Habilitation report

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On the basis of the scientific publications submitted for the competition relating to an academic position "Professor", 4.2. Chemical sciences, 01.05.10. Bioorganic chemistry and chemistry of natural and physiologically - active substances

This habilitation report is based on **27 scientific papers**, most of them published during the time post DSc degree (the criteria W & G). The publications that are part of Scopus are **24** with a first and the only one corresponding author - **11** scientific papers, including a **scientific book** published on the basis of the DSc-thesis (2021). There are papers (**9**) published in a Journal (*JOP*) and the Proceeding books (*SPIE Proceed*.) as a result of the scientific reports, and both of them are registered in Scopus. The list of publications also included **2** papers in two Journals that only have impact factor and are part of other data-bases. The citations involved in the present competition are **249** (Sonix) and include the time starting from the year 2022 till June 2025, so as not to cover the previous citations, which totally are > **950** with **H**-index: **17 (Scopus) 18** incl. other data-bases.

In summary, the scientific works included for the competition are as follows: (**Q**, number of papers): *Biomedicines; Gels; Int. J. Mol. Sci; Can. Gene Ther., Viruses* (**Q1**, **5**), *Inorganics; Front. Biosci. - Landmark Ed.; Curr. Iss. Mol. Biol.; J. Mol. Struct.; J. Fluor., J. of Biomed. Photonics & Eng. (**Q2**, **7**), Bulg. Chem. Comm.*; *Sylwan – 2 броя* (**Q4**, **3**), *SPIE Proceed.* (**SJR**, **5**), *J. of Physics: Conf. Series* (**SJR**, **4**) and two valuable papers, published by invitations in Journals that possess only IF > 3 (*J. Biomed. Discoveries и Arch. Biotech. Biomed.*, **2**).

I. INTRODUCTION

The scientific topic of the authors' interests can be titled with the name of the method known as "Photodynamic therapy" (PDT). This scientific work is based on the research and development of novel photosensitive compounds for biomedical applications within PDT and other applications that include light-associated phenomenon. The so-called photodynamic action has been observed and used for health care and cure antiquity and nowadays, it still remains actual for new challenging applications. PDT methodology includes the cascade photophysical processes that are continuing to the photochemical in the well-chosen conditions of the surrounding media. If the pathological conditions, such as tumor cells, pathogenic microorganisms, viruses, or others, are positioned in the vicinity of the photosensitizer and light, and not to forget the need for enough oxygen satiety for the process that has resulted in the fast, gentle, and local therapeutic effect. The method has characteristics of a non-conventional, palliative procedure with the main applications in medicine and ecology.

Photosensitive compounds for PDT have characteristics of absorption in the spectral region starting from visible to near infrared wavelengths, which are typical for compounds with highly conjugated aromatic structure as well as the excitation spectrum of the proper light from the modern light sources which are created according the requirements of the method and not the last, the supplementary with oxygen (the pathogens and tumors are often hypoxic). As a result of photophysical processes that are leading to electron transition in photoactive molecules, highly toxic species such as molecular singlet oxygen and different reactive oxygen species may be produced. The mechanism of singlet oxygen generation is proven as the most productive mechanism for photosensitization. The pathogenic cells as a target for the cytotoxic oxygen species, the curative progression may occur.

The first clinically approved photosensitizers have porphyrin type structure (hematoporphyrin derivative and purified version known as Photofrin, and protoporphyrin – PPIX, as a result of the biosynthesis from ALA). Presently, porphyrins have been successfully applied for the diagnosis and therapy of cancers all over the world and also in our country. Moreover, PDT is a in the period of fast research and development, referring to novel photosensitizers, modern light sources, and novel approaches for oxygen saturation. PDT is invented as a perspective method for new applications. It is in the focus of the new scientific challenges because of the non-specificity of the mechanisms and the necessity of the different mechanisms of action against pathogens in the present reality of resistance and the emergency need for therapeutic approaches for acute illnesses.

In view of the new chemical structures of photosensitizers, the studies of the new original compounds on the basis of the porphyrin ring are in progress. They are demarcated as second- and third- generation photosensitizers. New results show a different success within the attempts to minimize the main limitation of the present photosensitizers. The disadvantages of the known photosensitizers, which need a special attention, are the high hydrophobicity, limited drug-delivery, insufficient uptake and selectivity, and last but not least the lack of dark toxicity. These problems are still existing and are among the main efforts that have to be done for improvements of PDT drugs.

Phthalocyanine complexes under research interest belong to the porphyrin-like structured compounds with flexible structure and unique photo- properties with enormous potential for application in many different technical fields, but more challenging are the biomedical applications. The scientific efforts on this research topic were started in the Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences in the early 80s (XX century) in the research group of Prof. M. Shopova. As a result of these studies, it was patented a porphyrin derivative for application in cancer PDT. During the next years, the studies have been continued in the research and development of the new generation photosensitizers among the phthalocyanine compounds with the aim to meet the necessary criteria for the ideal photosensitizers. In the same time, the development of the method has been forced for the application of PDI on pathogenic microorganisms because of the escalation of cases with drug-resistance occurrences.

II. Scientific topics, results and contributions

1. New phthalocyanine complexes as photosensitizers for PDT: synthesis and photo-properties

The new structures of photosensitizers that are based on the phthalocyanine ring and biologically-active and cationic substituents, and metals for coordination to obtain the complexes. The chosen metal ions, such as palladium, nickel, gallium, zinc, aluminium were used as proper metals for the photosensitizers in order to facilitate the transfer to the triplet excited state molecule. The new complexes, in comparison to the similar phthalocyanines in our previous investigations, have a non-peripheral position of the substituents of methylpyridiloxy groups to the ring symmetric molecule. The attempt to prepare the non-peripheral octa-substituted derivatives was not productive. The octa-substituted phthalocyanine complexes have the positive feature of the purity and structure without regioisomers. The cyclotetramerization occurs from the monomer at strong reaction conditions. The second pathway, which includes first ~~to~~ preparing the metal-free phthalocyanine then the complex, has the advantages of high purity, yields, and fewer chemicals to prepare the desired complexes. The substitution of methylpyridiloxy groups was chosen consider the cationic charge to nitrogen, which after quaternization leads to increase the hydrophilic nature of highly hydrophobic phthalocyanine. A number of cationic and hydrophilic complexes of Pd(II), Ni(II) and Al(III) were prepared following the synthetic pathway that was previously developed for peripherally substituted phthalocyanines. The applied synthetic scheme was on the basis of monomeric dinitrile with a final stage quaternization of nitrogen in the pyridiloxy group. Two reagents were used for the final reaction, namely methyl iodide or methyl sulfate for the complexes. For comparison purposes together with the new one, there was also obtained zinc and aluminium complexes for PDT applications.

The synthesis of non-peripherally substituted phthalocyanine complexes was carried out following the synthetic pathway that included a procedure of synthesis of a ligand molecule first and then the complex. This pathway shows the higher yields and the purity of compounds in comparison to another pathway of direct synthesis starting from a monomer such as a suitable dinitrile. An exception is the complexes of lutetium and gallium, which were not possible to prepare thought the metal-free phthalocyanine as a ligand molecule. This pathway is not effective because of the steric hindrance effects. However, even at high energy, resp. temperature of the reaction conditions, the coordination was not successful. The other metals were coordinated starting from the substituted metal-free phthalocyanines.

The second group of phthalocyanine derivatives were obtained as complexes of Lu(III) with substituents of biologically-active carbohydrates (galactopyranose or galactose), all chemically linked to four non-peripheral positions of the phthalocyanine ring molecule. The successful pathway was the direct cyclotetramerization with Lu-salt, reflux and the presence of a catalyst (DBU). The similar conditions were applied for zinc phthalocyanine with azido- group substituents. A further click was applied with -OH of a fragment and a formation of triazole. The formed double structure of two phthalocyanine molecules was tested as not suitable for PDT.

The properties of all novel phthalocyanines were examined in a comparative manner and the data suggested the prospective photosensitizers for PDT in comparison to the other known phthalocyanines. The main photo-physicochemical properties, such as quantum yield of the triplet state and singlet oxygen generation, have better values for the complexes of palladium, and lutetium. The fluorescent properties of the obtained complexes, as well as after conjugation, were determined with values that suggested proper emission intensity for the so-called non-invasive optical diagnosis. Photochemical properties that are of importance for PDT such as photostability and quantum yields of singlet oxygen, showed the optimal values for the complexes of palladium and lutetium. The scientific efforts in this experiment are underlined by the photosensitivity of the compounds by irradiation in oxygen surroundings.

2. Natural photosensitizers: (1) cobalamins – properties and contributions to the PDT method; (2) anthraquinones of plant origin as photosensitizers

2.1. Cobalamins: photophysicochemical and photobiological properties.

Cobalamins are well known as vitamin B12, which has characteristics of the complex molecular structure containing a corrin ring and molecule and fragments of deoxyadenosyl group or methyl group in axial positions, which substituents are in the natural forms of cobalamins. A nucleotide base of 5,6-dimethylbenzimidazole is part that happens in all four known forms of cobalamins.

The function of cobalamins in cancer therapy is known to support the uptake of chemo-drugs. The effect is because of the accumulation of the applied chemo-drugs in tumor cells by the mechanism that is based on a receptor molecule (CD320). On the other site, the deficiency of B12 is known to increase the risk of cancer development. This finding can be supported in the PDT procedure by the usage of B12 as a photosensitive compound together with a phthalocyanine photosensitizer in order to tune the uptake and to enhance the PDT efficiency. Based on the literature sources, at that time there were not available studies on the cobalamins for cancer PDT.

The investigations on cobalamin in the conditions of photosensitizer suggested the photo-safety of the used compounds in a wide concentration range. The next arrangement of studies aimed to evaluate the photo- properties and PDT efficiency of two forms of cobalamins, namely hydroxy - and cyanocobalamins, in the PDT study of two phthalocyanine photosensitizers. These studies were carried out *in vitro* on two breast cancer cell lines (MCF-7 и MDA-MB-231) in comparison to the normal cell line (MCF-10A). The examination of compounds for photo safety at non-specific conditions, namely a solar spectrum of light irradiation at terrestrial fluence rate (360-960 nm, Helios-iO; 10 J/cm^2) showed a wide concentration range of non-toxicity (up to 1000 μM) of the applied cobalamin on embryonal cells (BALB 3T3). Both studied Zn(II)-phthalocyanines (3ZnPc & 4ZnPc) that are differ in the position of the four substitution groups showed the structure – function dependence of the results for photo-toxicity at constant light parameters (LED 660 nm; 50 J/cm^2 and 100 mW/cm^2). The cobalamin contribution to PDT was in the lower dark toxicity of the powerful photosensitizer, such as 3ZnPc, as well as in different phototoxicity on both tumor cell

lines. It was assumed that the observed effect was due to the selective uptake of ZnPcs and the screening effect due to cobalamin on the experimental cells in respect to the irradiation.

It can be concluded that the so-called cobalamin-dependent PDT has a positive effect on lowering the toxicity of the applied photosensitizer, on the improvement of selectivity of the PDT response and on the promising probability for minimizing the main disadvantages and the side effects post-PDT procedure.

2.2. Anthraquinones of plant origin: an overview of the up-to-date knowledge and the perspectives in the studies of these compounds as photosensitizers

The green approach in all kinds of scientific fields features as a great potential approach in research and development of the photodynamic method. The focus is on the main content of the PDT, namely the photosensitive compound and the spectrum of irradiation, both can be described as green. Nowadays, there is still no clinically approved photosensitizer of plant origin with one exception such as hypericin (*Hypericum perforatum*), which after many years of research, reached the clinical stage in the studies.

Generally, the photosensitizers of plant origin have a good scientific basis owning to the literature that explores the therapeutic properties of compounds of plant origin. The further studies of these compounds as photosensitizers for PDT are the topic under the research interests. The initial work included the photophysical studies on the extracts of a medicinal plant, which is rich in anthraquinones. These results were reported during the 6th PDT Congress (2024, Istanbul).

3. Conjugates of phthalocyanine complexes with proteins and enzymes: photo-properties and PDT effects

There were obtained and UV-vis spectroscopically characterized the physical conjugates on the basis of phthalocyanine macrocycle and biologically-active collagen (hydrolyzed collagen) or a proteolytic enzyme α-Chymotrypsin. The both chosen macromolecules have the functionality of the drug carrier systems. The studies were carried out at physiological conditions with the means of UV-vis spectroscopic properties of the formed double structure for different ratios of concentrations between phthalocyanine dye and protein or enzyme. A stable structure was observed with the possible points of interactions due to charges and the lipophilic - hydrophilic nature. The conjugates were observed to have pH - stability in neutral media (pH 7.4-7.8). The investigations on the photophysical properties suggested that the phenomenon FRET is involved. It was observed at excitation of biomolecules (collagen or chymotrypsin) that contain fluorogenic amino acids tryptophan and tyrosine, and the typical fluorescence emission for phthalocyanines (> 690 nm) was recorded (exc: 280 nm/ 290 nm, em: > 690 nm). This observation can be in usage in optical diagnosis in parallel to PDT. The conjugates were studied in comparison manner to the pure phthalocyanines (GaPc1 & GaPc2) for photo safety (solar LED and BALB 3T3 cells) and for photodynamic efficacy (in vitro) using a human pigmented melanoma cell line and two normal skin origin cell lines (SH4 versus HaCaT, BJ).

In summary, the obtained data are as follows: (1) the photo-physical and chemical properties showed the values almost one order of magnitude lower than the same parameters measured for the pure phthalocyanine compounds; (2) the low cytotoxicity was observed after conjugation in both kinds of biomolecules with phthalocyanine photosensitizers; (3) an optimal PDT effect can be realized as conjugates at higher concentrations of photosensitizer; (4) the conjugate with chymotrypsin showed toxicity on normal keratinocyte cells; and (5) the main advantage to PDT is the improve parameters of phototherapeutic index (PIF) and selectivity index (SI) for the conjugates.

4. Research and development of the photodynamic method with phthalocyanines and other photosensitizers as treatment procedure for actual medical purposes

4.1. Antimicrobial photodynamic therapy

Photodynamic inactivation of pathogens is a well-accepted method as effective against pathogenic microorganisms, especially in the present reality of the increase multidrug resistance. The studies were carried out with complexes of Pd(II) - vs. Zn(II) - phthalocyanines on two clinical isolates of Gramm(-) strains of *Aeromonas hydrophila* which were evaluated with resistance towards the tested antibiotics. The applied PDI showed a full inactivation after the palladium complex of phthalocyanine at a concentration > 5 μM, which was shown not to depend on the strain susceptibility. It was observed the effect of non-specificity of the photodynamic action. This was assumed to be a result of the uptake capacity into bacterial cells in suspensions with different cell density. The fluorescence of compounds was evaluated as measurable to test the phthalocyanine accumulation into pathogenic cells. The difference in the PDI efficacy was observed as a result of the uptakes of the tested phthalocyanines into pathogenic bacterial cells. The visualization of the accumulation was performed by means of confocal fluorescence microscopy.

The PDI studies were carried out on a Gramm (-) bacterium *Flavobacterium hydatis*, which is known to cause a huge injury in the fish farms. The applied antibiotics are often non-effective and not to forget, they transfer to humans by table-fish. The studies were performed with Pd(II) – phthalocyanines with different positions of the functional substitution groups to the phthalocyanine ring. The main result of these experiments is referred to structure – function effects. The non-peripherally substituted analogues showed inactivation potential in lower concentrations than the peripheral analogue compounds.

4.2. Photodynamic inactivation on viruses

The application of the photodynamic method for prompt inactivation of viruses appears very actual since the last health crisis of pandemic years. The main contribution to this topic was the promising experimental data obtained together with the colleagues from the Institute of Microbiology – BAS. The studies on different stages of virus contaminations suggested the possibility of a positive outcome for an effective inactivation on every stage of viruses' incorporation into the host cells due to the PDI. Further experiments are in progress to prove the efficacy of the method for harsh to-treat non-enveloped viruses.

4.3.Photodynamic therapy on tumors

The first clinical PDT application that was approved for cancer treatment with porphyrin derivatives. During the last twenty-five years, anticancer PDT has still been actual because of the mechanism of the photodynamic action, which excludes the resistance development against light and oxygen species. The studies of the so-called third - generation photosensitizers have been focused on the main limitations of the present photosensitizers such as drug-delivery, selectivity, uptake, release and toxicity of the newly developed structures. Among them are phthalocyanines with substitution units that have positive discrimination to the above properties.

The efforts made in this direction suggested that the non-peripherally substituted phthalocyanines are more toxic (dark and light) as compared to the peripheral similar compounds. This observation can be minimized by conjugation with native biologically active macromolecules.

The studies on different cancers' PDT applications, most of which are published in the papers from scientific forums (13-20 G). These results were obtained because of the collaboration projects of the colleagues from IE – BAS through the bilateral projects with Russia. The personal contribution for the obtained novelties was in the preparative work of new or known photosensitive phthalocyanines, which were synthesized and evaluated as photosensitizers by means of photophysical and photochemical studies.

III. The future scientific studies for the period of five years

1. Sustainable, green approaches in the synthesis of phthalocyanines: research and development of "green" synthesis of phthalocyanines (together with Turkish scientist)

The increase pollution in the environment and the problems associated with the unhealthy situation tend the development and implementation of the green technology, which allow the lowering of toxic emissions. Considering the field of organic synthesis, it is also under the research efforts of creation of low toxicity synthetic pathways, minimum by-products, and the reaction conditions that are following the "green" standards. In common cases this means the usage of chemicals with low impact on the atmosphere and environment.

The literature during the past five years has documented the possible approaches for phthalocyanines' "green" synthesis. These include some new but also some old methods that are defined as green. The novel approaches, such as usage of low- toxicity solvents or lack of solvents, ionic solvents, and deep eutectic mixtures, have more advantages than the irradiations or melting. The economic effect is also of importance, which is part of the DESs. On the basis of the present knowledge, the usage of these solvents for the synthesis of phthalocyanine will be the task for the future studies.

2. Study on the possibility of exosomes (LncRNA) and the matrix metalloproteases (MMPs) as targets in tumor therapy (project with China)

The matrix metalloproteases (MMPs) are well-studied with an overquantification during the formation of tumors, and also, they are related to the development of metastasis. Presently, MMPs are in usage as diagnostic tools and the sign for an early detection of tumor development.

This property serves the MMPs to be in usage as targets for the research and development of novel inhibitors. The known low molecular weight drugs aim to inhibit the active center of MPPs. However, the efficacy is limited to the studies in vitro and in vivo, and the further clinical studies fail. It will be the aim of our future work to study the influence of any photosensitizers and the PDT method on the inhibition of these proteases. The up-to-date studies reported divergent effects, which need to be clarified. The same directions are the planned investigations on the exosomes as possible carrier systems of the photosensitizers.

3. Natural photosensitizers: chemical analyses, and studies of the main photo-properties for different PDT applications

The major direction in the studies on the natural photosensitizers included the following research tasks: (1) PDT characterization of anthraquinones from the plant, which is typical for the Balkan region; (2) studies of the main photo- properties and the examination of their potential as photosensitizers; (3) evaluations of the possible applications of the plant-originated anthraquinones as photosensitive compounds for PDT method (new project).

In conclusion, it can be said, that the research and developments included in this summary have been focused on the 3rd generation photosensitizers that are based on the phthalocyanine macrocycle. Several phthalocyanine derivatives were prepared after chemical modifications with different cell-specific and biologically-active substituents. After a proper conjugation with the balanced drug-delivery systems the stable physical conjugates were obtained and further evaluated as appropriate for the PDT. The novelties in the synthesis of phthalocyanines and the usage of the sustainable approaches and the “green” solvents will be part of the further research benefits. The natural photosensitizers have been under the research interest and the further studies are in progress for their introduction as photosensitizers within the visible “green” light spectrum. In addition, PDT can have a positive impact on the ecology by developing the method in direction the decontamination of natural pools, fish farms and agricultural products. PDT has been determined as a therapeutic approach without replacement due to its unique mechanism of action that has low coincidental for resistance development. Furthermore, the PDT method is still under development for the current life-threatening biomedical applications such as acute diseases that are referring to pathogenic microorganisms and malignances.

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