Հայաստանի Գիտությունների Ազգային Ակադեմիա
Национальная Академия Наук Армении
National Academy of Sciences of Armenia

Հայաստանի Կենսաբանական Հանդես
Биологический Журнал Армении
Biological Journal of Armenia

• Փորձարարական և տեսական հոդվածներ • Экспериментальные и теоретические статьи • • Experimental and theoretical articles •

Biolog. Journal of Armenia, 4 (72), 2020

pH AS THE IMPORTANT FACTOR FOR THE COMPLEXATION OF PORPHYRINS WITH CERULOPLASMIN FOR PHOTODYNAMIC THERAPY OF TUMOR

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Cancer cells have inverted pH_i /pH_e (intracellular and extracellular) gradient compared to a normal cell. Higher acid extracellular microenvironment is a feature of cancer tissue. The pH changes may affect the process of photodynamic therapy (PDT) of tumor. pH-dependent complexation of porphyrins and metalloporphyrins with serum protein ceruloplasmin (CP) was studied. The aim of the research was to model the low pH conditions of cancer cells *in vitro*. The study showed that by decreasing pH from 6.9 to 6.0, as a result of the change of the microenvironment of porphyrin/metalloporphyrin + CP complex, the conformational changes of the protein occur. The porphyrins are mainly located in the inner parts of the protein. By decreasing pH, the amount of porphyrins that bind to the surface of the protein increased, which is also indicate the conformational changes of the protein.

Cancer – porphyrins – ceruloplasmin – photodynamic therapy – pH

Քաղցկեղային բջիջներն ունեն շրջված $pH_{ներ}$/$pH_{արտ}$ (ներբջջային և արտաբջջային) գրադիենտ՝ համեմատած նորմալ բջիջների հետ: Բարձր թթվային արտաբջջային միկրոմիջավայրը քաղցկեղային հյուսվածքի բնորոշ հատկանիշն է: pH փոփոխությունը կարող է ազդել ուռուցքների ֆոտոդինամիկ թերապիայի (ՖԴԹ) գործընթացի վրա: Աշխատանքում ուսումնասիրվել է պորֆիրինների ու մետաղապորֆիրինների pH-ից կախված կոմպլեքսառաջացումը շիճուկային սպիտակուց ցերուլոպլազմինի (ՑՊ) հետ: Ուսումնասիրության նպատակն է մոդելավորել քաղցկեղային բջիջների ցածր pH-ի պայմանները *in vitro*: Ուսումնասիրությունը ցույց է տվել, որ նվազեցնելով pH-ը 6.9-ից 6.0՝ պորֆիրին/ մետաղապորֆիրին + ՑՊ կոմպլեքսի միկրոմիջավայրի փոփոխության արդյունքում տեղի են ունենում սպիտակուցի կոնֆորմացիայի փոփոխություններ: Պորֆիրինները հիմնականում տեղակայված են սպիտակուցի գլոբուլի ներքին հատվածներում: Նվազեցնելով pH-ը՝ սպիտակուցի մակերեսին կապված պորֆիրինների քանակությունը մեծանում է, որը նույնպես վկայում է սպիտակուցի կոնֆորմացիայի փոփոխության մասին:

Քաղցկեղ – պորֆիրիններ – ցերուլոպլազմին – ֆոտոդինամիկ թերապիա – pH

Раковые клетки имеют инвертированный градиент pH_i / pH_e (внутриклеточный и внеклеточный) по сравнению с нормальной клеткой. Более высокая кислотная внеклеточная микросреда является особенностью раковой ткани. Изменения pH может влиять на процесс фотодинамической терапии (ФДТ) опухоли. В работе изучалась pH-зависимость комплексообразования порфиринов и металлопорфиринов с сывороточным белком церулоплазмином (ЦП). Целью исследования является моделирование условий низкого pH в раковых клетках *in vitro*. Исследование показало, что при снижении pH с 6,9 до 6,0 в результате изменения

микроокружения комплекса порфирина/металлопорфирина + ЦП-а происходят конформационные изменения белка.

Порфирины в основном расположены во внутренних частях белка. При уменьшении pH увеличивается количество порфиринов, которые связываются с поверхностью белка, что также указывает на конформационные изменения белка.

Рак – порфирины – церулоплазмин – фотодинамическая терапия – pH

Cancer remains a worldwide problem, being the disease with the highest impact on health [2]. PDT has proven to be an effective cancer therapy [17] and is a promising alternative treatment for controlling malignant diseases [2]. It based on a photochemical reaction between a light activatable molecule or photosensitizer (PS), light, and molecular oxygen. When these three harmless components are present together, reactive oxygen species are formed. These can directly damage cells and/or vasculature, and induce inflammatory and immune responses [17]. To overcome PDT limitations including its undesirable side effects, PS's have been conjugated with biological molecules to achieve a more specific delivery and accumulation in cancer tissue [13].

The therapeutic success rate of current anticancer drugs is quite low, especially for solid tumors. The main reason is that most of the conventional or traditional anticancer agents are low molecular weight drugs that tend to diffuse indiscriminately to all tissues and organs, thus causing serious adverse effects. However, biocompatible macromolecules with a molecular mass, larger than 40 kDa, showed preferential accumulation in solid tumors compared with normal tissues or organs. This phenomenon is known as the enhanced permeability and retention (EPR) effect [10]. The protein carriers are inherently biocompatible and enjoy the benefits of a nanometric size object. The main advantage of these interactions is that they provide a favorable environment to keep the PS in the monomeric, photoactive state. Protection of the bound PS from the solvent may also result in enhanced chemical stability of the compound [1].

The microenvironment of the PS will undoubtedly change upon conjugation to a large protein, and this may result in increased PS aggregation and lower yield of excited singlet and triplet states [12]. The use of endogenous transport systems for drug delivery is very promising, as it will reduce the uptake of drugs by macrophages and allow intracellular delivery of biologically active molecules [8].

The pH_e of cancer cells is more acidic than normal cells (fig. 1), which is correlate with cancer cell survival [9, 4]. Generally, pH_e values of the normal tissues (brain tissues, subcutaneous tissues, etc.) are in the rage of 7.2-7.5. However, pH_e of tumor cells is mildly acidic in the range of 6.4-7.0. pH_e of cancer cells is 0.3-0.7 pH units lower than that of corresponding normal cells [4].

The main purpose of the present research is to model of pH the extracellular conditions of tumor *in vitro* and study the effect of pH changes on complexation ability of cationic porphyrins and metalloporphyrins with serum protein ceruloplasmin (CP). CP is a α2-glycoprotein that contains 90 % of circulating copper in the body [6]. It is a 132-kD protein containing six copper atoms [7]. In this study the CP serves as a carrier of porphyrins, given the phenomenon of EPR effect and complexation studies of other serum proteins with PS's that already conducted by other research groups [13, 1, 12, 8, 18]. The pH dependence of complexation is important for better understanding the mechanism of drug uptake by cancer cells microenvironment.

Materials and methods. **Protein:** Human ceruloplasmin was obtained from the blood plasma of a donor. The protein was additionally purified by method of gel filtration

chromatography on column with a Sephadex G-150. The CP was obtained in monomeric form in a homogeneous state with a high spectral index of purity: $I = A_{280} / A_{610} < 20$ [15].

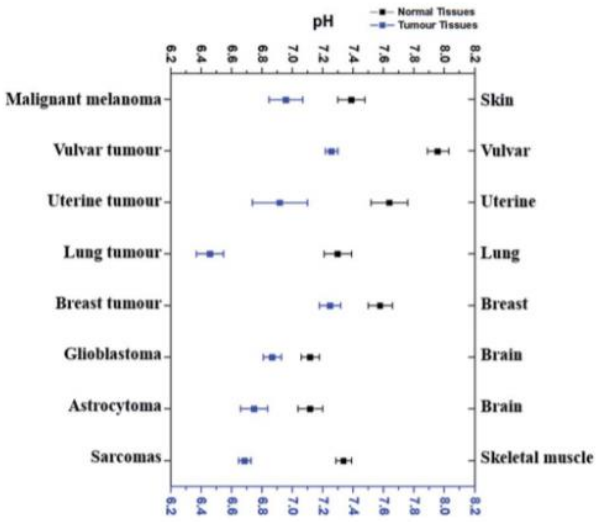

Fig. 1. The comparison of average extracellular pH values of different tumors with normal tissues [4]

Photosensitizers: The cationic porphyrins and metalloporphyrins, that are synthesized in Armenia and UK, selected here as objects of study (Fig. 2). They are as follows: 1) meso-tetra [4-N- (2'-oxyethyl) pyridyl] porphyrin (TOEt4PyP) (peripheral group R = $-CH_2-CH_2-OH$), 2) meso-tetra [4-N-butyl pyridyl] porphyrin (TBut4PyP) (peripheral group R = $-CH_2-CH_2-CH_2-CH_3$), 3) Zn-TOEt4PyP and 4) Zn-TBut4PyP. Also, we studied the interaction of ceruloplasmin with commonly used PS anionic chlorin e_6.

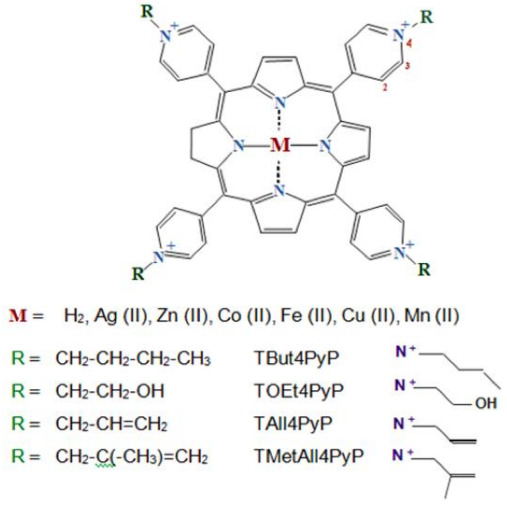

Fig. 2. Cationic porphyrins and metalloporphyrins. R- peripheral groups in the 4th position of the pyridyl ring, M- metal

Spectral studies: The complexation study was carried out by absorption and fluorescence spectroscopy methods in accordance with the work of Gyulkhandanyan A., et al [5]. The absorption spectra of porphyrins and their complexes with ceruloplasmin were recorded on a Shimadzu UV-VISIBLE Recording Spectrophotometer UV-2100 spectrophotometer (Japan) in a quartz cuvette (0.1 or 1 cm), in the range 200-800 nm. Changes in the absorption spectra were recorded for the Soret band (425-445 nm) of the porphyrin absorption. Fluorescence spectra were recorded on MPF 44 spectrofluorimeter (Perkin Elmer, USA). The luminescence kinetics of singlet oxygen was recorded on an FP-6500 spectrofluorimeter (JASCO, Japan) or Perkin-Elmer (USA) in a quartz cuvette (0.1 or 1 cm). All measurements were carried out at room temperature.

Changes of medium pH: The pH of the solution (0.01 M phosphate buffer /PBS/ pH 7.2) upon binding of PSs to CP was changed by adding a dilute solution of phosphoric acid (0.01 M or 0.001 M) to obtain the desired pH (from 6.9 to 6.0).

Results and Discussion. The study of complexation with CP for cationic porphyrins/metalloporphyrins and chlorin e_6 with a change in the medium of pH in the acidic region was carried out. As we can see from fig. 3, the decrease of pH causes the decrease of Soret band absorption in case of cationic and metalloporphyrins. But in case of chlorin e_6, the Soret band's absorption is slightly increases. These kinds of changes indicate the present of conformational changes of the protein.

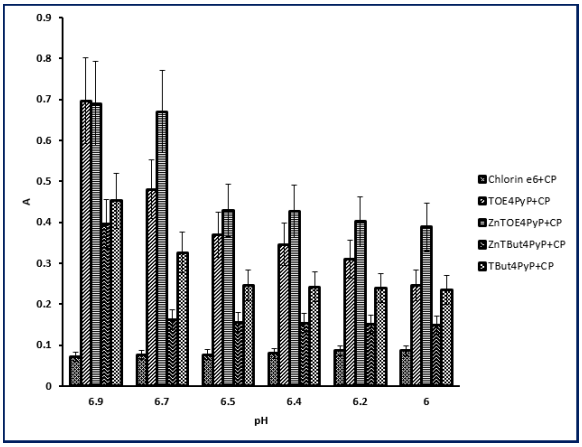

Fig. 3. Changes in the absorption (A) of complexes (CP +PS) at different pH values for the Soret band (425-445 nm)

A monomeric free-base porphyrin H_2-P in aqueous solution can add protons to produce mono H_3-P^+ and dications H_4-P^{2+} at very low pH, or loose protons to form the centrally monoprotic H-P^- at pH about 6.0 or aprotic P^{2-} species at pH $\geq$ 10. The change in spectra upon addition of acid or basic substances can generally be attributed to the attachment or the loss of protons to the two imino nitrogen atoms of the pyrroline-like ring in the free-base of porphyrin. The N-protonation induced a red-shifts [3]. In our study the absorption red shift is observed in case of cationic porphyrins and metalloporphyrins (from 1 to 10.5 nm) so it's possible that the N-protonation phenomena is the reason of this bathochromic effect (tab.1).

The aggregation and dimerization of porphyrins and metalloporphyrins is dependent strictly on ionic strength, pH and solvent composition, and so on. As predicted by Kasha, in case of J-type aggregates (side-by-side) the red-shift of spectra is observed, and in case of H-type (face-to-face)- blue-shift of the porphyrin absorption occurs [3, 16].

Table 1. The shift of Soret band peak for various PSs at different pH values (6.9 to 6.0)

Δλ of Soret Band, pH 6.9-6.0	TOEt4PyP +CP	ZnTOEt4PyP +CP	TBut4PyP +CP	ZnTBut4PyP +CP	Chlorin e_6+CP
	Δλ = 10.5 nm	Δλ = 1 nm	Δλ = 5.5 nm	Δλ = 5 nm	Δλ = -4 nm

So, the bathochromic (red shift) effect of cationic porphyrins absorption band can be a explained by the formation of J-type aggregates within the complex, and in case of anionic chlorin e_6 the hypsochromic shift (blue shift) indicates the formation of H-type aggregates.

The shift of absorption indicates that chromophores that located in the inner part of the protein in response to changes of pH are relocated to the surface of the protein globule. The opposite effects in case of anionic and in case of cationic PSs can be explained by the charge differences. Due to this the location of PS's with different charges in/on the protein is differ (fig. 4).

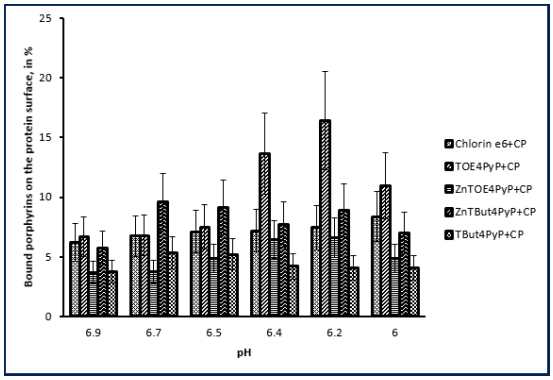

Fig. 4. Binding of porphyrins on the protein surface in complexes at different pH values, in %.

According to our study, the porphyrins are mainly located inside the protein globule. With decrease of pH the amount of porphyrins on the surface was increased which also indicate the pH-dependence conformational changes (fig. 4). It is important to note, that the complexation and the decrease of pH does not cause the dimerization of the protein according to chromatographic studies.

Thus, it has been shown that with a decrease of pH from 6.9 to 6.0 the conformation changes of the CP occur. The porphyrins are mainly buried in the cavity of the protein. Addition of acidic or basic substances (pH change) can lead to the attachment or the loss of protons to the two imine nitrogen atoms of the pyrroline-like ring in the free base of porphyrin.

REFERENCES

1. *dos Santos A.F., De Almeida D.R.Q., Terra L.F., Baptista M.S., Labriola L.* Photodynamic therapy in cancer treatment - an update review. J Cancer Metastasis Treat. 1-20, 2019.
2. *Giovannetti R.* The Use of Spectrophotometry UV-Vis for the Study of Porphyrins. Macro To Nano Spectroscopy. 87-108, (Ed. By Jamal Uddin), IntechOpen, 2012.

3. *Gyulkhandanyan A., Gyulkhandanyan L, Ghazaryan R., Fleury F., Angelini M., Gyulkhandanyan G., Sakanyan V.* Assessment of new cationic porphyrin binding to plasma proteins by planar microarray and spectroscopic methods. Journal of Biomolecular Structure and Dynamics, *31*, 4, 363-375, 2013.
4. *Hahn S.H., Jung S., Chang I.J.* Population Screening for Wilson Disease. Chapter 26, in "Clinical and Translational Perspectives on Wilson Disease", 287-296, (Ed. By Kerkar N. and Roberts E.A.), Elsevier Inc., 2019.
5. *Hally C., Delcanale P., Nonell S., Viappiani C., Abbruzzettiormac S.* Photosensitizing proteins for antibacterial photodynamic inactivation. Translational Biophotonics, 1-11, 2020.
6. *Hao G, Xu Z. Li L.* Manipulating extracellular tumor pH: an effective target for cancer therapy. RSC Advances, *39*, 8 , 22182-22192, 2018.
7. *Harris Z.L.* "Ceruloplasmin". Chapter 9, in "Clinical and Translational Perspectives on Wilson Disease", 77-84, (Ed. By Kerkar N. and Roberts E.A.), Elsevier Inc., 2019.
8. *Lebedeva N.S., Yurina E.S., Gubarev Y.A., Syrbu S.A.* Effect of pH on Albumin Binding with Hydrophobic Porphyrins. Russ. J. Gen. Chem., *89*, 565-569, 2019.
9. *Longhi A.* Manipulating pH in Cancer Treatment: Alkalizing Drugs and Alkaline Diet. Journal of Complementary Medicine & Alternative Healthcare, *2*, 1, 1-5, 2017.
10. *Maeda H., Islam W.* "Overcoming barriers for tumor-targeted drug delivery: the power of macromolecular anticancer drugs with the EPR effect and the modulation of vascular physiology", in "Polymer-Protein Conjugates From PEGylation and Beyond", 41-58, Elsevier Inc., 2019.
11. *Moan J., Rimington C., Evensen J.F., Western A.* "Binding of Porphyrins to Serum Proteins", in "Methods in Porphyrin Photosensitization. Advances in Experimental Medicine and Biology", *193*, 193-205, (Ed. By Kessel D.), Boston, MA, Springer, 1985.
12. *Orosz Á., Csik G.* "Peptide/protein conjugates of photosensitizers", in "Amino acids, peptides and proteins", *40*, 100-145, (Ed. By Rzadnov M., Hudecz F.), London, Royal Society of Chemistry, 2016.
13. *Pereira P.M.R., Carvalho J.J., Silva S., Cavaleiro J.A.S., Schneider R. J. Fernandes R., Tomé J.P.C.* Porphyrin conjugated with serum albumins and monoclonal antibodies boosts efficiency in targeted destruction of human bladder cancer cells. Org. Biomol. Chem., *12*, 1804-1811, 2014.
14. Ryden L. "Ceruloplasmin", Chapter 2, in "Copper Proteins and Copper Enzymes", Vol. III, 37-100, (Ed. by Prof. Rene Lontie), Taylor & Francis Group, Reissued 2018 by CRC Press.
15. *Sokolov A.V., Dadinova L.A. Petoukhov M.V., Bourenkov G., Dubova K.M., Amarantov S.V., Volkov V.V., Kostevich V.A., Gorbunov N.P., Grudinina N.A., Vasilyev V.B., Samygina V.R.* Structural Study of the Complex Formed by Ceruloplasmin and Macrophage Migration Inhibitory Factor. Biochemistry (Moscow), *83*, 6, 701-707, 2018.
16. *Thorpe S.L., Snyder G.N., Mammana A.* Spectroscopic study of porphyrin self-assembly: Role of pH, time, and chiral template. Chirality, 1-12, 2019.
17. *van Straten D., Mashayekhi V., de Bruijn H.S., Oliveira S., Robinson D.J.* Oncologic Photodynamic Therapy: Basic Principles, Current Clinical Status and Future Directions. Cancers, *9*, 2-19, 2017.
18. *Vashchenko G., MacGillivray R.T.A.* Multi-copper oxidases and human iron metabolism. Nutrients, *5*, 7), pp. 2289-2313, 2013.

Received on 13.10.2020