against-cancer?part=2.”

[72] P. Agostinis, K. Berg, K. A. Cengel, T. H. Foster, A. W. Girotti, S. O. Gollnick, S. M. Hahn, M. R. Hamblin, A. Juzeniene, D. Kessel, M. Koberlik, J. Moan, P. Mroz, D. Nowis, J. Piette, B. Wilson, and J. Golab, “Photodynamic Terapy of cancer: un update,” vol. 61, no. 4, pp. 250–281, 2012.

[73] N. Solban, I. Rizvi, and T. Hasan, “Targeted Photodynamic Therapy,” vol. 531, no. March, pp. 522–531, 2006.

[74] S. K. Golombek, J.-N. May, B. Theek, L. Appold, N. Drude, F. Kiessling, and T. Lammers, “Tumor targeting via EPR: Strategies to enhance patient responses.,” *Adv. Drug Deliv. Rev.*, 2018.

[75] B. Zhang, Y. Hu, and Z. Pang, “Modulating the tumor microenvironment to enhance tumor nanomedicine delivery,” *Front. Pharmacol.*, vol. 8, no. DEC, pp. 1–16, 2017.

[76] R. R. Allison, C. H. Sibata, T. Brody, R. Medicine, and L. W. Jenkins, “Oncologic photodynamic therapy photosensitizers : A clinical review,” *Photodiagnosis Photodyn. Ther.*, vol. 7, no. 2, pp. 61–75, 2010.

[77] J. L. Sessler and R. A. Miller, “New drugs with diverse clinical applications in radiation and photodynamic therapy,” vol. 59, no. 99, pp. 733–739, 2000.

[78] M. T. Huggett, M. Jermyn, A. Gillams, R. Illing, S. Mosse, M. Novelli, E. Kent, S. G. Bown, and T. Hasan, “Phase I / II study of verteporfin photodynamic therapy in locally advanced pancreatic cancer,” vol. 110, no. 7, pp. 1698–1704, 2014.

[79] “5-ALA Hexylester, 5-ALA Hexylesther, Aminolevulinic Acid Hexyl Ester, Hexaminolevulinate, Hexyl 5-Aminolevulinate, P 1206,” *R&D Drugs*, vol. 6, no. 4, pp. 235–238, 2005.

[80] A. R. Azzouzi, S. Lebdai, F. Benzaghou, and C. Stief, “Vascular-targeted photodynamic therapy with TOOKAD?? Soluble in localized prostate cancer: standardization of the procedure,” *World J. Urol.*, pp. 937–944, 2015.

[81] P. Mroz, A. Yaroslavsky, G. B. Kharkwal, and M. R. Hamblin, “Cell Death Pathways in Photodynamic Therapy of Cancer,” pp. 2516–2539, 2011.

[82] S. Elmore, “Apoptosis: A Review of Programmed Cell Death,” *Toxicol. Pathol.*, vol. 35, no. 4, pp. 495–516, Jun. 2007.

[83] J. O. Yoo and K. S. Ha, *New Insights into the Mechanisms for Photodynamic Therapy-Induced Cancer Cell Death*, 1st ed., vol. 295. Elsevier Inc., 2012.

[84] J. O. Yoo and K. S. Ha, *New Insights into the Mechanisms for Photodynamic Therapy-Induced Cancer Cell Death*, 1st ed., vol. 295. Elsevier Inc., 2012.

[85] J. M. Dąbrowski and L. G. Arnaut, “Photodynamic therapy (PDT) of cancer: from local to systemic treatment,” *Photochem. Photobiol. Sci.*, vol. 14, no. 10, pp. 1765–1780, 2015.

[86] D. Mittelman and J. H. Wilson, “The fractured genome of HeLa cells,” pp. 111–114, 2013.

[87] Y. Huang, M. Tanaka, D. Vecchio, M. Garcia-diaz, J. Chang, Y. Morimoto, and M. R. Hamblin, “Photodynamic Therapy induces an immune response against a bacterial pathogen,” *Expert Rev Clin Immunol*, vol. 8, no. 5, pp. 479–494, 2012.

[88] D. Tiahnhong, H. Ying-Ying, and M. R. Hamblin, “Photodynamic therapy for localized infections – state of the art,” *Photodiagnosis Photodyn Ther.*, vol. 6, no. 617, pp. 170–188, 2010.

[89] J. Y. Nagata, N. Hioka, E. Kimura, V. R. Batistela, R. S. S. Terada, A. X. Graciano, M. L. Baesso, and M. F. Hayacibara, "Antibacterial photodynamic therapy for dental caries: Evaluation of the photosensitizers used and light source properties," *Photodiagnosis Photodyn. Ther.*, vol. 9, no. 2, pp. 122–131, 2012.

[90] A. Sculean, A. Aoki, G. Romanos, F. Schwarz, R. J. Miron, and R. Cosgarea, "Is Photodynamic Therapy an Effective Treatment for Periodontal and Peri-Implant Infections?," *Dent. Clin. North Am.*, vol. 59, no. 4, pp. 831–858, 2015.

[91] S. H. Siddiqui, K. H. Awan, and F. Javed, "Bactericidal efficacy of photodynamic therapy against Enterococcus faecalis in infected root canals: A systematic literature review," *Photodiagnosis Photodyn. Ther.*, vol. 10, no. 4, pp. 632–643, Dec. 2013.

[92] R. Malik, A. Manocha, and D. K. Suresh, "Photodynamic therapy--a strategic review.," *Indian J. Dent. Res.*, vol. 21, no. 2, pp. 285–291, 2010.

[93] H. A. Khan, A. Ahmad, and R. Mehboob, "Nosocomial infections and their control strategies," *Asian Pac. J. Trop. Biomed.*, vol. 5, no. 7, pp. 509–514, 2015.

[94] F. Gomes, P. Teixeira, and R. Oliveira, "Mini-review: Staphylococcus epidermidis as the most frequent cause of nosocomial infections: Old and new fighting strategies," *Biofouling*, vol. 30, no. 2, pp. 131–141, 2014.

[95] S. Bhardwaj, K. Bhamre, J. Dhawale, M. Patil, and S. Divase, "Enterococcus faecium and Enterococcus faecalis, the nosocomial pathogens with special reference to multi-drug resistance and phenotypic characterization," *Int. J. Pharm. Sci. Pract.*, vol. 2, no. 1, pp. 1–10, 2013.

[96] R. Yin and M. Hamblin, "Antimicrobial Photosensitizers: Drug Discovery Under the Spotlight," *Curr. Med. Chem.*, vol. 22, no. 18, pp. 2159–2185, 2015.

[97] J. V. Fritz, P. Didier, J. P. Clamme, E. Schaub, D. Muriaux, C. Cabanne, N. Morellet, S. Bouaziz, J. L. Darlix, Y. Mély, and H. de Rocquigny, "Direct Vpr-Vpr Interaction in Cells monitored by two photon fluorescence correlation spectroscopy and fluorescence lifetime imaging," *Retrovirology*, vol. 5, pp. 1–17, 2008.

[98] J. P. Clamme, J. Azoulay, and Y. Mély, "Monitoring of the formation and dissociation of polyethylenimine/DNA complexes by two photon fluorescence correlation spectroscopy," *Biophys. J.*, vol. 84, no. 3, pp. 1960–1968, 2003.

[99] A. A. Gorman, I. Hamblett, C. Lambert, A. L. Prescott, M. A. J. Rodgers, and H. M. Spence, "Aromatic Ketone-Naphthalene Systems as Absolute Standards for the Triplet-Sensitized Formation of Singlet Oxygen, 02(1Ag), in Organic and Aqueous Media: A Time-Resolved Luminescence Study," *J. Am. Chem. Soc.*, vol. 109, no. 10, pp. 3091–3097, 1987.

[100] R. Schmidt, C. Tanielian, R. Dunsbach, and C. Wolff, "Phenalenone, a universal reference compound for the determination of quantum yields of singlet oxygen O2(1Δg) sensitization," *J. Photochem. Photobiol. A Chem.*, vol. 79, no. 1–2, pp. 11–17, Apr. 1994.

[101] N. Urbańska, M. Pietraszkiewicz, and J. Waluk, "Efficient synthesis of porphycene," *J. Porphyr. Phthalocyanines*, vol. 11, no. 08, pp. 596–600, 2007.

[102] N. Masiera, J. Buczyńska, G. Orzanowska, H. Piwoński, and J. Waluk, "Enhancing fluorescence by using pluronic block copolymers as carriers of monomeric porphycenes," *Methods Appl. Fluoresc.*, vol. 2, no. 2, p. 024003, Apr. 2014.

[103] H. Toledano, R. Edrei, and S. Kimel, "Photodynamic damage by liposome-bound porphycenes: comparison between in vitro and in vivo models.," *J. Photochem. Photobiol. B.*, vol. 42, no. 1, pp. 20–7, Jan. 1998.

[104] B. Zhivotosky and S. Orrenius, "Assessment of Apoptosis and Necrosis by DNA," *Curr Protoc*

Cell Biol., vol. 18, no. 3, pp. 1–23, 2001.

[105] M. Seervi and D. Xue, *Mitochondrial Cell Death Pathways in Caenorhabiditis elegans*, 1st ed., vol. 114. Elsevier Inc., 2015.

[106] D. V Krysko, T. Vanden, K. D. Herde, and P. Vandenabeele, "Apoptosis and necrosis : Detection , discrimination and phagocytosis," vol. 44, pp. 205–221, 2008.

[107] "https://www.lifesci.dundee.ac.uk/technologies/flow-cytometry-cell-sorting/techniques/cell-death-and-apoptosis." .

[108] "Cellular and Nuclear Degradation during Apoptosis," vol. 21, no. 6, pp. 900–912, 2010.

[109] M. Gil, J. a. Organero, J. Waluk, and a. Douhal, "Ultrafast dynamics of alkyl-substituted porphycenes in solution," *Chem. Phys. Lett.*, vol. 422, no. 1–3, pp. 142–146, Apr. 2006.

[110] C. Womb, "Phenalenone , a universal reference compound for the determination of quantum yields of singlet oxygen O # A ,) sensitization," vol. 79, pp. 11–17, 1994.

[111] F. Wilkinsonw, P. Helman, A. B. Ross, F. Wilkinson, W. P. Helman, and A. B. Ross, "Quantum Yields for the Photosensitized Formation of the Lowest Electronically Excited Singlet State of Molecular Oxygen in Solution Quantum Yields for the Photosensitized Formation of the Lowest Electronically Excited Singlet State of Molecular Oxygen in ," vol. 113, no. 1993, 2009.

[112] P. Alexandridis and T. A. Hatton, "DS thermodynamics , structure , dynamics , and modeling," *Colloids and Surfaces*, vol. 7757, 1995.

[113] R. W. Redmond, G. Valduga, S. Nonell, S. E. Braslavsky, K. Schaffner, E. Vogel, K. Pramod, and M. Köcher, "The photophysical properties of porphycene incorporated in small unilamellar lipid vesicles," *J. Photochem. Photobiol. B Biol.*, vol. 3, no. 2, pp. 193–207, 1989.

[114] M. Y. Kozlov, N. S. Melik-Nubarov, E. V. Batrakova, and A. V. Kabanov, "Relationship between pluronic block copolymer structure, critical micellization concentration and partitioning coefficients of low molecular mass solutes," *Macromolecules*, vol. 33, no. 9, pp. 3305–3313, 2000.

[115] B. Pucelik, L. G. Arnaut, G. Stochel, and J. M. Dabrowski, "Design of Pluronic-Based Formulation for Enhanced Redaporfin-Photodynamic Therapy against Pigmented Melanoma," *ACS Appl. Mater. Interfaces*, vol. 8, no. 34, pp. 22039–22055, 2016.

[116] S. Karrer, R. M. Szeimies, S. Ernst, C. Abels, W. Bäumler, and M. Landthaler, "Photodynamic inactivation of staphylococci with 5-aminolaevulinic acid or photofrin," *Lasers Med. Sci.*, vol. 14, no. 1, pp. 54–61, 1999.

[117] M. Grinholc, B. Szramka, J. Kurlenda, A. Graczyk, and K. P. Bielawski, "Bactericidal effect of photodynamic inactivation against methicillin-resistant and methicillin-susceptible Staphylococcus aureus is strain-dependent," *J. Photochem. Photobiol. B Biol.*, vol. 90, no. 1, pp. 57–63, 2008.

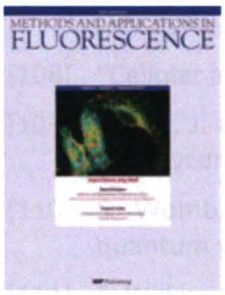
[118] B. Pucelik, R. Paczyński, G. Dubin, M. M. Pereira, L. G. Arnaut, and J. M. Dąbrowski, "Properties of halogenated and sulfonated porphyrins relevant for the selection of photosensitizers in anticancer and antimicrobial therapies," *PLoS One*, vol. 12, no. 10, pp. 1–22, 2017.

[119] R. Kreder, S. Oncul, O. A. Kucherak, K. A. Pyrshev, E. Real, Y. Mély, and A. S. Klymchenko, "Blue fluorogenic probes for cell plasma membranes fill the gap in multicolour imaging," *RSC Adv.*, vol. 5, no. 29, pp. 22899–22905, 2015.

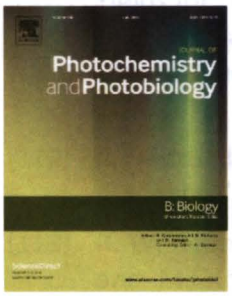
[120] M. K. Kuimova, S. W. Botchway, A. W. Parker, M. Balaz, H. A. Collins, H. L. Anderson, K. Suhling, and P. R. Ogilby, "Imaging intracellular viscosity of a single cell during photoinduced cell death," vol. 1, no. March, pp. 69–73, 2009.

[121] M. A. Izquierdo, S. A. Lermontova, and I. S. Grigoryev, “Supporting Information for : DUAL USE OF PORPHYRAZINES AS SENSITIZERS AND VISCOSITY MARKERS DURING PHOTODYNAMIC THERAPY,” 2015.

ACHIVEMENTS AND PUBLICATIONS RELATED WITH THE THESIS



N. Masiera, J. Buczyńska, G. Orzanowska, H. Piwoński, and J. Waluk, "Enhancing fluorescence by using pluronic block copolymers as carriers of monomeric porphycenes," *Methods Appl. Fluoresc.*, vol. 2, no. 2, p. 024003, Apr. 2014.
DOI: 10.1088/2050-6120/2/2/024003



N. Masiera, A. Bojarska, I. Gawryszewska, E. Sadowy, W. Hryniewicz, and J. Waluk, "Antimicrobial photodynamic therapy by means of porphycene photosensitizers," *J. Photochem. Photobiol. B Biol.*, vol. 174, no. July, pp. 84–89, 2017.
DOI: 10.1016/j.jphotobiol.2017.07.016

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