

REVIEW

BY PROF. DR. MILKA MILCHEVA MILEVA

Head of Laboratory "Biological Response Modifiers and Pathogenesis of Viral Infections" - Institute of Microbiology - Bulgarian Academy of Sciences

REGARDING: conducting a procedure for the defense of the dissertation work of Marieta Dimitrova Belcheva - doctoral student of self-study in the Department "Biotechnology", Laboratory "Bioremediation and Biofuels" - Institute of Microbiology - Bulgarian Academy of Sciences.

DISSERTATION ON TOPIC: "New photosensitizers and carbon composites as agents with antimicrobial effect"

SCIENTIFIC SUPERVISORS: Prof. Dr. Lyudmila Kabaivanova from Institute of Microbiology - Bulgarian Academy of Sciences
Prof. Dr. Boyko Tsintsarski from Institute of Organic Chemistry with Centre of Phytochemistry - Bulgarian Academy of Sciences

AREA OF HIGHER EDUCATION 4. Natural sciences, mathematics and informatics, Professional direction 4.3. Biological Sciences, Scientific specialty 01.06.12 "Microbiology"

This review was prepared in accordance with Order No. I-108/31.07.2024 of the Director of the Institute of Microbiology "Stephan Angeloff" - BAS (IMikB) by decision of the Scientific Council of IMikB, Protocol No. 4 of 04.23.2024. , according to which I am included in the scientific jury.

A review of the documents shows that the safeguarding procedure has been followed. The presented set of materials is in accordance with the requirements of Chapter II, Section II "Conditions and procedures for obtaining the educational and scientific degree "Doctor" from the Regulations for the conditions and procedures for obtaining scientific degrees and holding academic positions in the Institute of Microbiology "Stephan Angeloff" at the Bulgarian Academy of Sciences and includes all necessary documents.

I know personally the candidate in the competition; I don't have publications in common with her and I am not in a conflict of interest, according to the law.

BRIEF BIOGRAPHICAL DATA

Marieta Dimitrova Belcheva was born on September 19, 1974, in the city of Sofia. In 2001, he graduated from the Medical University - Sofia, Faculty of Medicine and obtained a master's degree in medicine. In the same year, he acquired another master's degree - in chemistry and physics at Sofia University "St. Kliment Ohridski".

In the period 2001-2014, he worked as head assistant, Head of the "Microbiology Laboratory" in the field of oral and clinical microbiology at the Sofia University. In 2017, she held the position of "Head

of the Microbiology Laboratory" at the Sofia Medical Center. In 2021, she was appointed as a specialist at the "Stefan Angelov" Institute of Microbiology - BAS.

During her work experience, Marieta Belcheva specialized in prestigious hospitals and clinics:
2002-2005 specialization in microbiology at the Department of Medical Microbiology, Medical University - Sofia;

2005 3 months of specialization /IF, FISH/ at the University of Zurich in Switzerland;

2010-2011 specialization in infectious diseases at the Medical Academy-Sofia:

2013 3 months specialization /RT-PCR/ at University Medical Center Groningen, Netherlands;

2023-2024 - 5 months specialization at University Clinic Münster, Germany

She is fluent in Russian, German and English.

THE DISSERTATION PAPER is dedicated to the antimicrobial action of newly synthesized, metal-containing phthalocyanine photosensitizers and carbon composites, with a view to their application as an alternative in the fight against infections in the maxillofacial region and as antibacterial agents in air purification

The oral microbiota is a collection of bacteria, viruses and fungi living in the mouth and is often referred to as a 'mini-immune system'. It plays the role of guardian of the entrance door of the body, because it leads directly to the respiratory and digestive systems, where there is a risk of viral and bacterial infections entering the body. Bacteria live and multiply not only on the teeth and between them. A huge amount of bacteria accumulates on the tongue and also on the cheeks. In many cases, they are the cause of diseases that spread in the area of the mouth, teeth and jaw from carious lesions, through periodontal diseases, to bacterial infections of the soft tissues.

In the foreground here also stands out a serious global problem in the treatment of all infectious diseases - antimicrobial resistance, which the World Health Organization has declared as one of the 10 biggest global threats to human health.

In this aspect, the topic of the dissertation work is extremely relevant and undoubtedly concerns each one of us. The presented results have a serious original contribution in this specific niche of science and clearly prove the connection between the conducted scientific research and their importance in medical practice.

An approach is proposed to solve current problems in medicine and clinical practice, while at the same time expanding knowledge on the therapeutic efficacy and antimicrobial action of newly synthesized metal-containing phthalocyanine photosensitizers and carbon composites. An original alternative way of fighting infections in the maxillofacial area and also of antibacterial air purification is used. Marieta Belcheva has taken advantage of the free form of the doctoral studies to conduct a serious and in-depth set of researches in a rather long period of time - from 2010 to 2024. The knowledge acquired from the two master's degrees gives her a stable basis for multifactorial logical analysis, discussion and the formation of complex conclusions from the obtained results. The experience of the two scientific supervisors for methodical selection and multifaceted interpretation is also indisputable here.

Approaches to combat bacterial infections in the oral cavity and their prevention have been used and optimized to obtain better bacterial reduction. At the same time, the emphasis falls on minimizing the negative consequences of the methods used today. The effect of photodynamic inactivation of new metal-containing phthalocyanine photosensitizers against some of the most common microorganisms was studied *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* и *Prevotella intermedia*.

An evaluation of the antimicrobial action of another class of compounds - newly synthesized metal-carbon composites - was carried out, and the criteria by which the effects were tracked and reported in both experimental schemes were precisely selected.

In preparing the dissertation, the doctoral student used the base and experience of leading Bulgarian and European universities and institutes. The research was conducted in the microbiological laboratories of the Faculty of Dental Medicine, Medical University - Sofia, Bulgaria; Department of Microbiology at the University Medical Center - Groningen, Netherlands; Diagnostic-consultative center Sofamed - Bulgaria; Institute of Clinical Microbiology of the University Clinic - Münster, Germany and "Bioremediation and Biofuels" Laboratory at the "Stephan Angeloff" Institute of Microbiology - Bulgarian Academy of Sciences.

Combined with the serious base of knowledge and skills acquired by Assistant Professor Marieta Belcheva from the two master's degrees, she has built a serious expertise and a multi-faceted interdisciplinary view on solving the scientific problems posed in the current dissertation work. Combining the experience of the teams of the two scientific supervisors, a multidisciplinary team with serious expertise in innovative approaches to the study of photosensitizers and carbon composites as agents with antimicrobial action has been created around the scientific issues of the dissertation work. I strongly hope that this collaboration will deepen and continue in the future and realize such interesting and targeted fundamental research having a projection in medical practice.

THE DISSERTATION PAPER is written on 148 standard pages, contains 56 figures and 4 tables. The work is structured according to the requirements and includes: Introduction, Literature review, Aim and objectives, Materials and methods, Results and discussion, Conclusions, Contributions, List of publications and participations related to the topic of the dissertation work, Literature (304 authors).

THE LITERATURE REVIEW is thorough and targeted, excellently structured.

Emphasis falls on data from the scientific literature regarding:

- The microbiological characteristics of the bacteria and their clinical manifestation in the macroorganism and in particular the maxillofacial region, which justifies their selection in the conducted studies;
- Basic mechanisms of resistance, as a prerequisite for the study of new solutions for therapy;
- The essence of photodynamic therapy is commented;
- The achievements of photodynamic therapy in the field of microbiology were examined, as a basis for comparison with the results of the conducted studies;
- Basic data on the nature, classification, production and use of activated carbon as a component of carbon composites;
- The studies with carbon composites in the field of microbiology are presented, as a basis for comparison with the obtained results.

MATERIALS

In this chapter, the biological materials, reference strains are described *Staphylococcus aureus* 1337, (MRSA); *Enterococcus faecalis* 391574; *Candida albicans* 74; *Aggregatibacter actinomycetemcomitans* ATCC 29523; *Porphyromonas gingivalis* ATCC 3247; *Prevotella intermedia* ATCC 49046; *Escherichia coli* ATCC 25922, as well as clinical isolates of *Streptococcus sanguis* CΦ3/9, *Enterococcus faecalis*

CΦ1/9, *Streptococcus salivarius* CΦ2/9, isolated from patients with chronic periodontitis at the Department of Conservative Dentistry at the Faculty of Dentistry, Medical University-Sofia (Sofia, Bulgaria) and randomly selected clinical strains *A. actinomycetemcomitans* (X295/13), *P. gingivalis* (X324/13) and *P. intermedia* (X490/13), obtained from patients at the Oral Microbiology Laboratory at the University Medical Center Groningen (Groningen, Netherlands); *S. aureus* (CM-E3822/21), isolated from the throat secretion of a patient of the microbiological laboratory of the Sofamed Diagnostic and Consultative Center.

IT WAS SHOWN:

- The design of the new biomaterial based on zinc phthalocyanine complexes, which clearly showed photodynamic activity;
- The design of the new biomaterial based on zinc phthalocyanine complexes, which clearly showed photodynamic activity;
- The synthesis of metal-carbon composites;
- Reagents and nutrient media used.

METHODS

Preparation of suspensions of reference microorganisms, determination of phototoxicity of microorganisms, methods of sampling materials from patients, isolation and identification of pathogenic bacteria, determination of phototoxicity in biofilms of microorganisms in the processing of extracted teeth, etc. are duly described.

RESULTS AND DISCUSSION

The results and discussion of the trials with the new metallo-phthalocyanine photosensitizers are discussed point by point depending on the biological model to which they are applied, namely reference strains, clinical isolates and biofilms. All results are excellently illustrated in tables and figures, with a very precise description and discussion.

IN THE DISSERTATION OF ASS. MARIETA BELCHEVA THE FOLLOWING CONCLUSIONS ARE NOTED:

1. In the conducted experiments with aerobic reference bacterial cultures, the best antimicrobial effect of the tested photosensitizers was observed when gallium phthalocyanine was used.
2. On the tested anaerobic reference bacterial cultures, the strongest effect of the action of the photosensitizers was found when using methylpyridyloxy zinc phthalocyanine and p-tetra-mercaptopyridine zinc phthalocyanine.
3. Zinc phthalocyanine was found to be most effective in treating reference yeast cultures.
4. The clinical aerobic bacterial isolates tested were most affected by zinc phthalocyanine, but the effect compared to the reference strains was weaker.
5. The effect on clinical anaerobic bacterial isolates was most pronounced with the use of tetra-methylpyridyloxy zinc phthalocyanine, p-tetra-mercaptopyridine zinc phthalocyanine and octa-mercaptopyridine zinc phthalocyanine, the effect being stronger compared to that of the reference strains.
6. In biofilm experiments, the best effect was observed using silicon phthalocyanine on a 48-hour biofilm of *E. faecals*.
7. The newly synthesized composite materials have very good potential for application as antibacterial agents, especially the copper composite.

8. The method of preparation of the activated carbon does not significantly affect the antibacterial properties. When applying the different methods, we found close values for their impact, which is most noticeable in the case of one-stage hydrolysis.
9. Best result at *E. coli* or 100% reduction in microbial count in the first minutes is observed when composites are used at a copper concentration of 7.5% and higher.
10. The obtained results show that the newly synthesized composite materials have a very good potential for application as antibacterial agents against staphylococci (*S. aureus*), especially the copper composite.
11. The physico-chemical characterization of the composites confirms the presence of metals and shows a carbon matrix with very good absorption properties and a low content of impurities.
12. Composites of activated carbon with metal nanoparticles combine the properties of metals and activated carbon, which contributes to increasing the quality of the final product and the application of its antibacterial properties, for example in hygiene devices and individual masks.

THERE ARE 6 CONTRIBUTIONS ALSO BEEN MADE, WITH THE FORMULATION OF WHICH I COMPLETELY AGREE.

1. The application of photodynamic inactivation with metal-containing phthalocyanine photosensitizers is a new optimized alternative to the methods used today in the fight against bacterial infections in the oral cavity and their prevention.
2. Carbon composites are synthesized using a new ecological technology from waste products as precursors.
3. Combining the properties of metals and activated carbon in metal-containing carbon composites contribute to increasing the qualities of the desired product and expanding the applications of its antibacterial properties.
4. The results obtained in the *in vitro* experiments can serve to prepare a protocol for further *in vivo* experiments.
5. The use of photosensitizers and newly synthesized activated carbon composites is a new approach to reduce the overuse of antibiotics in line with the modern trend of overcoming antibiotic resistance.
6. The development of new antibacterial materials is an important step in the fight against pathogenic organisms in humans and in the environment.

The abstract attached to the procedure documents fully reflects the content of the dissertation.

CRITICAL NOTES – some minor technical inaccuracies are noticeable, which I do not find essential.

EVALUATION OF THE SUBMITTED MATERIALS IN RELATION TO THE CRITERIA IN ACCORDANCE WITH ZRASRB

The publication activity of Assistant Professor Marieta Belcheva fully meets the requirements for obtaining the educational and scientific degree "Doctor". The research results are reflected in 3 scientific articles, of which 2 in journals with a general impact factor and 4.74. A citation is also attached, but I found two more, probably issued after the filing of the documents to start the procedure. This fact suggests the increased interest in the problems investigated in the dissertation.

According to the current procedure for the defense of the dissertation work for the awarding of the educational and scientific degree "Doctor", assistant professor Marieta Dimitrova Belcheva has covered all the criteria in accordance with the ZRASRB and the Regulations for its implementation at the Institute of Microbiology - BAS.

CONCLUSION: The dissertation presented to me for review represents a complex and original study on the antibacterial protective effects of newly synthesized photosensitizers and carbon composites. The excellent knowledge of the literature, the application of a wide range of modern methods, the well-formulated and executed tasks, the in-depth analysis and the excellent presentation of the obtained results, as well as the sufficient scientific productivity of the doctoral student give me reason to confidently support and recommend to the honorable members of the scientific jury awarded the Master of Medicine, Master of Chemistry and Physics, Assistant Professor Marieta Dimitrova Belcheva, the educational and scientific degree "Doctor".

/ Prof. Dr. Milka Mileva /

22/09/2024

Sofia