Abstract:

The subject of the dissertation is fluorophores for potential use in microscopy and spectroscopy, with an emphasis on fluorophores that can be effectively excited by two photons.

This doctoral dissertation is divided into two main sections: a literature review and an experimental section. The literature section consists of four main chapters: 1) Two-photon absorption, including a subsection on Two-photon Fluorescence Microscopy (2PFM); 2) Fluorophores for 2PFM; 3) Amyloids, including a subsection on two-photon fluorescent probes for amyloid detection. Its purpose is to outline the motivations and scientific gap addressed by the dissertation. The literature review aims to explain the importance of research on specific fluorescent markers, as well as the broader scientific and social context: 1) The importance of fluorescence microscopy with an emphasis on 2PFM in medical and biological sciences; 2) How to respond to the limitations of organic fluorophores in bioimaging with DNA-stabilized silver nanoclusters (Ag_N-DNA); 3) The importance of 2PFM in amyloid research; 4) The current state of knowledge on the principles of constructing markers for amyloids, with an emphasis on two-photon probes; 5) The importance of research on amyloids, which is related to their connection with human diseases. It was important for the author of the dissertation to demonstrate how fundamental studies on the optimization of two-photon absorbers, as well as a deeper understanding of their optical properties upon binding to amyloids, can be effectively translated into preclinical research — the driving force behind clinical studies and the development of effective medical therapies. Fluorescent probes are designed either to find direct application or to serve as a starting point for further modification. The hypotheses of this doctoral dissertation are as follows: **Hypothesis 1:** BF2-functionalized benzothiazoles can serve as fluorescent amyloid probes. Modification of the localization of functional groups in the molecules has an impact on optical properties of studied fluorophores bound to amyloid fibrils; **Hypothesis 2:** 2PA of fluorophores bound to amyloids is different than the one of free molecules and can be modulated based on their chemical and consequently electronic structure; **Hypothesis 3**: Atomically-precise nanoclusters Ag_N-DNA can have high two-photon absorption cross-section (σ_2) and two-photon brightness ($\sigma_{2,B}$) exceeding 50 GM, which will make them competitive potential probes for bioimaging. In addition to the hypotheses, the goals of the doctoral dissertation were also formulated: **Goal 1**: Prove that new fluorophore scaffold based on BF moiety interact with amyloids and have improved optical properties (red-shifted absorption and emission and high values of σ_2), as compared to commercially available standard Methoxy-X04; **Goal 2**: Determine relationship between the modulation of 2PA of studied fluorophores bound to amyloid with the structure of fluorophores; **Goal 3**: Proposing new NIR-emitting nanoparticles, which are water-soluble two-photon absorbers and confirm their optical properties.

The second part of the dissertation, i.e., the experimental part, refers to the specific objectives and hypotheses of the dissertation, with a final paragraph on the summary and further prospects resulting from the research conducted as part of this dissertation. Experimental part focuses on three articles, two of which concern organic markers for amyloids, and one focuses on silver nanoclusters as new potential two-photon markers. Each article is a separate subsection, which presents a description of the research objective, a discussion of key results, and a summary.

Each summary indicates which hypotheses and goals of the doctoral dissertation are addressed by a given scientific article. A detailed description of all results, discussions, and the experimental part is included in the attached articles and supplementary materials.

Answering the hypotheses and achieving the goals of this dissertation required the use of a number of spectroscopic methods, such as one-photon absorption measurements, fluorescence and fluorescence excitation spectra, Fluorescence Quantum Yield (FQY), and fluorescence lifetime. Fluorescence techniques were also used to determine specific parameters such as dissociation constant (K_d), photostability of systems, and interactions of organic dyes with amyloids. Nonlinear optical measurements, i.e., two-photon excited luminescence, were used to determine σ_2 and $\sigma_{2,B}$. Atomic force microscopy (AFM) and transmission electron microscopy (TEM) were used to confirm the formation of amyloids from native proteins.

Article 1, entitled "BF2-Functionalized Benzothiazole Amyloid Markers: Effect of Donor Substituents on One- and Two-Photon Properties" presented in section III "Published Work - Description of own research" describes the effect of the position of the electron donor group (-methoxy) in the core composed of BF2-functionalized benzothiazole on one- and two-photon properties after binding with amyloids. The research addresses the limited understanding of the influence of the structure of the fluorophore on the modulation of optical properties after binding with amyloids. In particular, in the field of two-photon absorption modulation, there is a lack of systematic work addressing this issue. An innovative aspect of the work is the determination of cross sections for two-photon absorption before and after binding with amyloids, as the literature presents measurements of cross sections determined only in solvents. The work responds to hypotheses 1 and 2, and **goal 2**.

Article 2, entitled "Two-photon brightness of NIR-emitting, atomically precise DNA-stabilized silver nanoclusters", presented in section III "Published Work - Description of own research", focuses on the two-photon absorption of four representatives of silver nanoclusters stabilized with DNA oligonucleotides (Ag_N-DNA). For the first time in the literature, I presented two-photon absorption cross-section and two-photon brightness of atomically precise Ag_N-DNA. The research addresses the limitations of organic two-photon absorbers, such as NIR emission (>700 nm) characterized by high fluorescence quantum yields and water solubility. The work addresses hypothesis 3 and **goal 3**, that Ag_N-DNA have two-photon brightness above 50 GM, which provides a basis for their use in bioimaging.

Article 3, entitled "A novel O,N,O-coordinated organofluoroboron probe for amyloid detection: insight from experiment and theory," presented in section III "Published Work - Description of own research", focuses on presenting a new structure that interacts with amyloids and has better optical properties such as fluorescence shifted towards longer wavelengths, higher cross sections for two-photon absorption, and a wider range of two-photon excitation, even in the range above 1000 nm, than the commercially available standard for two-photon microscopy for staining amyloids – Methoxy-X04. The work also presents a solution for modifying the structure based on O,N,O-coordinated organofluoroborane in order to increase fluorescence quantum yield and two-photon absorption cross-section. The research responds to hypothesis

2 and **goal 1** of developing a new marker for amyloids that would be competitive with the commercially available standard.

At the end of the dissertation, a possible perspective for the further development of the research contained in this doctoral dissertation is presented, as well as a general overview of the topics discussed.