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Photosensitizing potential of tailored magnetite hybrid nanoparticles functionalized with levan and zinc (II) phthalocyanine

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ABSTRACT

Phototherapies, including photodynamic therapy (PDT), have been widely used in the treatment of various diseases, especially for cancer. However, there is still a lack of effective, safe photosensitizers that would be well tolerated by patients. The combination of several methods (like phototherapy and hyperthermia) constitutes a modern therapeutic approach, which demands new materials based on components that are non-toxic without irradiation. Therefore, this study presents the synthesis and properties of novel, advanced nanomaterials in which the advantage features of the magnetic nanoparticles and photoactive compounds were combined. The primary purpose of this work was the synthesis of magnetic nanoparticles coated with biocompatible and antitumor polysaccharide - levan, previously unknown from scientific literature, and the deposition of potent photosensitizer – zinc(II) phthalocyanine on their surface. In order to better characterize the nature of the coating covering the magnetic core, the atomic force microscope analysis, a contact angle measurement, and the mechanical properties of pure levan and its blend with zinc(II) phthalocyanine films were investigated. This magnetic nanomaterial revealed the ability to generate singlet oxygen upon exposure to light. Finally, preliminary toxicity of obtained nanoparticles was tested using the Microtox® test – with and without irradiation.

1. Introduction

Photodynamic therapy (PDT) has been widely used in clinics for a variety of pathologies, such as dermatological and ophthalmic diseases and cancers. According to the “World Cancer Report 2014”, the number of cancer cases in 2025 will increase to 19 million per year and 24 million by 2030, while the number of deaths will increase from 8.2 to 13 million per year [1]. Therefore, it is crucial to search for new materials and methods that can contribute to cancer treatment. PDT usually reveals fewer side effects than standard therapies. Moreover, this kind of therapy is accompanied by a lesser amount of resistance expressed by cancer cells [2]. Similarly, PDT used against microorganisms also constitutes a prospective for antimicrobial therapy. So far, no specific microbial resistance has been reported for antimicrobial PDT, even after repeated treatments with low doses of photosensitizers [3,4]. The basis for PDT is a photodynamic reaction, which requires three essential factors: the photosensitizer, oxygen, and light. The photodynamic effect is triggered by the excitation of the photosensitizer with light. The excited photosensitizer reacts with oxygen thus generating reactive oxygen species (ROS), such as singlet oxygen (¹O₂), free radicals, and peroxides. ROS appearing in the tissue in a controlled way during PDT can be applied in the treatment of certain types of cancers and precancerous lesions [5,6].

Although photodynamic therapy has been used for years, there is still a demand for novel, useful and safe photosensitizers. The most studied photosensitizers for medical applications are porphyrinoids with zinc(II) phthalocyanine (ZnPc) being an example [7]. ZnPc, although a 1st generation photosensitizer, has been widely studied in PDT due to its high quantum yield of singlet oxygen generation, low toxicity, and a strong absorbance in the red light region allowing for a deeper tissue permeability [8,9]. Despite some success in the treatment of cancer, ZnPc reveals some limitations, most notably related to its...
hydrophilic nature. This property leads to poor solubility of the photosensitizer molecules and causes aggregation in the biological media. Another limitation pertains to the partial retention of the photosensitizer in healthy cells or difficult ecretion of the photosensitizer from normal tissues [10]. All the aspects mentioned above lead to a significant decrease in PDT efficiency. This issue can be solved by the application of magnetic nanoparticles, which offer many advantages allowing for the optimization of photosensitizer administration and, thus, improvement of PDT protocol [11].

The last decade has witnessed an extremely rapid development of nanotechnology in various branches of science, especially in medicine and pharmacy, including pharmaceutical technology and medicinal chemistry [12]. The so-called nanomedicine is based on the use of nanomaterials, mainly nanoparticles, in the diagnosis, treatment, and prevention of diseases. Nanomaterials have been studied for these particular therapeutic and diagnostic applications due to their possessing some specific properties, such as small size, biocompatibility, wide chemical affinity, non-toxicity, and also a broad range of prospective modifications, which would allow a further increase in their desired functionality [13]. In PDT, the magnetic properties of magnetite (Fe₃O₄) nanoparticles can be applied for the improvement to the localization of the therapeutic agents in tumor cells [14]. A combined therapy, using photoactive compounds and non-toxic nanoparticles, can be considered as interesting modality aiming to meet this challenge. According to literature reports, the combination of PDT and magnetic hyperthermia is an effective approach and may enhance the therapeutic effect, therefore enabling significant tumor regression [15-17]. It should also be noted that magnetic nanoparticles based on magnetite, that present superparamagnetic properties, constitute a particular class of nanomaterials. Pure magnetite, however, reveals colloidal instability, especially in neutral pH; thus, its suspensions require a specific stabilization and surface modification [18].

That being said, the coating of magnetite with macromolecules, in particular with polymers, allows for easy surface design. Such modification triggers its further utility, especially in comparison with the coatings of a low molecular weight [19]. It goes without saying the materials applied in biomedicine and pharmacy should express good biocompatibility and be non-toxic. Therefore, it is worth noting that these criteria are fulfilled by some polysaccharide polymers, such as chitosan, starch, or dextran [10]. Polysaccharides comprise a large family of naturally occurring polymeric carbohydrates. Their high chemical reactivity, as well as easy modification, are among the desired features of these molecules. Polysaccharide-based coatings offer many advantages, such as high biocompatibility with human body fluids. According to the latest reports, materials based on polysaccharides can also be utilized in the fight against viruses, including the SARS-CoV-2 causing COVID-19 coronavirus disease responsible for the global pandemic outbreak in 2020 [20]. Levan (Lev), a natural polysaccharide, is a promising material for use in pharmaceutical technology due to its outstanding physicochemical properties, like low viscosity, compatibility with salts and surfactants, and high loading capacity for water and chemicals [21,22]. Furthermore, this type of fructan exhibits a strong antioxidant effect and offers protection against oxidative stress associated with atherosclerosis. In addition, it is biodegradable, non-toxic, and presents the ability to form adhesion bonds with various compounds due to a large number of hydroxyl groups in the structure. Lev (Fig. 1) appears in several microorganisms and consists of glycosidic β-(1,2) major and β-(1,2) minor linkages. The isolated microbial levan has a molecular weight of less than 100 million Da [23,24]. Interestingly, Lev was also studied as an antitumor agent [22]. In the literature, various polysaccharides coating magnetic nanoparticles have been so far described, such as chitosan, starch, dextran, and sodium alginate [25-29], but to our best knowledge, there have been no reports presenting the surface modification of magnetic nanoparticles with levan [30].

2. Materials and methods

2.1. Materials

Levan from *Erwinia herbicola*, iron (III) chloride hexahydrate, iron (II) sulfate hydrate, 1,3-diphenylisobenzofuran (DPBF), (±)-epichlorohydrin, glycerol, diiodomethane (pure for analysis), and zinc (II) phthalocyanine (ZnPc) were purchased from Sigma–Aldrich (Darmstadt, Germany) and used without further purification. Acetic acid, sodium hydroxide, and solvents were purchased from POCh Gliwice (Gliwice, Poland). All solutions were prepared with deionized water.

2.2. Synthesis of levan and levan-zinc (II) phthalocyanine (Lev-ZnPc) films

Neat levan from *Erwinia herbicola* (0.3 g) was stirred in acetic acid solution (C = 1%, 30 mL) at room temperature. Zinc (II) phthalocyanine (0.05 g, 0.09 mmol) in DMF (5 mL) was added, and the stirring was continued for another 30 min. Then, epichlorohydrin (0.23 mL, 2.5 mmol) was added, and the mixture was stirred at 50 °C for 2 h. Next, the solution was cast into polystyrene Petri dishes to evaporate the solvent.

The film of levan without the photosensitizer was prepared in the same manner but without the addition of the zinc(II) phthalocyanine 2.3. Synthesis of levan-coated magnetic nanoparticles (Lev-Fe₃O₄) and zinc (II) phthalocyanine combined with levan-functionalized magnetic nanoparticles (Lev-ZnPc-Fe₃O₄)

Levan from *E. herbicola* (0.3 g) in acetic acid solution (C = 1%, 30 mL) was added to a solution of zinc (II) phthalocyanine (0.05 g) in DMF (5 mL). The resulting mixture was mechanically stirred at room temperature for 30 min. Then, iron (III) sulfate hydrate (2.02 g, 0.1 mol).
2.4. Analysis and characterisation

2.4.1. Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR)

Structures of the obtained materials were characterized by the Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR)–Spectrum Two™ (Perkin Elmer, Waltham, MA, USA). Spectra were recorded in the region from 4000 to 450 cm⁻¹.

2.4.2. Scanning, transmission electron microscopy, and atomic force microscopy (SEM, TEM, AFM)

The morphology of Lev and Lev-ZnPc was studied with a scanning electron microscope 1430 VP LEO Electron Microscopy Ltd. and atomic force microscopy (AFM) (MultiMode NanoScope IIIa Veeco Metrology Inc., USA) technique. Roughness parameters: arithmetic mean, Rₐ, root mean square, Rₛ, and the highest peak value, Rₚ, were calculated for 5 × 5 µm² scanning area.

Surface morphology and the size of the obtained Lev-ZnPc-Fe₃O₄ nanoparticles were analyzed by transmission electron microscope Tecnai F20 X-Twin, FEI Europe, equipped with energy dispersive X-ray spectrometer (EDX) Edax.

2.4.3. Thermogravimetric analysis (TGA)

Thermal gravimetric analysis of the prepared nanomaterials and neat levan was accomplished on a TA Instruments (SDT 2960 Simultaneous DSC-TGA) thermogravimetric analyzer. TGA measurements were performed at a 10 °C/min heating rate in the range from ambient to 800 °C in the atmosphere of nitrogen.

2.4.4. X-ray diffraction (XRD) measurement

X-ray diffraction pattern (XRD) were recorded with X’PertPRO diffractometer (Malvern Panalytical, Almelo, Holland) using a CuKα radiation (wavelength of 1.540 Å, nickel filtered) and High Score software.

2.4.5. Contact angle and surface free energy

The contact angle (θ) of the Lev and Lev-ZnPc was measured at constant temperature (24 °C) by the sessile drop method using DSA10 goniometer (Kruss GmbH, Germany) equipped with a camera. The polar glycerol and nonpolar diiodomethane were applied as test liquids. Each θ is an average of at least 7 measurements. The surface free energy (γₛ) was calculated by the Owens–Wendt method [35].

2.4.6. Mechanical properties

The mechanical properties of pure levan and zinc (II) phthalocyanine-levan films were determined using the EZ-Test E2-LX Shimadzu texture analyzer (Shimadzu, Kyoto, Japan). Six sample strips of levan and zinc (II) phthalocyanine-levan were cut and clamped between pneumatic grips. The force (N) was recorded during the extension at 20 mm/min. The tensile strength, Young’s modulus (E), and elongation were determined.

2.4.7. Dynamic light scattering (DLS)—particle size analysis

A Malvern Nano Zetasizer ZS90 instrument (Malvern, UK) was applied. Measurements were performed at a wavelength of 633 nm, using a detection angle at 25°.

2.4.8. Magnetization measurement

Magnetization measurements were performed using a Quantum Design Superconducting Quantum Interference Device (SQUID) magnetometer (model MPMS3, San Diego, CA, USA) in Laboratory of Magnetic Measurements, Faculty of Chemistry, University of Wroclaw (Poland). M/H curves were recorded at a temperature of 298 K (T).

2.4.9. Specific surface area and pore size analysis

Structural parameters like the specific surface area (Brunauer-Emmett-Teller surface area Sₘₚₑ) and pore structure of Lev, Lev-ZnPc, Lev-Fe₃O₄, and Lev-ZnPc-Fe₃O₄ samples were examined using the low-temperature nitrogen adsorption method. The relevant isotherms of all samples were measured at ~196 °C using an automatic adsorption instrument, ASAP 2010 (Micromeritics, USA). Before gas adsorption measurements, the materials were outgassed in a vacuum at 50 °C for 8 h. The pore size distribution (PSD) was derived from adsorption branches by the nonlocalized density functional theory (NLDFT) method.

2.4.10. Aggregation measurement

The aggregation behavior study of the obtained Lev-ZnPc-Fe₃O₄ nanoparticles were performed at different concentrations in DMF. Measurements of UV–Vis absorption were carried out on a UV-1800 spectrophotometer (Shimadzu, Japan). The experiments were performed according to the literature procedures [36].

2.5. Photoactivity of prepared materials

2.5.1. Singlet oxygen generation measurement

Singlet oxygen generation ability measurements of the obtained Lev-ZnPc-Fe₃O₄ nanoparticles were performed at ambient temperature using DMF as a solvent, following the previously described procedure [37]. Mixtures of DPBF, which is a singlet oxygen chemical quencher (C = 1.478 × 10⁻⁸ mol/dm³), and the Lev-ZnPc-Fe₃O₄ nanoparticles (C = 4%) were exposed to light-emitting diode [38] (PrevaLED® Core G7 Food and Fashion LED modules OSRAM, Germany), emitting radiation at an absorption maximum of 615 nm, and changes in the UV/Vis spectra were studied. The intensity of the emitted radiation was equal to 0.21 µW/cm² and was measured with an electronic radiometer IL 1400A (Newburyport, USA). Zinc(II) phthalocyanine was used as a reference with the quantum yield of singlet oxygen formation according to literature data of 0.56 in DMF [39]. Measurements of UV–Vis absorption was carried out using a UV-1800 spectrophotometer (Shimadzu, Japan).

2.5.2. Toxicity assessment under irradiation

The toxicity of the materials was evaluated by applying solutions or suspensions of the materials to Microtox® acute toxicity test – 81.9% Screening Test, which was performed using Microtox® M500 equipment according to the protocols distributed by the producer (ModernWater plc) [40,41]. Distilled water was used as the solvent for all the materials. Two sets of experiments were conducted: dark toxicity and light toxicity test. For assessment of light-related toxicity, an additional red-light diode (λ_max = 665 nm) was mounted above the Microtox® M500 cuvette incubator, which was used to irradiate the samples upon mixing with Aliivibrio fischeri bacteria. The light intensity at the surface of the tested solutions was set to 7.0 mW/cm² (Radiometer RD 0.2/2 with TD probe, Optel). Cell viability was calculated according to bioluminescence emitted by the bacteria as measured with Microtox® M500 with Modern Water MicrotoxOmni 4.2 software.
3. Results and discussions

3.1. Synthesis of Lev-Fe₃O₄ and Lev-ZnPc-Fe₃O₄ nanoparticles

According to the literature, various polysaccharides have so far been used as coatings for magnetic nanoparticles, but to date, no reports are presenting a modification of the magnetic core surface with levans. In the Scopus database, the data concerning “magnetic nanoparticles coated with levans” did not yield any results as for 29th February 2020 [30]. It is worth noting that levans-coated magnetic nanoparticles (Lev-Fe₃O₄) were obtained as a new magnetic material for the first time in the herein presented study. The Lev-Fe₃O₄ nanoparticles were synthesized by co-precipitation of ferric and ferrous ions in the alkaline solution (pH = 14). For Lev coating, the epichlorohydrin was selected as a crosslinking agent due to the hydroxyl groups present in levans molecules. Initial research indicated that the obtained levans-coated magnetic nanoparticles might become the basis of a new system for binary selective anticancer therapy. Next, the levans-functionalized magnetic nanoparticles were combined with zinc(II) phthalocyanine (Lev-ZnPc-Fe₃O₄) to give a complex system. Due to the presence of zinc(II) phthalocyanine photosensitizer, the deposition of ZnPc on Lev-Fe₃O₄ nanoparticles was carried out in the absence of light. The synthetic strategy for the preparation of the Lev-ZnPc-Fe₃O₄ nanoparticles is presented in Fig. 2. It seemed that it would be possible to strengthen the antitumor effect by the combination of magnetic hyperthermia and clinically approved photodynamic therapy (PDT), which are known to induce a relatively good anticancer response independently.

3.2. Characterization of Lev-Fe₃O₄ and Lev-ZnPc-Fe₃O₄ nanoparticles

The Lev-Fe₃O₄ and Lev-ZnPc-Fe₃O₄ nanoparticles were characterized using various techniques to define the structure, morphology, size, and composition of the prepared magnetic nanomaterial.

The structure of the Lev-Fe₃O₄ and Lev-ZnPc-Fe₃O₄ hybrid nanoparticles was confirmed by ATR-FTIR spectroscopy. For comparison reason, the spectra of neat magnetite, levans, and zinc (II) phthalocyanine were also recorded (Fig. 3). In the spectrum of levans, the band at 3303 cm⁻¹ was assigned to OH stretching vibration of the polysaccharide, while the bands at 2903 cm⁻¹ and 2885 cm⁻¹ were assigned to C-H stretching vibration of fructose rings. The absorption bands appearing at 1123 cm⁻¹, 1012 cm⁻¹, and 925 cm⁻¹, represent the C – O – C symmetric bending vibrations of fructofuranose rings and glycosidic linkages [42]. In the ZnPc spectrum, the characteristic bands over the region 430–1600 cm⁻¹ were revealed. The bands at 1283 cm⁻¹, 1162 cm⁻¹, 1058 cm⁻¹, and 748 cm⁻¹ were assigned to the stretching vibrations within ZnPc macrocycle. A band at 1114 cm⁻¹ corresponds to the in-phase stretching of Zn-N bond coupled with benzene ring expansion, whereas a band at 1328 cm⁻¹ is due to in-phase Zn-N stretching coupled with benzene ring deformation. The band at 1482 cm⁻¹ is the result of the C-N-C bridge stretching [43,44]. For Lev-ZnPc-Fe₃O₄ nanoparticles, there were observed O-H vibration bands from the fructofuranose rings at 3257 cm⁻¹ and C-H stretching vibration at 2878 cm⁻¹ from the fructose rings. Furthermore, the bands observed at 1383 cm⁻¹ and 1414 cm⁻¹ were assigned to the C-N-C groups of the photosensitizer. All these bands confirmed the presence of the levans and zinc(II) phthalocyanine in the hybrid material. Moreover, the signal at 574 cm⁻¹ corresponds to the Fe-O group of magnetite [45].

The morphology and size of the obtained nanoparticles were studied using transmission electron microscopy. Prepared nanomaterials were rather spherical in shape with an average diameter of 10–15 nm. As shown in Fig. 4, the Lev-ZnPc-Fe₃O₄ nanoparticles aggregate. To determine the structure of the magnetic core, the electron diffraction was applied. On the SADP image (Fig. 4e), it can be seen that the obtained nanomaterials contain zinc nanoparticles present in cubic Crystal structure that was additionally confirmed by the XRD experiment. The XRD pattern (Fig. 4d) of the sample demonstrated six characteristic peaks for magnetite, marked by their indices ((2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1), (4 4 0)). These results were consistent with the standard JCPDA data (JCPDA card No. 19-629) [46] and indicated that the co-precipitation of the coating of the magnetic nanoparticles did not occur in the phase change of magnetite [47].

Furthermore, the prepared nanomaterial displayed a narrow size distribution with a hydrodynamic diameter of 15 nm, as indicated by the dynamic light scattering (DLS) measurement (Fig. 5). The polydispersity index of Lev-ZnPc-Fe₃O₄ nanoparticles was 0.174.

For a better characterization of the surface morphology, the adsorption-desorption isotherms were measured for the prepared composites (Fig. 6a). The primary adsorption data were the basis for the calculation of the so-called structural parameters, i.e. specific surface area and pore size distribution. The nitrogen isotherms only for Lev-ZnPc-Fe₃O₄ sample resulted in type-IV shape. The hysteresis loop in the range of 0.4–0.98 relative pressure represents the H2 type (according to IUPAC classification). The H2 hysteresis contains more complex pore networks consisting of pores with ill-defined shape and wide pore size distribution. The specific surface areas were calculated by Brunauer-Emmett-Teller (BET) surface adsorption method and were 2.5 m² g⁻¹, 5.6 m² g⁻¹, 9.5 m² g⁻¹ and 93.2 m² g⁻¹ for Lev, Lev-ZnPc, Lev-Fe₃O₄, and Lev-ZnPc-Fe₃O₄, respectively. Lev-ZnPc-Fe₃O₄ presents the highest surface area as compared to other nonporous materials. The pore size
distribution curve was calculated using a non-local density functional theory (NLDFT) method and was depicted in Fig. 6b. The PSD function of the Lev-ZnPc-Fe₃O₄ sample may be attributed mainly to the presence of micropores and small mesopores.

The magnetic properties of the Lev-ZnPc-Fe₃O₄ nanoparticles were studied using the Superconducting Quantum Interference Device (SQUID) technique at room temperature. As can be seen in Fig. 7, the saturation magnetization value of the prepared nanomaterial was 37.82 emu/g, while the neat magnetite was about 77 emu/g. Less magnetization of nanoparticles coated with levan and phthalocyanine (Lev-ZnPc-Fe₃O₄) results from the presence of a layer surrounding the magnetite core. The obtained values are comparable with the literature dimensions of the obtained Lev-ZnPc-Fe₃O₄ nanoparticles, which were consistent with superparamagnetic behavior and the nanoscale theory (NLDFT) method and was depicted in Fig. 6b. The PSD function of the Lev-ZnPc-Fe₃O₄ sample may be attributed mainly to the presence of micropores and small mesopores.

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The thermal stability of the samples was studied using thermogravimetric analysis (TGA). The observation of different stages of degradation was performed using thermogravimetric analysis (DTG). The analysis was performed for temperatures ranging from room temperature to 800 °C in the atmosphere of nitrogen. The results obtained for levan, ZnPc, and the magnetic nanoparticles are presented in Fig. 8 and listed in Table 1. As shown in Fig. 8, levan, and zinc(II) phthalocyanine underwent two degradation stages. At the first stage of levan degradation, approximately 9% of the initial weight loss occurred between 20 and 115 °C, due to the loss of moisture content. The main decomposition of Levan was observed between 190 °C and 300 °C, with 69% weight loss. It is probably caused by the breaking of the main chain linkages, as well as the breaking of bonds in the ring units. The first step in the decomposition of the zinc(II) phthalocyanine started in the range between 20 and 470 °C and was associated with about 10% weight loss. The second stage related to the main decomposition of ZnPc appeared about 470 °C. The 45% weight loss in this stage corresponds to the loss and fragmentation of the one unit present in the peripheral environment of the phthalocyanine molecule. The results obtained for both compounds are well-correlated with the literature data [51–54].

In Fig. 8, in the TG curve measured for the Lev-ZnPc-Fe₃O₄ nanoparticles, three degradation stages can be distinguished. In the first one appearing in the range between 20 and 155 °C, about 13% weight loss is observed, which is attributed to the evaporation of adsorbed water. The second step occurs in the range between 155 and 523 °C with about 15% weight loss, and the last one starts at 523 °C and corresponds to 9% weight loss. The carbonaceous residue at 800 °C correlates with about 63% weight loss.

Furthermore, at temperatures above 650 °C, no changes in TG and DTG curves were observed. This means that thermally stable carbonaceous coating was created at the surface of the nanoparticles, which protects the Fe₃O₄ against further transformation at very high temperatures [55].

To better understand the nature of the coating covering the magnetic core of the obtained nanoparticles, the AFM analysis, the contact angle measurement, and the mechanical properties of neat levan film and its combination with zinc (II) phthalocyanine were studied. The topographical atomic force microscope was used for morphological assessment of the samples and surfaces and allowed to obtain the topographic images and the roughness of scanned compounds. The AFM images of the Lev and Lev-ZnPc samples are shown in Fig. 9. While the surface of both samples is rough, Lev-ZnPc is more so, as evidenced by the values of roughness parameters (Table 1). Such rough topography may be advantageous for cell adhesion [56].

The obtained results of the contact angle measurements are shown in Table 2. As can be seen, the contact angle for levan with the non-polar liquid is almost the same as the contact angle of the polar liquid, and the dispersion component (γD) is higher than the polar component (γP), indicating the hydrophobic nature of the sample. However, the contact angle for the levan-zinc(II) phthalocyanine system is significantly smaller than for pure levan, which is indicative of the higher hydrophilicity of novel material.

The mechanical properties of the samples are shown in Table 3. The obtained results indicated that the deposition of photosensitizer in levan layer increases the tensile strength, Young’s modulus, and elongation compared with pure levan film. The Young’s modulus of Lev-ZnPc composite is 25% greater than that of neat levan, the elongation is 61% greater, and the tensile strength is approximately doubled. According to X. Chen [54], the neat levan films revealed poor mechanical properties, with toughness lower than 2 MPa and elongation of 2.21%, due to a highly branched and compact structure of levan that prevented significant intermolecular entanglement. The addition of zinc (II) phthalocyanine to the levan layer had a positive effect on levan’s mechanical properties. Therefore, despite the previously proposed application of such kind of hybrid material for anticancer PDT, it can also be applied for antimicrobial photodynamic therapy. Especially in dentistry, it can be relevant to endodontic treatment, where temporary...
dressings are used to disinfect dentine tubules [57]. Another possible application of the hybrid material concerns pharmaceutical technology and the preparation of dressings for the treatment of skin lesions [58].

3.3. Photochemical properties

3.3.1. Aggregation measurement of Lev-ZnPc-Fe$_3$O$_4$ nanoparticles

The formation of aggregates is a very common problem in photodynamic therapy, which affects the efficiency of singlet oxygen generation. Therefore, the ability of prepared Lev-ZnPc-Fe$_3$O$_4$ nanoparticles to form aggregates was studied using UV–Vis spectrophotometry, where several spectra were recorded at certain intervals of time. Plots of absorbance (A) against the concentration of magnetic nanoparticles were presented in Fig. 10. The equation $A = f(c)$ is a linear function which is in agreement with the Lambert-Beer law. Moreover, the differences in concentration did not cause any changes in the absorption bands. These results indicate that no aggregation of nanoparticles appears in this range of studied concentrations.

3.3.2. Singlet oxygen generation measurement of Lev-ZnPc-Fe$_3$O$_4$ nanoparticles

Studies of singlet oxygen generation ability are very important in assessing the potential effectiveness of new photosensitizing agents for photodynamic therapy. The singlet oxygen quantum yield ($\Phi_\Delta$) was determined using equation (1):
Fig. 6. (a) Nitrogen adsorption–desorption isotherms (b) Differential pore size distribution \(\frac{dV}{dw}\) vs. pore width plots obtained using NLDFT method of Lev-ZnPc-Fe\(_3\)O\(_4\) nanoparticles.

Fig. 7. Magnetic hysteresis loop of neat Fe\(_3\)O\(_4\) and Lev-ZnPc-Fe\(_3\)O\(_4\) nanoparticles.

Fig. 8. The TGA-DTG curves of the levan, zinc(II) phthalocyanine, and Lev-ZnPc-Fe\(_3\)O\(_4\) nanoparticles.
Table 1
Thermal parameters of the Lev, ZnPc, and the obtained magnetic nanoparticles.

| Sample          | First stage | Second stage | Third stage |       |
|-----------------|-------------|--------------|-------------|-------|
|                 | $T_{\text{max}}$ [°C] | $\Delta m$ [%] | $T_o$ [°C] | $T_{\text{max}}$ [°C] | $\Delta m$ [%] | $T_o$ [°C] | $T_{\text{max}}$ [°C] | $\Delta m$ [%] |
| Lev             | 47          | 9            | 190         | 229   | 69       | –           | –           | –           |
| ZnPc            | 270         | 10           | 470         | 632   | 45       | –           | –           | –           |
| ZnPc-Lev-Fe$_3$O$_4$ | 73          | 13           | 155         | 229   | 15       | 523         | 645         | 9           |

Fig. 9. AFM images of (a) Lev and (b) Lev-ZnPc films.

Table 2
Surface characterization of Lev and Lev-ZnPc samples.

| Sample | Roughness parameters [nm] | Average Contact Angle [$\theta$, °] | Surface Free Energy [mJ/m$^2$] |
|--------|---------------------------|------------------------------------|-------------------------------|
|        | $R_s$ | $R_a$ | $R_{\text{max}}$ | Measuring Liquid | Glycerin | Diiodomethane | $\gamma_s$ | $\gamma_s^d$ | $\gamma_s^p$ |
| Lev    | 4.20  | 3.17  | 31.0             | Glycerin                  | 73.4     | 71.1          | 45.33      | 28.61        | 16.72        |
| Lev-ZnPc | 9.16  | 7.76  | 48.0             | Glycerin                  | 45.6     | 61.9          | 27.59      | 11.48        | 16.11        |
where $\phi_{\Delta \text{ref}}$ represents the singlet oxygen quantum yield of the reference compound, $a$ and $a_{\text{ref}}$ are the regression coefficients of a straight line of the sample and reference compound, respectively.

The ability of the Lev-ZnPc-Fe$_3$O$_4$ to generate singlet oxygen species was estimated using a chemical method with zinc (II) phthalocyanine as a standard and DPBF as a singlet oxygen quencher. The UV–Vis spectrum (Fig. 10b) of the sample and DPBF blank in DMF solution were measured at ten-seconds intervals under a LED irradiation. The absorbance of the applied quencher at 409 nm decreased almost to zero in the presence of the prepared magnetic nanoparticles under irradiation. In comparison, the absorbance band at 670 nm remained unchanged, thus indicating the photostability and effective mediation of singlet oxygen generation by Lev-ZnPc-Fe$_3$O$_4$. The singlet oxygen quantum yield of the reference ZnPc is $\phi_\Delta = 0.56$ [39]. The singlet oxygen quantum yield measured for the obtained nanomaterial is $\phi_\Delta = 0.41$, which is 71% the value obtained for the standard. The quantum yields vary in the range of 0.3–0.8 for most photosensitizers, which means that the obtained value of $\phi_\Delta$ is relatively high. Moreover, the nature of the resulting Lev-ZnPc connection does not modify the photodynamic reaction of the hybrid material, which explains its photosensitizing activity. In addition, the obtained material combines the features of

| Sample   | Mechanical properties |
|----------|-----------------------|
|          | Tensile strength [MPa] | Elongation [%] | Young’s modulus [MPa] |
| Lev      | 0.98                  | 2.47           | 39.72                 |
| Lev-ZnPc | 1.98                  | 3.98           | 49.62                 |

![Fig. 10. (a) Absorption spectra and correlations between absorbance and concentration for Lev-ZnPc-Fe$_3$O$_4$ nanoparticles, (b) absorption changes during the determination of singlet oxygen quantum yield.](image-url)
both the photosensitizer and the nanomaterial intended for hyperthermia (see magnetization study).

4. Acute toxicity study

Toxicity of the prepared hybrid materials was evaluated by applying their suspensions to Microtox® acute toxicity test – 81.9% Screening Test, which was performed using Microtox® M500 equipment [40,41]. Microtox acute toxicity test is based on the measurement of A. fischeri bioluminescence change when exposed to toxic samples. The bioluminescence is correlated with the level of metabolism and, finally, cell viability. Microtox test can be used to determine the potential toxicity of the assessed materials and photosensitizers, which has been presented many times recently [32,41,59]. Suspensions of all obtained nanoparticles were prepared for the test: levan-coated nanoparticles (Lev-Fe3O4), levan, and photosensitizer (Lev-ZnPc-Fe3O4), as well as neat magnetite nanoparticles (Fe3O4). Toxicity for levan (Lev) suspension was also tested. The test was performed for suspensions in conditions not exposed to light and after irradiation with light at 665 nm of intensity 7.0 mW/cm², which corresponds to the clinically used doses in PDT. The results for suspensions at various concentrations of the materials are shown in Fig. 11.

As can be seen, a reduction in cell survival after irradiation is observed only for Lev-ZnPc-Fe3O4 nanoparticles. For levan, the cell viability is equal both in the dark and after exposure to light. For neat Fe3O4 nanoparticles, there was even an increase in cell survival observed after exposure to light as compared to the dark test. Importantly, the dark cytotoxicity associated with Fe3O4 nanoparticles was strongly reduced by the levan coating. The reduction of cell viability for ZnPc-bearing nanoparticles after 5 min of exposure to light was about 20% higher than that noted in the dark (EC50 values, respectively 3.8 and 5.1 mg/mL). It should be remembered that the amount of phthalocyanine is only about 3% by mass of nanoparticles, which gives 0.03 mg of photosensitizer per 1 mg of magnetic nanomaterial. The relationship between the increase in material toxicity and the concentration of nanoparticles with photosensitizer is shown in Fig. 12.

The initial toxicological results obtained constitute an excellent introduction to further research on the use of this material in PDT therapy. Currently, the cytotoxicity study on various cancer cell lines is ongoing and will be the subject of subsequent scientific reports.

5. Conclusion

In summary, levan-coated magnetic nanoparticles and levan-coated magnetite nanoparticles with zinc(II) phthalocyanine nanoparticles were obtained, both 15 nm diameter in size, and characterized with spectroscopic methods (ATR-FTIR) and electron microscopy techniques (SEM, TEM, AFM). The diffractometric analysis (XRD) proved that the core of the prepared material consists of pure magnetite. The thermogravimetric analysis of the Lev-ZnPc-Fe3O4 nanoparticles indicated that they are thermally stable at least up to 800 °C as the carbonaceous coating was created on their surface. Moreover, it was found that phthalocyanine constitutes about 3%, and the levan layer about 21% of the nanoparticle mass. The polymer shell coating MNPs (levan decorated with phthalocyanine) exhibits a hydrophilic character, and better mechanical properties than pure levan, which is particularly beneficial for applications in hyperthermia.
for biological applications. The singlet oxygen quantum yield value of 0.41 for the obtained Lev-ZnPcFe3O4 nanoparticles is high. Also, the aggregation study of the prepared nanomaterials showed that the differences in concentration did not cause any changes in the absorption bands. The Microtox© cytotoxicity test indicated that the reduction of cell survival for Lev-ZnPc-Fe3O4 nanoparticles after 5 min of exposure was about 20% higher than without light. For Lev-Fe3O4, no differences in survival in the dark or after light exposure were observed. This result can be considered as a good introduction for further research on the use of this novel material in PDT. In vitro and in vivo studies for the potential use of the prepared photosensitive nanomaterial as promising nano-agents are ongoing.

CRediT authorship contribution statement

**Dorota Chelmniak-Dudkiewicz**: Formal analysis, Investigation, Writing - original draft. Patryk Rybczynski: Formal analysis, Investigation, Visualization. Aleksander Smolarkiewicz-Wyczaschowski: Formal analysis, Investigation. Dariusz T. Mlynarczyk: Formal analysis, Investigation. Katarzyna Węgrzynowska-Drzymalska: Formal analysis, Investigation. Anna Ilnicka: Formal analysis, Investigation. Tomasz Goslinski: Formal analysis, Investigation, Writing - review & editing. Michal P. Marszall: Formal analysis, Investigation. Marta Ziegler-Borowska: Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing, Supervision.

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