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PHOTOBLEACHING OF NON-COVALENT COMPLEXES OF FOLIC ACID AND PHOTOSENSITIZERS

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Photodynamic therapy (PDT) is an alternative treatment for the control of malignant diseases. It is based on the action of a photosensitizer (PS) molecule, which, upon being excited by light in a determined wavelength, reacts with oxygen and generates reactive oxygen species in target tissues, leading to cell death. We perform the non-covalent binding of PS's with folic acid (FA) that have specific interactions with receptors, which are overexpressed on tumor cells to improve the targeted nature of PDT. The photobleaching of complexes and their components also was studied. The study showed that PS's were actively bonded with FA non-covalently, with the molar ratio of components up to [FA]/ [PS] = 10.8, depending on the structure of the PS. As the duration of illumination increases, the photostability of the samples decreases. 20 % glycerin had a stabilizing effect on all samples. Therefore, the presence of glycerin in the solution is advisable to obtain stable non-covalent complexes.

Photodynamic therapy – porphyrins – folic acid – photobleaching

Ֆոտոդինամիկ թերապիան (ՖԴԹ) չարորակ հիվանդությունների բուժման այլընտրանքային մեթոդ է: Այն հիմնված է ֆոտոսենսիբիլիզատորի (ՖՍ) մոլեկուլի ազդեցության վրա, որը որոշակի երկարության ալիքի լույսով գրգռման դեպքում փոխազդում է թթվածնի հետ ու թիրախային հյուսվածքում առաջացնում թթվածնի ակտիվ ձևեր, որոնք հանգեցնում են բջջի մահվան: ՖԴԹ-ի թիրախային բնույթի բարելավման համար ՖՍ-ները ոչ կովալենտ կապվել են ֆոլաթթվի հետ (ՖԹ), որը մենահատուկ փոխազդում է քաղցկեղային բջիջների վրա գերէքսպրեսվող ընկալիչների հետ: Նաև ուսումնասիրվել է կոմպլեքսների ու նրանց բաղադրիչների ֆոտոբլիչինգը (լուսագունաթափում): Ուսումնասիրությունը ցույց է տվել, որ ՖՍ-ները ոչ կովալենտ կապով ակտիվորեն կապվում են ՖԹ-ի հետ՝ մինչև [ՖԹ]/[ՖՍ] = 10.8 մոլային հարաբերությամբ՝ կախված ՖՍ-ի կառուցվածքից: Լուսավորման տևողության աճին զուգահեռ նյութերի լուսակայունությունը ընկնում է: 20 % գլիցերինն ունեցել է կայունացնող էֆեկտ բոլոր նմուշների դեպքում: Ուստի կայուն կոմպլեքսների ստացման համար նպատակահարմար է գլիցերինի առկայությունը լուծույթում:

Ֆոտոդինամիկ թերապիա – պորֆիրիններ – ֆոլաթթու – ֆոտոբլիչինգ

Фотодинамическая терапия (ФДТ) – альтернативный метод лечения злокачественных заболеваний. Он основан на воздействии молекулы фотосенсибилизатора (ФС), который при возбуждении светом определенной длины волны реагирует с кислородом и генерирует активные формы кислорода в тканях-мишенях, что приводит к гибели клеток. Чтобы улучшить нацеленность ФДТ, было выполнено нековалентное связывание ФС с фолиевой кислотой (ФК), специфически взаимодействующих с рецепторами, которые сверхэкспрессируются на опухолевых клетках. Было также изучено фотообесцвечивание комплексов и их компонентов. Исследование показало, что ФС активно связываются с ФК

нековалентно с молярным соотношением компонентов до [ФК]/[ФС] = 10.8, в зависимости от структуры ФС-а. С увеличением длительности освещения фотостабильность образцов снижается. 20% глицерин оказал стабилизирующее действие на все образцы. Следовательно, присутствие в растворе глицерина целесообразно для получения стабильных нековалентных комплексов.

Фотодинамическая терапия – порфирины – фолиевая кислота – фотообесцвечивание

Cancer is a worldwide health problem. Photodynamic therapy (PDT) is currently an alternative treatment for the control of malignant diseases. It is based in the uptake of a photosensitizer (PS) molecule, which upon being excited by light in a determined wavelength, reacts with oxygen and generates reactive oxygen species (radicals, singlet oxygen, triplet species) in target tissues, leading to cell death [6]. Various PS's based on porphyrins, chlorins, bacteriochlorins, and phthalocyanines [3]. PDT nowadays used worldwide in the treatment of tumors including skin basal cell carcinoma, lung, esophagus, bladder, head and neck, brain, ocular melanoma, ovarian, prostate, renal cell, cervix, pancreas and bone [10].

One of the ways to improve the targeted nature of therapy is the conjugation of the PS with molecules known to have specific interactions with receptors, which are overexpressed on tumor cells. Numerous cancer cell lines over-express folic acid (FA) receptors because of their fast growth and cell division [14][7]. Targeted PDT using (PS+FA) complexes appears to be a promising treatment for cancer.

Photochemical stability of PSs is the ability to withstand many cycles of excitation and back relaxation. The irreversible loss of original optical properties occurring as a result of these cycles is called photodegradation or photobleaching. It depends on dye structure, medium conditions, the intensity of excitation light [4] may affect the mechanism of photobleaching [6]. Photobleaching causes a loss of absorption of chromophores under light exposure. It is commonly accepted that the more stable the PS the better it will perform, mainly because it can endure more cycles of singlet oxygen ($^1O_2$) production [15].

The aim of this study was to gain non-covalent complexes of FA and PS's for targeted PDT and determine the photostability of free components and complexes.

Materials and methods.

Chemicals

1.1. Folic acid. Folic acid was obtained from the ACROS Organics (Folic Acid, 97% pure; Product code 216630100, *CAS Number*: 59-30-3).

1.2. Photosensitizers. The cationic porphyrins and metalloporphyrins, that are synthesized in Armenia and UK, selected here as an object of study. They are as follows: 1) zinc meso-tetra [4-N- (2'-oxyethyl) pyridyl] porphyrin (Zn-TOEt4PyP), 2) zinc-meso-tetra [4-N-butyl pyridyl] porphyrin (Zn-TBut4PyP), 3) TOEt4PyP. Also, we use the commonly used PS anionic Photosens for experiments (sulfonated aluminum phthalocyanine).

Complexation

The 0.01 M FA solution in 0.1 M phosphate buffered saline (PBS) was prepared according to [12]. FA and PS were mixed in a ratio of 4/1 and incubated for 48 hour, in the dark, at 5°C. Glycerin was added to the mixture of FA and PS to make it 20% in the final volume. After additional 48 hour incubation with glycerin, the unbonded components were purified by using Al_2O_3 (aluminum oxide) column chromatography with 0.1 M PBS containing 20 % glycerin as eluent. The absorption spectra of porphyrins and their complexes with FA were recorded on a Shimadzu UV-VISIBLE Recording Spectrophotometer UV-2100 (Japan) in a quartz cuvette (0.1 or 1 cm), in the range 200-850 nm. Fluorescence spectra were recorded on MPF 44 spectrofluorimeter (Perkin Elmer, USA). PS concentration was calculated by the UV-Vis spectrum from extinction coefficients [9] and the folic acid concentration was determined by fluorescence spectroscopy.

Photostability

Photostability studies were conducted by illuminating under similar conditions and following the absorbance changes over time of the PS's and their complexes with FA.

PS's, FA and non-covalent complexes (PS+FA) were irradiated by tungsten lamp with a range of a wavelength 380-1000 nm using an irradiance of 30 mW/cm^2, to a total of 1-hour duration. Changes in the absorption spectra were recorded for the Soret band (425-445 nm) of the porphyrin absorption and for FA absorption at 280 and 350 nm. The absorptions for samples were recorded during the irradiation and after irradiation for 0 min, 5 min, 15 min, 30 min and 1 hour.

Results and Discussion. FA is considered an unstable compound and in the complexes with PS's FA spectrum changed over time. 20 % glycerin was selected as a stabilizer to obtain stable complexes. Photostability of samples was studied in the presence and absence of glycerin. Vitamin A (and its derivatives), Thiamin, Riboflavin, Niacin, Pantothenic Acid, Cyanocobalamine, Folic Acid, Vitamin E, Vitamin D and Vitamin K are protected against loss of activity when various levels of glycerin are present [8]. It has been observed that the replacement of water by glycerin improved the FA stability in liquid [16]. Therefore, glycerin was selected as a stabilizing agent for non-covalently bonded complexes (PS+FA).We obtain stable non-covalent complexes of FA with cationic porphyrins TOEt4PyP, Zn-TOEt4PyP, Zn-Tbut4PyP and anionic PS Photosens. Therefore, glycerin was selected as a stabilizing agent for non-covalently bonded complexes (PS+FA). FA exhibits good stability in 0.1 M PBS that contains 20% glycerol. Therefore, later the complexes with FA were obtained in the presence of 20 % glycerin.

Free folic acid had one fluorescence emission peak: excitation 370 nm, emission 450 nm. Peaks of FA emission are observed at 433/470 nm in complexes with PS.These changes in the shape of the FA peak (two peaks) and a shift both towards short wavelengths (-17 nm) and towards long wavelengths (+20 nm), apparently, indicate the binding of FA to PS's non-covalently (fig. 1).

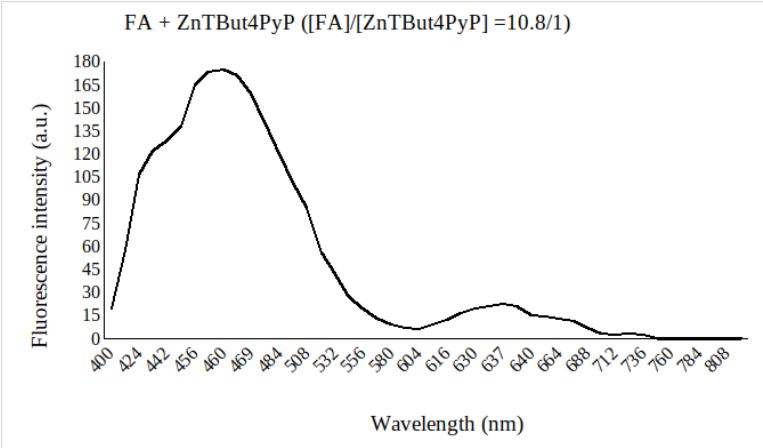

Fig.1. Fluorescence spectrum of (FA + Zn-TBut4PyP) complex.

Afterwards, we perform a photostability study of (FA+PS) complexes and free components (FA and PS's) before illumination and after 5 minutes, 15 minutes, 30 minutes and 1 hour of continuous exposure. Fig. 2 presents the results of the photobleaching.

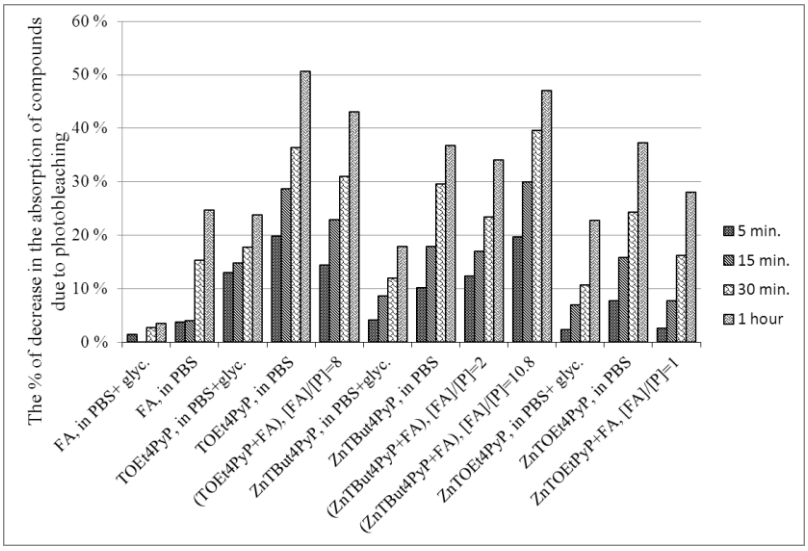

Fig. 2. Photobleaching of FA, PS's and their complexes. The calculations were performed with respect to unlit samples (before illumination).

Two absorption bands of FA appeared in the spectral ranges 200–300 and 300–400 nm, which were assigned to the π-π*and n-π*electronic transitions, respectively, of the pterin and p-amino benzoyl acid moieties of FA [2]. FA absorption peaks at 280 nm and 350 nm are characterized for photobleaching study. After 30 minutes of FA illumination in 0.1 M PBS containing 20% glycerol, the absorption at 280 nm decrease by 2.7 % (hypochromic shift) and at 350 nm increaseby 20.7% (hyperchromic shift). As a result of 1 hour illumination, the absorption at 280 nm decrease by 3.45% (hypochromic shift) and at 350 nm increase by 20.7% (hyperchromic shift). Illumination of folic acid in 0.1 M PBS containing 20% glycerin also led to the shift of bands to longer wavelength (bathochromic shift) by 2.5 nm and 16 nm, at 280 nm and 350 nm, respectively (fig. 3).

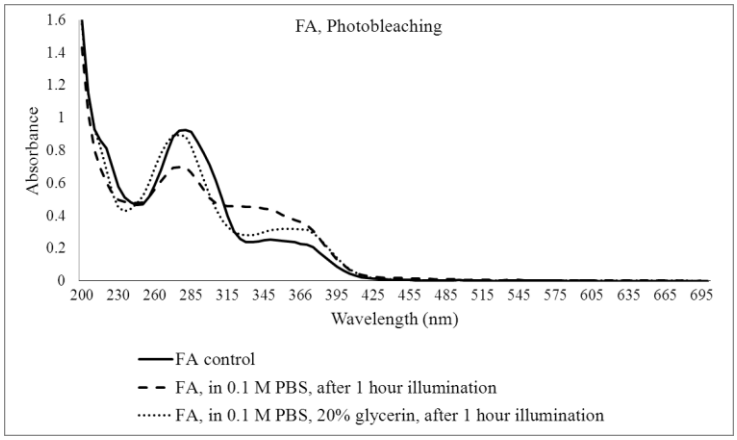

Fig.3. FA after 1 hour of photobleaching in the PBS with and without glycerin.

In case of FA in the 0.1 M PBS, without glycerin, we observe no wavelength shifts at 280 nm and 350 nm after 1-hour illumination. However, the absorption changes (decrease in absorption at 280 nm and increase of absorption at 350 nm) due to illumination are more significant than in a solution containing 20% glycerin. 30 minutes of illumination result in a 5.7-times greater reduction in absorption at 280 nm (by 15.35 %) and a 1.9-fold greater elevation in absorption at 350 nm (by 39 %) compared with the 20 % glycerol containing FA in 0.1 M PBS. As a result of 1 hour illumination, the absorption decrease at 280 nm was 7.14 times higher (24.65%). Likewise, at 350 nm the intensity increased (by 42.83%) about 2 times higher than at 20 % glycerol containing FA solution in 0.1 M PBS (fig. 3).

In the study of [12] was noted that during UV exposure FA is converted into p-aminobenzoyl-L-glutamic acid (PGA) and 6-formylpterin (FPT), which is then further oxidized to pterin-6-carboxylic acid (PCA).

We can conclude that 1 hour illumination by a lamp with an irradiance of 30 mW/cm^2 cause the absorbance decrease of the band assigned to the π-π^* electronic transition, while the absorbance of the band assigned to the n-π^* electronic transition progressively increase in FA solutions with and without glycerin. These changes can indicate the photolysis of FA in aerobic condition, and as a result two photodegradation products, pterine-6-carboxylic acid (PCA) and p-amino-benzoyl-L-glutamic acid (PGA), are formed. Similar changes were observed by Baibarac et al [2] after UV irradiation and also the mechanism of this process was proposed by Akhtar et al [1].

The absorption changes of FA in the presence of 20% glycerin, in 0.1 M PBS is less than for FA solution with no glycerin, which indicate that glycerin increases the photostability of FA to photolysis, which is of great importance for the use of FA in targeted PDT. The stabilizing property of glycerin for FA in 0.1 M PBS is also obvious by the fact that after 15 minutes of illumination, the absorption changes at 350 nm were negligible after additional 15 and 30 minutes of illumination (figure 2). Conversely, in a glycerin-free solution, the absorption changes and the formation of photoproductsare continuous as the illumination duration increases.

We choose porphyrins in 0.1 M PBS, containing 20% glycerol in one case and no glycerin in the other, as porphyrin controls which have the same concentration as in complexes.

The presence of 20% glycerin in the solution of Zn-TBut4PyP dissolved in 0.1 M PBS, resulted in 2.46 and 2 times less photobleaching of the molecule compared to a porphyrin control solution in 0.1 M PBS that did not contain glycerin, after 30 minute and 1 hour illumination, respectively. This may be explained by the photostabilization of the porphyrin molecule by glycerin.

In our study, Zn-TBut4PyP forms two types of complexes with folic acid: high molecular weight complexes with molar ratio of components [FA]/[PS] = 10.8 and low molecular weight complexes with molar ratio [FA]/[PS] = 2. High molecular weight complex which contains more FA is more susceptible to photobleaching than low molecular weight complex, 1.7 and 1.38 times more, after 30 minute and 1 hour illumination, meaning that the photoproducts from the one chromophore cause the decay of a neighboring chromophore as the result of their interaction. The complex of Zn-TBut4PyP and FA is 1.9 times more susceptible to photobleaching at 440 nm of porphyrin Soret band than the Zn-TBut4PyP that contains 20 % glycerin. We assume that the illumination of complex causethe formation of 6-formylpterin (FPT) and pterin-6-carboxylic acid (PCA), that can act as PS and therefore increase the rate of photobleaching of Zn-TBut4PyP in the complexes: as the concentration of FA increases, it led to formation of more FPT and PCA and consequently more intense photobleaching of porphyrin in the complexes. In turn, the decrease in FA absorption at 280 nm in the

high-molecular complex with Zn-TBut4PyP was 3.9 and 4.1 times greater than in the case of folic acid containing 20% glycerol, after 30 minute and 1 hour illumination, respectively. Also, the absorption elevation at 350 nm is 1,3 and 1,7 times less than in the case of folic acid with 20% glycerol, after 30 minute and 1 hour illumination, respectively. In the high molecular complexes of ZnTbut4PyP and FA, there is a shift of band at 280 nm to shorter wavelength by 6,5 nm and to longer wavelength at 350 nm by 10,5 nm. These changes may indicate that non-covalent binding of folic acid to porphyrin affects the production of photoproducts as a result of FA photolysis in the complexes.

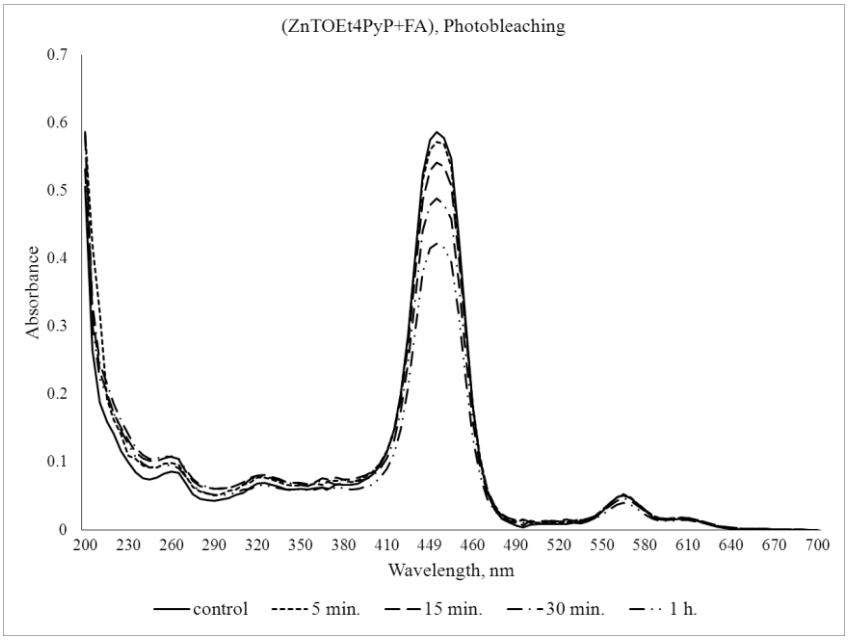

Fig.4. Photobleaching of (ZnTOEt4PyP+FA) complex.

Zn-TOEt4PyP in 0.1 M PBS with 20 % glycerin, photobleached 2.27 and 1.6 times less than the glycerin free Zn-TOEt4PyP, , after 30 minute and 1 hour illumination, respectively. Therefore, in this case,likewise, glycerin had a stabilizing effect from illumination.

Zn-TOEt4PyP non-covalently binds with FA with molar ratio FA/P=1. Absorption of the (Zn-TOEtPyP+FA) complex at 440 nm was significantly reduced by 1.5 and 1.2 times more than in the case of porphyrin in 0.1 M PBS containing 20% glycerol, after 30 minute and 1 hour illumination, respectively, which means that the complex with FA is more susceptible to photobleaching than porphyrin in 20 % glycerin.

In 20% glycerin, TOEt4PyP is photobleached twice as less than in the glycerin free solution, after both 30 minute and 1 hour illumination. TOEt4PyP forms non-covalent complexes with FA, molar ratio FA/P=8. In complex, TOEt4PyP photobleaches ~1,8 times more than TOEt4PyP in 20 % glycerin, after both 30 minute and 1 hour illumination, which may be the result of FA action on the TOEt4PyP in complex.

Anionic Photosens is a mixture of di-, tri-, and tetra-substituted fractions of sulfonated aluminum phthalocyanine with the number of sulfo group 3,4 [13]. The complexes of Photosens and FA were obtained with the molar ratio of components

FA/Photosens = 5.8. Absorption peak for Photosens is in the range of 676–679 nm. The Photosens in 20 % glycerin is 2,8 and 1,8 times more photostable than without glycerin, after 30 minute and 1 hour illumination, respectively. At the same time, the absorption decreases of Photosens and its complex due to photobleaching is insignificant (by 7,75 and 5,1% following 1 hour illumination).

FA forms non-covalent stable complexes with ZnTOEt4PyP, ZnTBut4PyP, TOEt4PyP and Photosens with different ratio of components for targeted PDT.

Illumination led to the loss of the absorbance in all bands of PS's (200 to 700 nm). In the case of FA, illumination led to the loss of the absorbance at 280 nm accompanied by an elevation of the absorbance at 350 nm. Increasing the illumination duration leads to gradual reduction in the absorption of photosensitizer in control samples and in complexes with FA. The formation of the new peaks at visible region did not occur due to illumination.

The illumination can lead to the formation of 6-formyl pterin and pterin-6-carboxylic acid (PCA) that act as a PS and therefore the complexes photobleached more as FA concentration increased in complexes.

The addition of 20 % glycerin led to photostabilization of complexes and their free components.

List of abbreviations

ՖԴԹ - ֆոտոդինամիկ թերապիա
ՖԹ - ֆոլաթթու
ՖՍ - ֆոտոսենսիբիլիզատոր
ФДТ - фотодинамическая терапия
ФК - фолиевая кислота
ФС - фотосенсибилизатор
FA - folic acid
FPT - 6-formylpterin
PBS - phosphate buffered saline
PCA - pterin-6-carboxylic acid
PDT - photodynamic therapy
PGA - p-aminobenzoyl-L-glutamic acid
PS – photosensitizer
TOEt4PyP - meso-tetra [4-N- (2'-oxyethyl) pyridyl] porphyrin
Zn-TBut4PyP - zinc-meso-tetra [4-N-butyl pyridyl] porphyrin
Zn-TOEt4PyP - zinc meso-tetra [4-N- (2'-oxyethyl) pyridyl] porphyrin

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