Novel Photosensitizer Based on Bacteriopurpurinimide and Magnetite Nanoparticles

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A method of O-propyloxime-N-propoxybacteriopurpurinimide (dipropoxy-BPI) immobilization on magnetite nanoparticles was developed, which allows to overcome the photosensitizer aggregation, maintain high fluorescence of nanoparticles with loaded PS, and give to the latter the ability to dissolve in water. Biological tests on two lines of prostate cancer cells showed that the dipropoxy-BPI intracellular accumulation efficiency depends on tumor cells type and PS dosage forms.

Keywords: Photosensitizer, bacteriopurpurinimide, magnetite nanoparticles, photodynamic therapy, fluorescence diagnosis, magnetic resonance imaging.

Новый фотосенсибилизатор на основе бактериопурпуринимида и наночастиц магнетита

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Разработан способ иммобилизации O-пропилоксим-N-пропоксибактериопурпуринимида (дипропокси-БПИ) на наночастицы магнетита, позволяющий преодолеть агрегацию фотосенсибилизатора, сохранить высокую флуоресценцию наночастиц с загруженным ФС и придать последним способность растворяться в воде. Биологические испытания на двух линиях клеток рака простаты показали, что эффективность внутриклеточного накопления дипропокси-БПИ зависит от типа опухолевых клеток и лекарственной формы ФС.

Ключевые слова: Фотосенсибилизатор, бактериопурпуринимид, наночастицы магнетита, фотодинамическая терапия, флуоресцентная диагностика, магнитно-резонансная томография.
Introduction

Photodynamic therapy (PDT) of cancer is non-invasive method of oncotherapy, which comprises the selective accumulation of photosensitizers (PS) in the tumor tissue followed by the generation of active oxygen forms under the light irradiation of zone of interest by certain wavelength corresponding to PS absorption.[1-3] A tumor necrosis occurs in the process of this method implementation, whereas a normal tissue, where the PS concentration is much lower, does not damage. The majority of PS, used by the current moment, are natural or synthetic macroheterocyclic compounds including porphyrins, chlorins, phthalocyanines and their metal complexes.[4-5] The most preferable photosensitizers should absorb light in the range of 700-800 nm, where the absorption of endogenous chromophores is minor and penetrating light power in the tissue is high. Bacteriochlorophyll $a$ derivatives are promising IR-PS, however the lacking selectivity of its accumulation in the tumor tissue limits the therapeutic potential of these compounds.[6] Currently, there are two approaches to increase of PS tumor tropism. On the one hand, creating PS conjugates with vector molecules implements active targeting to tumor cells by receptor-mediated endocytosis.[7-9] On the other hand, PS immobilization onto the nanoparticles of different nature provides passive targeting, comprising the macromolecules extravasation from defective tumor vessels and their retention in the interstitium due to impaired lymphatic drainage tumor system (EPR-effect).[10-13]

Nanostructures based on the bacteriochlorin PS and the magnetic iron oxide nanoparticles, proposed in this paper could be considered as perspective theranostic agent for photodynamic therapy and MRI diagnosis. Among various iron oxides nanoparticles the researchers focus most of their attention to the magnetite nanoparticles, due to their low toxicity, the possibility of functionalization by biomolecules and relative aggregative stability.[14] 

Experimental

Materials and Methods

PS electronic-absorption spectra were recorded by a spectrophotometer Shimadzu UV-1800. Size of nanoparticles was measured using a Malvern Zetasizer by dynamic light scattering method. Organic solvents were purified by standard methods. $O$-Propyloxime-$N$-propoxybacteriopurpurinimide was derived from bacteriochlorophyll $a$, which was isolated from Rhodobacter capsulatus biomass in accordance with the previously described method.[15] Preparation of photosensitizer and its immobilization on the nanoparticles were conducted under an inert atmosphere and protect from light. In biological researches the preparations were analyzed by fluorescence microscopy EVOS (life technologies); Lens PlanFluor 60x/0.75. Images processing was carried out in the program ImageJ. Plotting of dependency diagrams of fluorescence intensity on the cells incubation time with the sample and calculation of the standard deviation values were performed in Microsoft Office Excel 2007 software.

Preparation of Nanoparticles

Iron oleate (800 mg), sodium chloride (10 mg), oleic acid (110 µl) and water (60 µl) were mixed with 1-octadecene (10 ml). The resulting solution was degassed by passing argon for full oxygen removal. Then the solution was heated to 320 °C at a rate 3.3 °C/min, held at this temperature for 120 min, and then cooled to room temperature. For the precipitation of the particles, isopropanol (30 ml) was added into the solution. The precipitated magnetite nanoparticles were isolated by centrifugation, washed three times with isopropanol and dissolved in hexane. Previously, according to this procedure, magnetite nanoparticles in the octapod-form were obtained.[16]

Photosensitizer Immobilization

$O$-Propyloxime-$N$-propoxybacteriopurpurinimide (3.0 mg) was dissolved in methylene chloride (1 ml) and added to 5 ml of magnetite nanoparticles solution in hexane (2.5 mg/ml). After incubation for 10 min, the solution was evaporated, nanoparticles were redissolved in hexane (3 ml) and passed through a filter.
having a pore diameter of 450 nm. Adding to the filtrate 3 ml of Pluronic F127 aqueous solution (15 mg/ml) gave a two-phase system that was stirred for 48 hours under argon, centrifuged with separation of the water-soluble nanoparticles loaded with PS. The latter were dissolved in deionized water (3.5 ml) and passed through a syringe filter with a pore diameter of 220 nm. The content of the nanoparticles and photosensitizer in the obtained solution is 2.9 mg/ml and 295 µg/ml, respectively.

**Preparation of Dipropoxy-BPI Micellar Solution**

The Pluronic F127 aqueous solution was prepared by mixing 90 mg of solubilizer with 2 ml of water. After stirring for 40 min dipropoxy-BPI (0.6 mg) dissolved in 2 ml of methylene chloride was added to the solution. Argon was bubbled through the resulting mixture heated to 41 °C until complete evaporation of the organic layer.

**Preparation and Testing of Cell Cultures in vitro**

Studies were carried out on cell lines LNCaP and PC-3 (human prostate cancer). Cells were grown in RPMI-1640 medium (gibco) supplemented with 10 % fetal bovine serum (FBS) (Sigma) and 1 % solutions of L-glutamine (200 mM, gibco), penicillin (10 U/ml) and streptomycin (10 µg/ml, gibco), and also vitamin solution for RPMI (Sigma). Cells were cultured under 37 °C and 5 % CO2. Cells were seated on the glass in Petri dishes at a concentration of 400000/ml for LNCaP and 200000/ml for PC-3. After one day, investigated PS samples with final concentration of 47 µg/ml were added to the cells in the culture medium. Fixation of the cells was carried out at 15, 30, 45, 60 and 120 min, preliminary washing medium without serum. As a fixator 3.7 % formaldehyde solution in PBS (1:10) was used. Fixation of the cells was carried out for 20 min, and then washed with PBS.

**Results and Discussion**

As the photosensitizer for immobilization on magnetite nanoparticles (nanostructured PS form) previously obtained bacteriochlorophyll a derivative – O-propyloxime-N-propoxybacteriopurpurinimide (dipropoxy-BPI) was chosen, which has an absorption maximum at 800 nm, thus providing the possibility of use for the treatment of deep-seated tumors at nanomolar concentrations of the drug. Furthermore, natural pigment has low dark toxicity, biodegrades and excretes rapidly enough. In current work, magnetite nanoparticles were chosen as means of dipropoxy-BPI delivery into the tumor tissue. Due to their superparamagnetic properties, in addition to the known benefits of nanoscale materials, magnetite nanoparticles could be utilized as contrast agents in magnetic resonance imaging. Photosensitizer immobilization on the surface of such nanoparticles would allow fluorescent tumor imaging, MRI diagnosis and treatment by PDT.

Spherically shaped magnetite nanoparticles were prepared by standard methods starting from ferric oleate. The average particle size according to the TEM was 12 nm (Figure 2). At the initial stage of study dipropoxy-BPI immobilization was carried out on magnetite nanoparticles coated with Pluronic F127 – an amphiphilic block copolymer of polyethylene and polypropylene, hydrophobic dyes and medicines solubilizer. However, when using this approach PS aggregation, accompanied by long-wavelength absorption band shift in the region of 900 nm, fluorescence quenching, and suppression of the singlet oxygen generation, was observed. Another approach involved hydrophobic PS immobilization on magnetite nanoparticles coated with oleic acid. This method included the preparation of spherical magnetite nanoparticles from iron(III) oleate followed by incubation with PS in methylene chloride. The obtained nanoparticles, loaded with PS, were treated by an aqueous solution of Pluronic F127. Using this approach allowed to overcome the photosensitizer aggregation and keep its spectral properties (Figure 3). After PS
loading and nanoparticles coating with Pluronic F127 average hydrodynamic diameter of particles, determined by dynamic light scattering (DLS), was 125 nm (Figure 4). Concentrations of PS and nanoparticles in the resultant solution were 2.9 mg/ml and 295 µg/ml, respectively. Thus, PS loading on nanoparticles surface was 9.2 wt%.

The study of intracellular accumulation of nanostructured dipropoxy-BPI was carried out on the two cell lines – LNCaP and PC-3. To make a comparison the same micellar form of photosensitizer in Pluronic F127 was taken. As a part of study, diffuse cytoplasmic staining of both cell lines, which were incubated with PS, comparing to control cells, in which culture medium PS was not added, was revealed (Figures 5, 6). To compare the accumulation dynamics of two PS forms – micellar and nanostructured – cells fluorescence intensity calculation was done. The results of investigation are given in Figure 5. In the case of LNCaP cell line (Figure 7A) fluorescence intensity and, consequently, the PS concentration in the cells increases quickly enough in the first 60 min of the experiment. However, further PS accumulation rate in the cells of the line begins to decrease. It is also worth noting, that the difference in the accumulation rate of micellar and nanostructured PS forms in LNCaP cell line is negligible. For PC-3 cell line,

**Figure 5.** Fluorescent micrographs of prostate cancer cells LNCaP with micellar form of dipropoxy-BPI in Pluronic F127 (A-E) and with nanostructured form of PS (F-J) at 15, 30, 45, 60 and 120 min after the pigment addition to the cells, and also the photograph of intact cells (K).

**Figure 6.** Fluorescent micrographs of prostate cancer cells PC-3 with the micellar form dipropoxy-BPI in Pluronic F127 (A-E) and c nanostructured form of PS (F-J) at 15, 30, 45, 60 and 120 min after the pigment addition to the cells, and also the photograph of intact cells (K).

**Figure 7.** Kinetics of micellar (1) and nanostructured (2) photosensitizer accumulation in LNCaP (A) and PC-3 (B) cell lines.
the accumulation of the two PS forms is most active in the first hour of co-culture. Subsequently, the PS content in the cells do not seriously change (Figure 7B). However, it should be noted, that a significant difference in accumulation efficiency of micellar and nanostructured PS forms for the cell line PC-3 was found. The latter occurred to be twice as much. This fact could be explained due to morphology differences of LNCaR and PC-3 cells, and different particle sizes. Herewith, conclusions on the PS particle size influence on its tumor tropism could be made after experiments on tumor-bearing animals.

Conclusion

As a result of current study nanostructured photosensitizer was obtained, which is considered as theranostic agent with combination of photodynamic potential of bacteriochlorophyll α derivative (diproxy-BPI) with cancer diagnostic abilities by magnetic resonance imaging due to the presence of magnetite nanoparticles in the structure.

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