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Novel Nanostructural Hybride Materials for Photodynamic Theraphy

Maria Nowakowska,[*1] *Kinga Nawalany,*[1] *Mariusz Kępczyński,*[1] *Zdzisław Krawczyk*[2]

Summary: Photodynamic theraphy (PDT) is a clinically approved method for treatment of cancer and some other diseases. It employs the combination of a drug (photosensitizer) and light to induce photoxicity towards the cancerous cells. The efficiency of currently used photosensitizers is limited due to their aggregation in aqueous media and low chemical purity; usually a mixture of various isomers is used. This paper presents the results of our studies on the development of nanostructural materials for PDT. They are constructed from porphyrin (Po) which is covalently attached to the chain of hydrophylic polymer such as poly(methacrylic acid) (PMA) or poly(ethylene glycol) (PEG) and solubilized in lipid bilayer of liposome vesicles. The attachment of Po to the polymer chain improves its solubility in water while the solubilization in liposome carriers helps the dye to penetrate the cell membranes. Physicochemical and photophysical properties of those systems were determined. The *in vitro* studies on cancer cell lines demonstrated that the photosensitizers are efficiently accumulated in the cells. Their dark toxicity is negligible, while phototoxicity is very high.

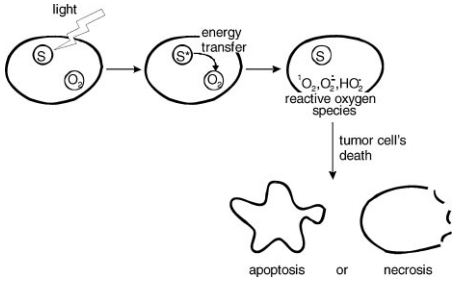
Keywords: photodynamic theraphy; polymeric photosensitizers; porphyrins; tumor cells

Introduction

Photodynamic theraphy (PDT) is a clinically approved method for treatment of cancer and some other diseases.[1] It is considerably less damaging than other medical procedures such as radiation therapy or surgery. PDT involves the administration of a photosensitizer which is then activated using an external light source in the presence of oxygen (see Scheme 1). The photosensitizer is preferentially accumulated in malignant tissue. The molecules of photosensitizer located in tumor tissue are excited with the use of properly chosen light source: lamp or laser (directly or via optical fibers). The electronically excited molecules are quenched by molecular oxygen what results in formation of very reactive cytotoxic oxygen species, mainly singlet oxygen. Singlet oxygen participates in oxidation of biological material.

Most of the photosensitizers which are currently clinically used are derivatives of porphyrin. All of them are large hydrophobic molecules which tend to aggregate in aqueous environment. The aggregation decreases the efficiency of photosensitizer performance. To ensure the expected efficiency, the dose of the photosensitizer is usually increased, which makes the side effects more probable. Considering all above, one can conclude that there is a need for considerable improvement. The improved photosensitizer should be characterized by the following features: (i) be hydrophilic to ensure the solubility in water and low tendency for aggregation; (ii) be liophilic enough to allow penetration to the cell membrane; (iii) absorb the light from

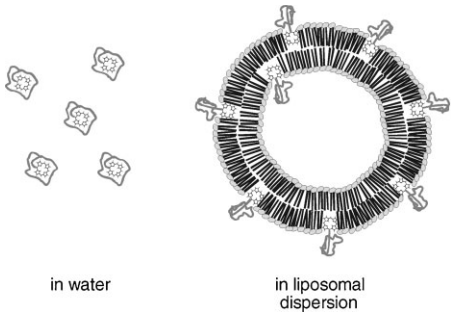
[1] Faculty of Chemistry, Jagiellonian University, 30-060 Kraków, Ingardena 3, Poland, Fax: (+48) 634 05 15; E-mail: nowakows@chemia.uj.edu.pl
[2] Comprehensive Cancer Centre, 44-101 Gliwice, Wybrzeże Armii Krajowej 15, Poland



Scheme 1.
Mode action of the photosensitizer (S) in tumor cell.

the visible region, possibly in long wavelength as it can penetrate deeper into the tumor; (iv) be photochemically stable; (v) efficiently generate singlet oxygen; (vi) be preferentially taken-up by cancer cells; (vii) have negligible dark cytotoxicity.

Although it is difficult to find the system which would fulfill all of these requirements, there are a lot of attempts reported in literature to prepare more efficient and at the same time more save photosensitizers. In this paper we will present our contribution to that field of research. We have taken a following general approach. Porphyrins were used as photochemically active dyes. They were covalently attached to the hydrophilic polymer chain and, if necessary solubilized in liposome bilayer. The liposomes were used as carriers of the photoactive molecules. Scheme 2 shows the structure of liposome-polymer system.



Scheme 2.
Visualisation of the solubilisation of porphyrin attached to the polymer chain in lipid bilayer and formation of liposome-porphyrin-polymer hybride.

Physicochemical and photopysical properties of such hybrids were determined. The *in vitro* tests carried out on human colon adenocarcinoma (line Hct-116) cell lines allow to determine the inclusion of the photosensitizers into the cell. The results are very promising.

Experimental Part

Poly(methacrylic acid)-5-(4-acryloyloxyphenyl) 10,15,20-tritolylporphyrin (PMA-Po) with no more than one porphyrin chromophore at the end of the polymeric chain was synthesized by anionic polymerization.[2] The PMA chains contain 94 repeated units ($M_n = 8\,400$ g/M). Poly (ethylene glycol)-5-(4-hydroxy methyl-phenyl)-10,15,20-tritolylporphyrin (PEG_{8000}-Po) ($M_n = 8\,000$ Da) with the same, as in the case of PMA-Po, porphyrin chromophores at the end of the polymeric chain was obtained by the method described in literature.[3] Series of pegylated 5,10,15,20–tetrakis(4-hydroxyphenyl)porphyrin with PEG chains of 350, 2000 and 5000 Da were synthesized as described in literature.[4,5]

Model Studies

In order to determine the effect of attachment of polymer chain on physicochemical and photophysical properties of Po as well as its ability to interact with liposomes the model studies were performed. Well defined PMA-Po, of low polydispersity and containing one Po chromophore attached at one chain end of PMA was prepared and studied.[2] The measurements of the absorption and emission spectra indicated that the attachment of the Po to the polymer chain did not influence considerably its spectral and photophysical properties. Porphyrin-poly(methacrylic acid) (PMA-Po) is very well soluble in water. The properties of that system are strongly dependent on pH due to the pH induced conformational changes of PMA chain and acid-base equilibria of Po chromophores. The studies on aggregation of Po were carried out at pH 8 (76.5% of

carboxylic groups dissociated and 97% of Po present in neutral form). The aggregation process was described using simple equilibrium monomer (Po) - dimer (D) model:

$$2\mathrm{Po} \rightarrow \mathrm{D}; \quad K_\mathrm{D} = [\mathrm{D}]/[\mathrm{Po}] \tag{1}$$

Based on the electronic absorption spectra of PMA-Po in aqueous solutions of various concentrations the equilibrium constant for dimerization was determined, $K_\mathrm{D} = 6.9 \times 10^4\ \mathrm{M}^{-1}$. The value of K_D is relatively low, and comparable with these for water-soluble tetraarylporphyrines (TArPs). The superiority of PMA-Po lies in fact that in a case of TArPs the negative charges on their sulphonate or carboxylic groups are distributed symmetrically around the pyrrole ring while in a case of Po-PMA they are located on the polymer chain. Such location of the charges is of great importance for interaction with lipid vesicles.

In the next step the interaction of PMA-Po with liposomes was studied. The partitioning of PMA-Po between the lipid vesicles and aqueous phase can be quantitatively described by an effective binding constant, K_b, which is defined as follows:

$$K_\mathrm{b} = \mathrm{c_L}/(\mathrm{c_w}\ [\mathrm{L}]) \tag{2}$$

The partitioning of PMA-Po was examined as a function of pH in the range from 6.5 to 9.2 using the spectroscopic titration technique. The values of K_b are dependent on pH and, what is important, the highest values are obtained for neutral pH, $K_\mathrm{b} = 127 \pm 14\ (\mathrm{mg/mL})^{-1}$. The K_b values for PMA-Po are much higher than these for commonly used photosensitizers: e.g. hematoporphyrin derivative ($12.2 \pm 0.3\ (\mathrm{mg/mL})^{-1}$ [6]), or Photofrin II ($9.2 \pm 0.8\ (\mathrm{mg/mL})^{-1}$ [6]). In order to predict the phototoxicity of liposome-PMA-Po system, the efficiency of singlet oxygen generation was determined. The quantum yield of singlet oxygen formation (Φ_Δ) by PMA-Po was found to be equal to 0.70 ± 0.03 in methanol and 0.88 ± 0.05 in liposome dispersion. The values are very high and comparable with these for TArPs in organic solvents.

The results of the model studies have suggested that the covalent attachment of Po to the hydrophilic polymer chain can produce promising photosensitizers characterized by: good solubility in water, lower aggregation of chromophores, efficient partitioning to the liposome vesicles and high efficiency of singlet oxygen generation.

Towards a System for Medical Applications. Pegylated Porphyrins

In the next step we have decided to prepare the system which would be more acceptable for medical applications. Thus we have replaced PMA chain by poly (ethylene glycol) (PEG). PEG was chosen as it is widely used as support in biomedical applications due to its low protein resistance, low toxicity and non-immunogenity.[7] It is used for steric stabilization of liposomes (preparation of Stealth Liposomes). Several photosensitizers with porphyrin covalently attached to PEG chain were synthesized and studied. They varied in type of Po chromophore attached to PEG, in length of PEG chain and in a number of PEG chains attached to single Po molecule. The first one was synthesized by covalent attachment of 5-(4′-acryloyloxyphenyl)-10,15,20-tri(p-tolyl)porphyrin (Po) to one end of the PEG chain ($\mathrm{M_n} = 8\,000$ Da). The $\mathrm{PEG_{8000}}$-Po is well-soluble in water and in organic solvents such as chloroform, methanol, DMSO and DMF. The polymer displays the absorption spectrum characteristics for porphyrin. The aggregation of the Po chromophores was studied, as described above, and found that the equilibrium constant for dimerization was low, $K_\mathrm{D} = 5 \times 10^3\ \mathrm{M}^{-1}$. That value is lower than these for water-soluble TArPs with non-ionic or negative periphery groups. Interaction of PEG-Po with liposomes was followed using several techniques. The dynamic light scattering (DLS) indicated that the addition of $\mathrm{PEG_{8000}}$-Po to the liposomes results in increase in their size. The studies carried out with the use of transmission electron microscopy confirmed

the data obtained by DLS. Interesting results were obtained from the measurements of zeta potential. It was found that the addition of PEG-Po (3.3 mol%) to liposomal dispersion results in pronounced reduction of zeta potential.

That observation can be explained assuming the adsorption of PEG-Po onto liposome bilayer. The PEG chains are exposed to the bulk solution shielding the negatively charged liposomal surface. That is expected to be important for penetration of cell membranes (*vide supra*). The presence of the hydrophilic polymer PEG layer at the liposome surface leads to polymer-coated, sterically stabilized liposomes (SSL). The partitioning of PEG_{8000}-Po between the lipid vesicles and aqueous phase was quantitatively studied using the fluorescence titration technique. The binding constant (K_b) was determined and found to be, $K_b = 24.6 \pm 0.9$ mg/mL. One can notice that the binding constant is considerably lower that that for PMA-Po. That observation can be explained considering the changes in the polarity of aqueous phase induced by PEG. The previous studies by Arnold et al.[8] have shown that the aqueous phase becomes less polar upon addition of PEG due to the hydrogen bond formation between PEG and water what reduces the rotational mobility of water molecules.

Then the effect of length of PEG chain on the physicochemical properties and photodynamic efficacy of photosensitizers was studied. For these studies, more hydrophilic porphyrin, 5,10,15,20 -tetrakis(4-hydroxyphenyl)porphyrin (mTHPP) was used. PEG chains of various molecular weights (350 Da (PEG_{350}-Po), 2000 Da (PEG_{2000}-Po) and 5000 Da (PEG_{5000}-Po)) were covalently attached to the porphyrin ring. The dimmerization constants of Po chromophores in aqueous solutions were determined and found to decrease with an increase in a chain length of PEG. This can be explained assuming that the porphyrin molecules are protected by the polymer chain. Thus their ability for direct interactions and π-stacking leading to the aggregation is reduced. To check that hypothesis we have performed the light scattering measurements and atomic force microscopy (AFM) observation. The DLS experiments have shown that hydrodynamic diameters of the objects presented in aqueous solutions of the pegylated porphyrins are considerably greater than these expected for individual PEG coil in aqueous solution. Moreover, they are weekly dependent on the molecular weight

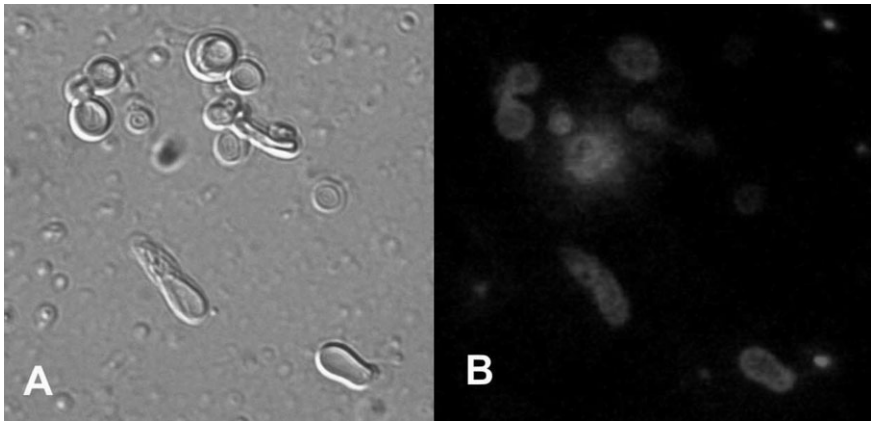


Figure 1.
Differential interference contrast (DIC) (A) and fluorescence microscopy (B) images of the liposomes with incorporated the pegylated porphyrin (PEG_{5000}-Po).

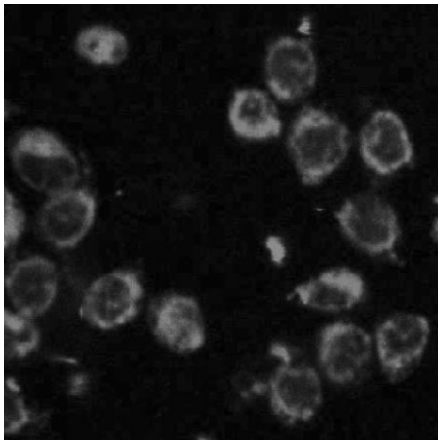


Figure 2.
Intracellular fluorescence image of human colon adenocarcinoma cell line (Hct116) treated with PEG_{5000}-Po (1 μg/mL, after 3 h) in confocal laser scanning microscope.

of PEG. That suggests that pegylated porphyrins form polymer clusters in the aqueous solution. The AFM images obtained for dry and hydrated pegylated porphyrins confirmed the presence of the well defined, separated objects in these systems. The hydration results in considerable increase in the volume of the macromolecules. That can be explained taking into account that approximately three water molecules are associated with one ethyl oxide unit.[9]

Interaction of the pegylated porphyrins with liposomes was visualized by fluorescence microscopy. Figure 1 shows, as an example, that liposomes treated with PEG_{5000}–Po exhibit a strong fluorescence of Po chromophores. This is a clear evidence that the porphyrin chromophore attached to the end of PEG chain can enter to the lipid bilayer. The effective binding constants of the pegylated porphyrins to liposomes were determined. The values of K_b were shown to be strongly dependent on the length of PEG chain. The binding constant is the highest for the system with the shortest PEG chain. But as for PEG_{8000}-Po, the values were much lower than these for the free porphyrin or PMA-Po. The lower partitioning of the pegylated porphyrins into liposomal membrane can be explained considering the presence of the polymer clusters in the aqueous solution.

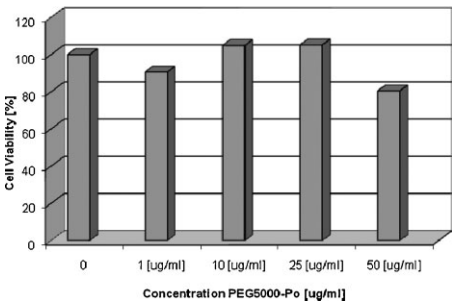


Figure 3.
Viability of cells HCT 116 after solubilization of PEG_{5000}-Po at various total concentrations.

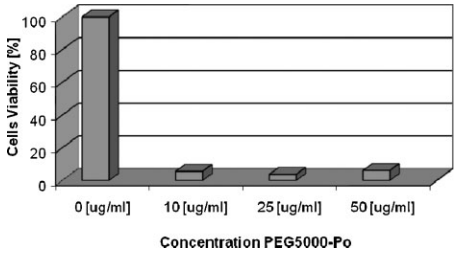


Figure 4.
Viability of Hct 116 cells after their irradiation absorbed by the solubilized PEG_{5000}-Po.

In the next step the *in vitro* studies on colon cancer cell line HCT 116 were performed. The cellular uptake of Po and the pegylated Po solubilized in liposomes were studied using the confocal laser scanning microscopy (Figure 2).

The growing intensity of intracellular fluorescence of porphyrins indicated its accumulation in the cells. It was observed that the photosensitizers (PEG-Po) encapsulated in liposomes were much more readily uptaken by the cells than these dissolved in a buffer solution. The cytotoxicity of these systems were than determined and found to be rather low (see Figure 3).

Finally, the phototoxicity of the sensitizers was evaluated. The cells containing sensitizers were irradiated for 2 min 30 s

with the red light and their viability was determined 24 hours after irradiation. It was found that all the photosensitizers studied are highly phototoxic.

Conclusions

Novel photoactive nanostructural materials useful for PDT were developed and studied. They used porphyrin as a chromophore. In order to decrease the aggregation of hydrophobic Po molecules in water, that dye was attached to the chain of hydrophilic polymers such as PMA or PEG. To improve the ability of the photosensitizers for penetration of the hydrophobic cell membrane the polymer-Po were solubilized in liposome vesicles. It was found that such a hybrid structures have very promising photophysical properties, especially very high efficiency of singlet oxygen formation. The *in vitro* tests have demonstrated that these photosensitizers accumulate readily in living cells, display low dark toxicity while very high phototoxicity.

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