SYNTHESIS AND PHOTOPROPERTIES OF A SUBSTITUTED ZINC(II) PHTHALOCYANINE–*N*-(2-HYDROXYPROPYL)METHACRYLAMIDE COPOLYMER CONJUGATE

Zhong-wei GU[a], John D. SPIKES[b], Pavla KOPEČKOVÁ[a] and Jindřich KOPEČEK[a,*]

[a] *Department of Bioengineering,*
2480 MEB, University of Utah, Salt Lake City, Utah 84112, U.S.A.
[b] *Department of Biology,*
University of Utah, Salt Lake City, Utah 84112, U.S.A.

Received April 2, 1993
Accepted June 7, 1993

Dedicated to Professor Otto Wichterle on the occasion of his 80th birthday.

In cancer photodynamic therapy (PDT), improved efficiency of photosensitizer delivery to tumors may be obtained by binding them to targetable water soluble polymeric carriers. However, attachment of photosensitizers to macromolecular carriers may alter their spectral and photosensitizing properties. In this study, a new monosubstituted phthalocyanine derivative, *N*-glycyl zinc(II) 4,9,16,23-tetraaminophthalocyanine (G-TAPC-Zn) was synthesized by the reaction of zinc(II) 4,9,16,23-tetraaminophthalocyanine (TAPC-Zn) with *N*-*tert*-butoxycarbonyl-glycine *N'*-hydroxybenzotriazole ester followed by deprotection of the *tert*-butoxycarbonyl (BOC) group. G-TAPC-Zn contains an aliphatic amino group suitable for attachment to water soluble polymeric carriers. By aminolysis of a polymeric precursor, an *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer containing oligopeptide (GFLG) side-chains terminated in *p*-nitrophenyl ester groups, with G-TAPC-Zn a polymeric derivative of the latter (P-GFLGG-TAPC-Zn) was synthesized. Spectral data indicated that in aqueous solutions P-GFLGG-TAPC-Zn formed aggregates. The degree of aggregation decreased with increasing concentration of detergents or organic solvents in buffer solutions. Consequently, the release of the drug from the carrier catalyzed by thiol proteinases, papain or cathepsin B, took place only in the presence of detergents or organic solvents, i.e., under conditions with a lower probability of aggregate formation. Binding of G-TAPC-Zn to HPMA copolymers decreased the quantum yield of singlet oxygen generation from 0.24 to 0.063 and significantly increased its resistance to photobleaching.

The combination of light and certain absorbing molecules, called photosensitizers, in the presence of oxygen, can lead to rapid cell destruction. It is most widely believed that the generation of singlet oxygen is ultimately responsible for the majority of such

---

* The author to whom correspondence should be addressed.

phototoxic effects, although other reactions, e.g., formation of radicals do indeed occur[1-3]. A reactive excited state of molecular oxygen, singlet oxygen, lies only 90 kJ mol^{-1} above the triplet ground state, enabling photosensitizers to efficiently catalyze its formation[1]. Because the lifetime of singlet oxygen is short (it varies from 10^{-6} s in an aqueous environment to 10^{-4} – 10^{-5} s in a hydrophobic environment[4]) its diffusion path in the absence of reactants is short. Consequently, the site of action of a photosensitizers is largely determined by its location[1]. Selective delivery of photosensitizers to tumor cells followed by irradiation are the rationale of PDT (refs[5,6]).

Attachment of drugs to macromolecules renders them lysosomotropic[7]. Consequently, the attachment of photosensitizers to water soluble polymeric carriers results in the localization of the carrier bound photosensitizer in the lysosomal compartment of the cell. Additionally attachment of targeting moieties, such as antibodies, or saccharides may direct the targetable photoactivatable polymeric drug to a subset of cells. A double targeting effect can be achieved by the subsequent localized application of light.

Phthalocyanines[8] have been suggested as effective photosensitizers for PDT(refs[9,10]). They absorb in the red region of the spectrum, permitting deeper light penetration into tissues when compared to porphyrins or chlorins. They have large extinction coefficients, high quantum yields of triplet formation and are nontoxic in the dark[9,10].

In this study, a new modified phthalocyanine, *N*-glycyl Zn(II) 4,9,16,23-tetraaminophthalocyanine (G-TAPC-Zn) was synthesized, and attached to *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers via oligopeptide (GFLG) side-chains. The rate of phthalocyanine release from the polymeric carrier catalyzed by thiol proteinases (papain and cathepsin B) was evaluated. Spectral, photophysical, photosensitizing, and photobleaching properties of G-TAPC-Zn and of its polymeric conjugate were also evaluated.

EXPERIMENTAL

Materials and Methods

4-Nitrophthalic acid, zinc sulfate and *N*-hydroxybenzotriazole were from Aldrich Chemical Co., GF, LG, *N*-*tert*-butoxycarbonyl-glycine, and papain were from Sigma. Cathepsin B (from bovine spleen) was a kind gift from Dr M. Baudyš. Highly purified Rose bengal, used as a standard for the measurements of quantum yields of singlet oxygen photogeneration, was kindly provided by Dr D. C. Neckers. All other chemicals used were obtained in the purest from commercially available. All solvents were of AR grade and were used directly without further purification.

All amino acids except glycine were of L-configuration. Single letter abbreviations were used for amino acids: G glycine; F phenylalanine; L leucine. Abbreviations BOC and ONp were used for *tert*-butoxycarbonyl and *p*-nitrophenoxy, respectively.

Buffers (aqueous): B 0.1 M NaH_2PO_4/Na_2HPO_4, pH 7.4; C 0.058 M Na_2HPO_4, 0.005 M citric acid, 0.002 M ethylenediaminetetraacetic acid (EDTA), pH 7.0; CTAB-B: 0.01 M cetyltrimethylammonium bromide (CTAB) in buffer B; D 0.051 M Na_2HPO_4, 0.024 M citric acid, 0.002 M EDTA, pH 5.0.

Fourier transform infrared spectra (KBr; $\tilde{\nu}$, cm^{-1}) were recorded on a Beckman FT 2100 apparatus. ^1H NMR spectra were recorded in CD_3SOCD_3 on a Varian VXR-500 instrument (500 MHz); chemical shifts are given in ppm (δ-scale). Absorption and fluorescence properties were measured with Perkin–Elmer Lambda 6 and/or Lambda 9 spectrophotometers, and a MPF-66 fluorescence spectrophotometer. Elemental analyses (C, H, N) were performed by Atlantic Microlab, Inc. or Galbraith Laboratories, the content of Zn was determined on a Perkin–Elmer 3100 atomic absorption spectrophotometer. The weight- and number-average molecular weights of HPMA copolymers were estimated by size exclusion chromatography on a Superose 6 column using a Pharmacia Fast Protein Liquid Chromatography system (0.05 M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) + 0.5 M NaCl, pH 8, as eluent) calibrated with poly(HPMA) fractions.

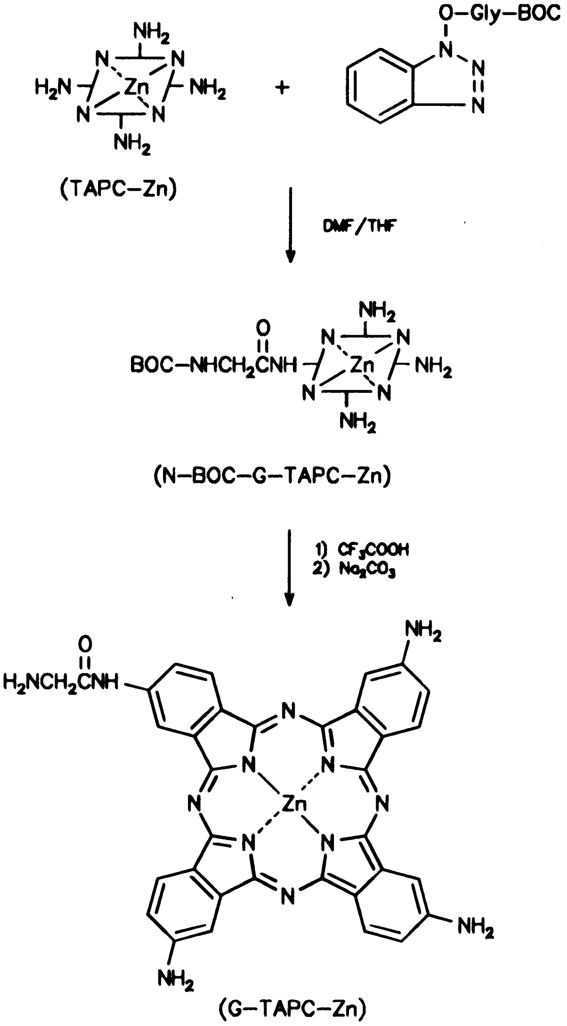
Synthesis

Zinc(II) 4,9,16,23-tetraaminophthalocyanine (TAPC-Zn). TAPC-Zn was prepared by a two step synthesis as previously described[11] (Scheme 1). First, zinc(II) 4,9,16,23-tetranitrophthalocyanine (TNPC-Zn) was prepared from 4-nitrophthalic acid, zinc sulfate heptahydrate, ammonium chloride, ammonium molybdate and excess urea in 82% yield. In the second step, the nitro groups of TNPC-Zn were reduced with sodium sulfide. Briefly, a mixture of finely groud TNPC-Zn (1 g), sodium sulfide nonahydrate (5 g), and water (25 ml) was stirred under reflux for 24 h. The solid product was isolated by centrifugation, treated with 1 M hydrochloric acid (75 ml), then with 1 M sodium hydroxide (50 ml). The dark green zinc complex was purified by repeated treatment with water, and dried in vacuo over phosphorous pentoxide; yield 0.9 g (90%). For $C_{32}H_{20}N_{12}Zn$. 2 H_2O (673.4) calculated: 57.03% C, 3.56% H, 24.95% N, 9.71% Zn; found: 57.27% C, 3.54% H, 24.85% N, 10.31% Zn. IR spectrum: 3 420 – 3 220, 1 608 (NH_2), 1 340 – 1 250 (–C–N), 1 490 (aromatic ring), 1 520, 1 335 (NO_2). ^1H NMR spectrum: 6.21 s, 8 H (NH_2); 7.39, 8.40 and 8.88 d, 12 H (substituted benzene).

N-(tert-Butoxycarbonyl)glycine N′-hydroxybenzotriazole ester (N-BOC-G-HBA). A solution of dicyclohexylcarbodiimide (4.17 g, 0.02 mol) in THF (50 ml) was added dropwise to a solution of *N*-BOC-glycine (3.50 g, 0.02 mol) and *N*-hydroxybenzotriazole (HBA) (2.76 g, 0.02 mol) in THF (50 ml) cooled to 0 °C; the reaction mixture was stirred 2 h at 0 °C and another 2 h at room temperature (RT). *N,N′*-Dicyclohexylurea that precipitated was filtered off, and the filtrate was evaporated to dryness. The white solid residue was recrystallized from THF–hexane (1 : 1, v/v) to give 3.66 g (63%) of N-BOC-G-HBA; m.p. 125 – 126 °C. For $C_{13}H_{16}N_4$ (292.3) calculated: 53.36% C, 5.47% H, 19.16% N; found: 53.43% C, 5.57% H, 19.16% N. IR spectrum: 3 450 – 3 420 (–NH–), 1 750 (COO–N), 1 715 (O–CONH), 2 975 – 2 850 (C–CH_3), 1 630 (substituted benzene), 855 (O–N). ^1H NMR spectrum: 1.38 s, 9 H (*t*-Bu); 3.58 d, 2 H (–CH_2–); 7.05 t, 1 H (–NH–); 7.41, 7.54, 7.70 and 7.98 t, 1 H; t, 1 H; d, 1 H; d, 1 H (substituted benzene).

N-(tert-Butoxycarbonyl)glycyl zinc(II) 4,9,16,23-tetraaminophthalocyanine (N-BOC-G-TAPC-Zn). A solution of N-BOC-G-HBA (0.13 g, 0.446 mmol) in THF (10 ml) was added to a solution of TAPC-Zn (300 mg, 0.446 mmol) and triethylamine (0.05 g, 0.49 mmol) in DMF–THF (15 ml : 25 ml) and the reaction mixture was stirred at RT for 24 h. All volatiles were rotoevaporated, the dark green solid residue was washed with diethyl ether and chloroform (to remove unreacted N-BOC-G-HBA and *N*-hydroxybenzotriazole), treated in a Soxhlet apparatus with ethyl acetate for 48 h, and dried in vacuo at 60 °C to give 350 mg of N-BOC-G-TAPC-Zn (95%). IR spectrum: 1 695 (O–CONH, CONH). ^1H NMR spectrum: 1.47 s, 9 H (*t*-Bu); 6.26 d, 6 H (NH_2).

N-Glycyl zinc(II) 4,9,16,23-tetraaminophthalocyanine (G-TAPC-Zn). A solution of N-BOC-G-TAPC-Zn (0.35 g, 0.421 mmol) in trifluoroacetic acid (15 ml) was stirred 1 h at 0 °C and an additional 1 h at RT. The reaction mixture was alkalized with aqueous sodium carbonate to pH 9 – 9.5, and the precipitate washed with water until sodium carbonate and sodium trifluoroacetate were removed. After drying in vacuo, 260 mg of G-TAPC-Zn were obtained (85%). A solution of G-TAPC-Zn (100 mg, 0.0685 mmol) in DMF (50 ml) was refluxed for 2 h in the presence of zinc acetate (90 mg, 0.205 mmol). The volume of the mixture was reduced by rotoevaporation to 5 ml, and poured into water (200 ml). The precipitated dark green solid was extensively washed with water, isolated by centrifugation and dried in vacuo at 60 °C; $\varepsilon_{722} = 1.19 \cdot 10^5$ l mol^{-1} cm^{-1} (DMSO). For $C_{34}H_{23}N_{13}Zn \cdot 2\,H_2O$ (730.4) calculated: 8.95% Zn; found: 8.22% Zn. IR spectrum: 1 670 (-CONH-). ^{1}H NMR spectrum: 6.37 d, 8 H (NH_2); 7.68 s, 1 H (CO-NH); 7.38, 8.41, and 8.92 m, 4 H; m, 4 H; m, 4 H; (in the ring).



SCHEME 1

Synthesis of monomers. *N*-(2-Hydroxypropyl)methacrylamide (HPMA, ref.[12]) and *N*-methacryloylglycylphenylalanylleucylglycine *p*-nitrophenyl ester (MA-GFLG-ONp, ref.[13]) were prepared as previously described.

Synthesis of polymer precursor. Poly[HPMA-*co*-(MA-GFLG-ONp)] was prepared by radical precipitation copolymerization[14] of HPMA (0.73 g, 96 mole %) and MA-GFLG-ONp (0.12 g, 4 mole %) in acetone at 50 °C for 24 h using 2,2′-azobis(isobutyro)nitrile as the initiator. The ratio of monomers to initiator and solvent was 12.5 : 0.6 : 86.9 wt.%. The copolymer was filtered off, washed with acetone and dry diethyl ether and desiccated. The copolymer was then reprecipitated from a methanolic solution into an excess of acetone, washed and dried. The content of ONp groups determined spectrophotometrically (ε_{270} = 0.95 . 10^4 l mol^{-1} cm^{-1}, DMSO) was 3.34 mole %. The weight-average molecular weight (31 000) and polydispersity (1.5) were determined, after aminolysis of the copolymer with 1-amino-2-propanol, by size exclusion chromatography as described above.

Synthesis of HPMA copolymer–N-glycyl Zn(II) 4,9,16,23-tetraaminophthalocyanine conjugate (P-GFLGG-TAPC-Zn). An amount of 500 mg (0.106 mmol ONp groups) of poly[HPMA-*co*-(MA-GFLG-ONp)] was dissolved in DMSO (8 ml). A solution of G-TAPC-Zn (0.093 g, 0.127 mmol) in DMSO (2 ml) was added, and the reaction mixture stirred for 24 h at RT. D,L-1-Amino-2-propanol (50 μl) was added to aminolyze the remaining ONp groups, and the mixture was stirred for additional 30 min. The copolymer was precipitated into an excess of acetone–diethyl ether (3 : 1, v/v), filtered, washed with acetone and diethyl ether, and desiccated. The copolymer was then dissolved in 15 ml methanol and applied to a Sephadex LH-20 column (50 × 3 cm). The polymer band was collected, evaporated to dryness, dissolved in deionized water (250 ml), and filtered. The filtrate was frozen and lyophilized to give 0.47 g of P-GFLGG-TAPC-Zn. This copolymer, poly[HPMA-*co*-MA-GFLGG-TAPC-Zn], contained 2.06 mole % G-TAPC-Zn as determined spectrophotometrically (ε_{722} = 1.19 . 10^5 l mol^{-1} cm^{-1}, DMSO).

Enzymatic Cleavage of P-GFLGG-TAPC-Zn

Papain (EC 3.4.22.2) and bovine spleen cathepsin B (EC 3.4.22.1) were used in the cleavage experiments. The concentrations of the enzymes in stock solutions were determined spectrophotometrically using ε_{277} = 5.78 . 10^4 l mol^{-1} cm^{-1} in buffer C and ε_{281} = 5.15 . 10^4 l mol^{-1} cm^{-1} (ref.[15]) in buffer D, respectively. The activity of papain (in buffer C, 107.7 U mg^{-1}) and cathepsin B (in buffer D, 13.5 U mg^{-1}) were determined using N^{α}-benzoyl-L-arginine *p*-nitroanilide as substrate[16].

Cleavage of P-GFLGG-TAPC-Zn by papain. The solution of enzyme, ethylenediaminetetraacetic acid (EDTA) and cetyltrimethylammonium bromide (CTAB) was bubbled with N_2 on ice for 5 min. Glutathione was added and the solution was preincubated for 5 min at 37 °C to activate the enzyme. Stock polymer solution was added, the sample flushed with N_2 and incubated in the dark at 37 °C. The final concentrations in the incubation mixtures were: papain 1.29 . 10^{-5} mol l^{-1}, glutathione 5.08 . 10^{-3} mol l^{-1}, EDTA 2.0 . 10^{-3} mol l^{-1}, G-TAPC-Zn 2.34 . 10^{-4} mol l^{-1}, CTAB 1.2 . 10^{-2} mol l^{-1}. A separate sample was used for each time interval. At a chosen time, 0.40 ml of sample was diluted with 2.1 ml of buffer C and applied on a Sephadex PD-10 column equilibrated with buffer C and 1 ml fractions were collected. The absorbance at 706 nm of each fraction was recorded and the percent of cleaved G-TAPC-Zn was calculated from the decrease of absorbancy of the polymer fractions.

Cleavage of P-GFLGG-TAPC-Zn by Cathepsin B. Buffer D (pH 5.0) was used. The procedure was similar to that described for papain. Final concentrations in the incubation mixtures were: cathepsin B 2.35 . 10^{-5} mol l^{-1}, glutathione 5.15 . 10^{-3} mol l^{-1}, EDTA 2.0 . 10^{-3} mol l^{-1}, G-TAPC-Zn 2.34 . 10^{-4} mol l^{-1}, CTAB 1.2 . 10^{-2} mol l^{-1}.

Photophysical Measurements

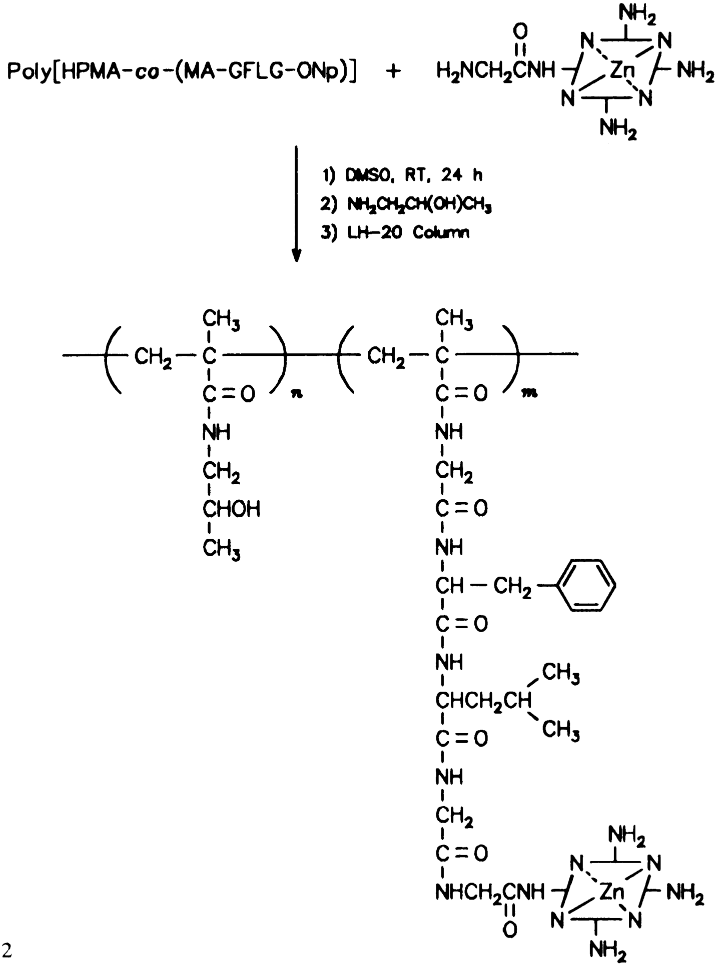
Quantum yields of oxygen uptake during photosensitized reactions, defined as (initial rate of uptake of oxygen molecules)/(initial rate of absorption of photons), were measured using a recording oxygen electrode system as described elsewhere[17]. Estimates of quantum yields of singlet oxygen generation by the illuminated sensitizers were made using a modification of the method of Verlhac et al.[18], similar to that of Murasecco et al.[19], as described elsewhere[17]. Furfuryl alcohol was employed as the substrate for these measurements, as described in the text. The dye, Rose bengal, was used as a standard[20]. Disappearance of sensitizer molecules during photobleaching was assayed spectrophotometrically after different periods of illumination[21]. Illumination for oxygen uptake and photobleaching measurements was provided by a 500 W incandescent lamp with bandpass filters corresponding approximately to the absorption peaks of the sensitizers. Incident light fluence rates, as measured with a calibrated thermocouple, were ~ 5 mW cm^{-2}. Errors in the various photochemical measurements were ~ ±10%. Reaction mixtures for the photochemical measurements were prepared by adding 50 μl of DMSO solution of sensitizers to 3.95 ml of aqueous buffered solution. All measurements were carried out at 25 ± 0.5 °C.

RESULTS AND DISCUSSION

The relationship between the structure and biological properties of HPMA copolymer – anticancer drug conjugates has been studied in detail[22,23]. Several attempts to bind photosensitizers to polymeric carriers with the aim to improve tumor accumulation and efficacy of photodynamic therapy were reported[15,24 – 27]. Because of restricted penetration of light into tissues, there is an urgent need in PDT to use photosensitizers that absorb at higher wavelengths, such as phthalocyanines and naphthalocyanines. However, these compounds are hydrophobic and do not dissolve in water. Binding to polymeric carriers would improve their solubility in water, targetability, and decrease side-effects, such as skin phototoxicity.

Synthesis of Polymer Bound Phthalocyanines

Wöhrle et al.[28 – 30] synthesized water soluble derivatives of tetraphenylporphyrins, phthalocyanines, and naphthalocyanines. Attachment of these photosensitizers to poly(methacrylic acid), and poly(vinyl pyrrolidone-*co*-methacrylic acid) produced photosensitizers attached to negatively charged carriers. To obtain uncharged carriers, the remaining carboxylic groups in poly(vinyl pyrrolidone-*co*-methacrylic acid) were converted to methyl ester groups using diazomethane[29]. Positively charged carrier – photosensitizer conjugates were synthesized by reacting chloromethylated polystyrene with tetrasubstituted photosensitizers followed by quaternization of remaining chloromethyl groups with trimethyl amine[30]. In contrast to anionically charged polymers, the cationically charged polymers did not show aggregation of the photosensitizer moieties in polar solvents. In all attachment reactions[28 – 30], crosslinking occurred, since symmetrical tetrasubstituted photosensitizers containing four OH or NH_2 groups were used. When low concentrations of photosensitizers were used in the polymer analogous reaction, the gel-point was not reached and branched water soluble polymer conjugates were obtained. To avoid the crosslinking reaction during attachment of photosensitizers, e.g., phthalocyanines to polymeric carriers, unsymmetrically substituted phthalocyanine derivatives[31,32] have to be synthesized which contain only one potential attachment point, in spite of the difficulties in purifying these compounds[33]. To this end, we have attempted to synthesize a monosubstituted phthalocyanine derivative, G-TAPC-Zn, which contains an aliphatic amino group (Scheme 1). By reaction of G-TAPC-Zn with an HPMA copolymer containing tetrapeptide (GFLG) side-chains terminated in reactive *p*-nitrophenyl ester groups, a polymeric phthalocyanine conjugate, P-GFLGG-TAPC-Zn, was prepared (Scheme 2). G-TAPC-Zn, as other phthalo-



SCHEME 2

cyanines[33], exhibits strong aggregation effect. Purification is very difficult, since TAPC-Zn and G-TAPC-Zn eluted in identical bands during flash chromatography and produced identical spots on TLC. Based on the differences in the solubility of N-BOC-G-TAPC-Zn and TAPC-Zn, the latter was removed by extraction with ethyl acetate. However, we can not exclude that the G-TAPC-Zn contained a small amount of di-substituted (diglycine) derivative, as indicated by the widening of the size exclusion chromatogram of P-GFLGG-TAPC-Zn in CTAB containing buffer when compared to the aminolysed polymeric precursor (results not shown).

Spectral Properties

In aqueous buffer, G-TAPC-Zn had a broad, low, double absorption peak in the red (Fig. 1, curve *3*), characteristic of aggregated phthalocyanines[9]. In contrast, in DMSO (Fig. 1, curve *1*), it had a sharp peak at 720 nm ($\varepsilon = 1.08 \,.\, 10^5$ l mol^{-1} cm^{-1}) with a shoulder on the short wavelength side, as is observed for phthalocyanines monomeric in solution[9]. The spectrum in CTAB-B also showed some monomerization of the sensitizer, with a peak at 711 nm (Fig. 1, curve *2*). On binding to the copolymer, the red peak in DMSO was characteristic of monomeric phthalocyanines and was shifted slightly to 717 nm (Fig. 2, curve *1*). The spectrum in CTAB-B also showed some monomerization, with a peak at 706 nm (Fig. 2, curve *2*). In aqueous buffer,

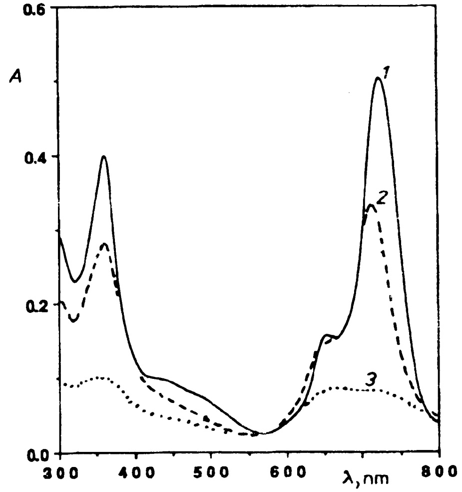


Fig. 1

Spectra of G-TAPC-Zn ($5 \,.\, 10^{-6}$ mol l^{-1}) in different solvents: *1* DMSO; *2* CTAB-B; *3* buffer B

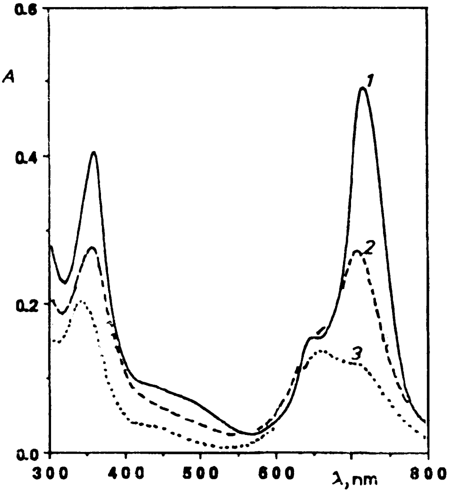


Fig. 2

Spectra of P-GFLGG-TAPC-Zn ($5 \,.\, 10^{-6}$ mol l^{-1}) in different solvents: *1* DMSO; *2* CTAB-B; *3* buffer B

P-GFLGG-TAPC-Zn had a low, broad red peak at 658 nm with a shoulder on the long wavelength side (Fig. 2, curve *3*), characteristic of the aggregated form. The spectra of G-TAPC-Zn and P-GFLGG-TAPC-Zn in other aqueous detergents (cetyl pyridinium chloride, sodium dodecyl sulfate, and Triton-100) were similar to those in CTAB-B (results not shown). The degree of unimerization increased with increasing content of CTAB in buffer B (Fig. 3) and with increasing content of DMSO in the mixtures of DMSO and buffer B (Fig. 4). The high concentrations of detergent or organic solvent necessary to prevent aggregation (Figs 3 and 4) indicate the strength of the association of P-GFLGG-TAPC-Zn side-chains.

Enzymatic Cleavage

Polymer bound drugs will be taken up by cells by endocytosis, thus restricting their localization to the lysosomal compartment. When irradiated, singlet oxygen will be evolved resulting in the eventual destruction of the organelle and the release of the degradative enzymes into the cytoplasm[1]. Theoretically, there is no need to bind photosensitizers to polymeric carriers via a bond enzymatically cleavable in the lysosomes. However, we have shown previously[15], that the photosensitizing efficiency of a HPMA copolymer–chlorin e_6 conjugate increased upon the release of the photosensitizer (chlorin e_6) from the polymeric carrier catalyzed by cathepsin B, indicating the influence of polymer aggregation on the process of singlet oxygen generation. Consequently, we bound G-TAPC-Zn via an oligopeptide sequence GFLG, stable in the blood stream, but degradable by lysosomal cysteine proteinases[13,16]. To determine the cleavability of phthalocyanine from the HPMA copolymer, we incubated P-GFLGG-TAPC-Zn with papain and cathepsin B. Papain is a plant enzyme, however, its speci-

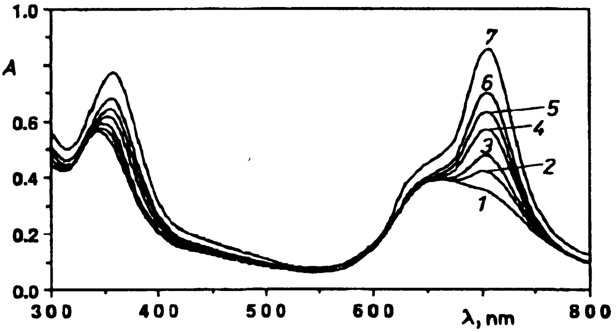


FIG. 3
Spectra of P-GFLGG-TAPC-Zn ($9.3 \cdot 10^{-6}$ mol l^{-1}) in buffer B containing different amounts of CTAB. CTAB content (10^{-3} mol l^{-1}); *1* 0, *2* 0.32, *3* 6, *4* 36, *5* 72, *6* 130, *7* 274

ficity is similar to that of cathepsin B, the major cysteine proteinase in the lysosomes. During incubation of P-GFLGG-TAPC-Zn with both enzymes in buffer solutions no release of phthalocyanine from the polymeric carrier was observed (results not shown). This was probably due to the formation of aggregates[34,35] (micelles) in which the hydrophilic polymeric backbone forms the shell of the micelle, whereas the hydrophobic side-chains are aggregated in the core of the micelle preventing the formation of the enzyme-substrate complex. The addition of a detergent (CTAB) decreased the activity of the enzymes, but the release of phthalocyanine was observed due to a lower degree of aggregation. After 48 h of incubation with papain 16.3% of G-TAPC-Zn was released, whereas cathepsin B released only 8.8% of G-TAPC-Zn under identical experimental conditions (Fig. 5). The decrease of the rate of cleavage at longer time intervals corresponded with the decrease of enzymatic activity as determined using N^{α}-benzoyl-L-arginine *p*-nitroanilide as substrate (results not shown). The conditions of enzymatic cleavage (Fig. 5) used did not prevent the formation of aggregates. This could contribute to the observed low rate of enzymatically catalyzed phthalocyanine release. Another factor may be the size of the leaving group. Both enzymes prefer hydrophobic amino acid residues in the P_2 and P_3 positions of their active site[16,36]. Consequently, the substrate should line-up in the active site in such a way that TAPC-Zn interacts with the S_2' subsite of the active site of the enzyme. Steric hindrance may render the S_2'–P_2' interactions energetically unfavorable and consequently the formation of the enzyme substrate complex more difficult. It is interesting to note that phthalocyanine in the P_2' position decreases the rate of cleavage by cathepsin B to a greater extent than the HPMA copolymer containing chlorin e_6 bound via a tetrapeptide sequence GFLG, where the smaller chlorin e_6 occupies the P_1' position[15]. However, even the modest rate of phthalocyanine release should improve the quantum yield of photooxidation reactions in the lysosomes of target cells, since irradiation may be ap-

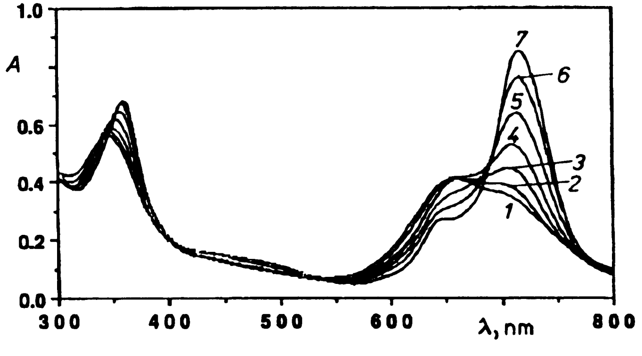


FIG. 4
Spectra of P-GFLGG-TAPC-Zn (9.3 . 10^{-6} mol l^{-1}) in mixtures of buffer B and DMSO. DMSO content (vol.%): *1* 0, *2* 30, *3* 50, *4* 60, *5* 70, *6* 80, *7* 100

plied 24 or 48 h after administration[15]. Moreover, aggregation of HPMA copolymers is concentration dependent[35] and the actual concentration in the lysosomal compartment may be substantially lower than the concentrations used in vitro.

Photoproperties

Fluorescence

As with other aggregated phthalocyanines[9], no fluorescence could be detected from G-TAPC-Zn or P-GFLGG-TAPC-Zn in buffer B. In DMSO, G-TAPC-Zn showed a single fluorescence peak at 749 nm (uncorrected) with a relative intensity of 530, while the single peak of P-GFLGG-TAPC-Zn was at 747 nm, with an intensity of 160. In CTAB-B as solvent, G-TAPC-Zn fluoresced at 731 nm, with an intensity of 540; P-GFLGG-TAPC-Zn emitted at 727 nm, with an intensity of only 80. The fluorescence properties of the sensitizers in other aqueous detergents (1-cetylpyridinium chloride, sodium dodecyl sulfate, and Triton-100) were similar to those observed in CTAB-B. As for the spectral data presented above, these results indicate that both sensitizers are aggregated in buffer B, but at least partially favor the unimer form in DMSO and aqueous detergents.

Photogeneration of Singlet Oxygen

Tumor damage during PDT with most photosensitizers, including porphyrins, chlorins and phthalocyanines, appears to be mediated largely by photogenerated singlet oxygen[4,9]. Thus, it is of interest to determine the quantum yields of singlet oxygen production by prospective PDT sensitizers on illumination. In the present work this was

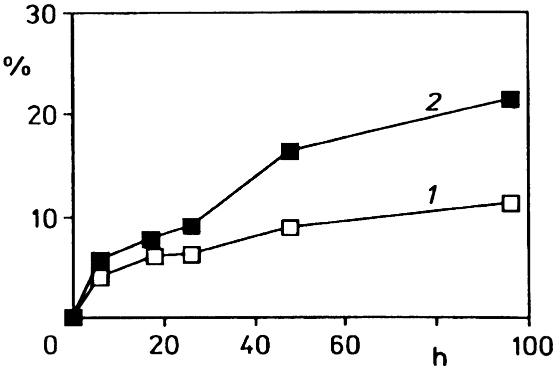


FIG. 5
Cleavage of P-GFLGG-TAPC-Zn (%) with *1* cathepsin B and *2* papain. For experimental details see Experimental

done using furfuryl alcohol (FA) as the substrate. This compound rapidly reacts chemically with singlet oxygen, and does not react appreciably with hydrogen peroxide or superoxide[17]. The quantum yields of oxygen uptake during the Rose bengal, zinc phthalocyanine tetrasulfonate (ZnPCS4), G-TAPC-Zn and P-GFLGG-TAPC-Zn sensitized photooxidation of FA as a function of the alcohol concentration are shown in Fig. 6. The yields for oxygen uptake leveled off at FA concentration range $5 \cdot 10^{-2} - 1 \cdot 10^{-1}$ mol l^{-1} with all of the photogenerated singlet oxygen being trapped by substrate in this range. Thus the ratios of the quantum yields of singlet oxygen generation will be the same as the ratios of the quantum yields of oxygen uptake. Using a value of 0.75 for Rose bengal as a standard[20], the quantum yields of singlet oxygen generation by the other sensitizers can be calculated to be 0.69 for ZnPCS4, 0.24 for G-TAPC-Zn and 0.063 for P-GFLGG-TAPC-Zn. For comparison, the yield with Photofrin II (PF II) is 0.16 (ref.[21]). Thus, the yield by G-TAPC-Zn is reduced to ~one-fourth by binding to the HPMA copolymer. However, if G-TAPC-Zn is freed from the copolymer by enzymatic hydrolysis in tumor cells, this would restore its full photosensitizing efficiency. We reported earlier, that the covalent binding of mesochlorin e_6 to a HPMA copolymer reduced its singlet oxygen yield to one-third[37]. The mechanism of this effect is not clear, but it may result from interference with the access of oxygen to the sensitizing moiety and the diffusion of singlet oxygen out of the copolymer structure[37].

The efficiency of singlet oxygen photogeneration by G-TAPC-Zn is somewhat greater than that of PF II, which has been used successfully for the photodynamic therapy of tumors in a large number of patients[4–6]. However, the efficiency of G-TAPC-Zn in the PDT of tumors may be much greater than that of PF II because of its strong red

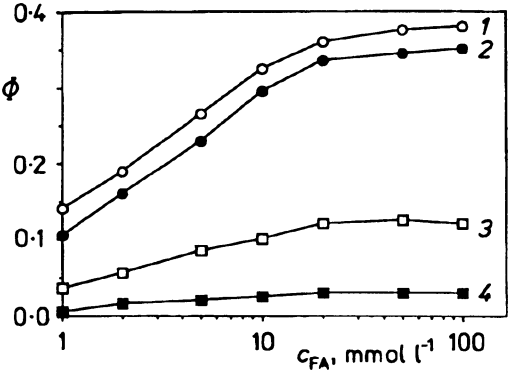


FIG. 6

Quantum yields of oxygen uptake (Φ) during the sensitized photooxidation of furfuryl alcohol (FA) as a function of FA concentration (c_{FA}). Concentration of reaction components (mol l^{-1}) in buffer B: $5 \cdot 10^{-6}$ in sensitizer, $2.2 \cdot 10^{-4}$ in oxygen, $1 \cdot 10^{-2}$ in CTAB (except for Rose bengal). Sensitizers used were: *1* Rose bengal; *2* ZnPCS4; *3* G-TAPC-Zn; *4* P-GFLGG-TAPC-Zn. Rose bengal, used as a standard, photogenerates singlet oxygen with a quantum yield of 0.75 (ref.[20])

peak absorbance (Fig. 1) compared to that of PF II, for which the molar absorption coefficient at the red peak (630 nm) is only ~ 3 000 l mol^{-1} cm^{-1}. Also, the longer wavelength of G-TAPC-Zn absorption permits the use of light in PDT of wavelengths that penetrate better into mammalian tissues[9].

Azide is an efficient quencher of singlet oxygen. Thus, if low concentrations of azide inhibit a sensitized photooxidation reaction, this strongly suggests that the reaction is mediated by singlet oxygen[2]. The G-TAPC-Zn-sensitized photooxidation of furfuryl alcohol was inhibited 50% by 0.77 mmol of azide, while the reaction with P-GFLGG-TAPC-Zn was inhibited 50% by 0.52 mmol of azide. These values are similar to those observed with porphyrins[21]. Thus, the photooxidation of FA with both sensitizers appears to be mediated by singlet oxygen, as has been observed for several kinds of substrates with other phthalocyanines[9].

Photooxidation of Biomolecules

The quantum yields of oxygen uptake during the G-TAPC-Zn- and P-GFLGG-TAPC-Zn-sensitized photooxidation of FA and several types of biomolecules that are known to be photooxidized with other sensitizers[2] are listed in Table I. Data for PF II are also shown. As may be seen, the yields with G-TAPC-Zn are similar to those observed with PF II. However, the yields with P-GFLGG-TAPC-Zn are only ~ 25% of those for G-TAPC-Zn, corresponding to its lower quantum yield of singlet oxygen photogeneration (Fig. 6).

TABLE I

Quantum yields of oxygen uptake (Φ) during the photooxidation of furfuryl alcohol (FA) and biological substrates sensitized by G-TAPC-Zn and P-GFLGG-TAPC-Zn. Concentration of reaction components (mol l^{-1}) in buffer B: 5 . 10^{-6} in sensitizer, 5 . 10^{-3} in substrate (except for bovine serum albumin, which was 1 wt.%), 2.2 . 10^{-4} in oxygen (air saturated), and 1 . 10^{-2} in CTAB (for G-TAPC-Zn and P-GFLGG-TAPC-Zn only)

Substrate	Φ		
	G-TAPC-Zn	P-GFLGG-TAPC-Zn	Photofrin II[a]
FA	0.082[b]	0.021[b]	0.073
Cysteine	0.034	0.010	0.046
Histidine	0.052	0.011	0.053
Methionine	0.021	0.005	0.020
Tryptophan	0.033	0.005	0.026
Bovine serum albumin	0.028	0.007	–

[a] Data from ref.[21]. [b] Data from Fig. 6.

Self-Sensitized Photobleaching of Free and Copolymer Bound G-TAPC-Zn

If a sensitizer used in PDT bleaches too rapidly, its concentration will decrease to below the level required for tumor destruction. If it bleaches at an appropriate rate, however, its concentration in the normal tissues surrounding the tumor could be decreased to below the threshold level, while enough sensitizer could remain in the tumor tissue to mediate its photodestruction[21]. Thus, it is of importance to determine the photobleaching behavior of sensitizers with potential application in PDT.

The quantum yields of photobleaching of the two sensitizers, as measured in DMSO and in CTAB-B, are listed in Table II. The initial quantum yields of photobleaching for both G-TAPC-Zn and P-GFLGG-TAPC-Zn were approximately two-fold higher in CTAB-B than in DMSO. With porphyrins and chlorins, the yields increase roughly with an increase in the dielectric constant of the solvent used[21,37]. Since the dielectric constants of DMSO and water are 45 and 78.5, respectively[37], the same relationship may hold for the present sensitizers. The initial photobleaching yields in both solvents are approximately three-fold greater for the free G-TAPC-Zn than for the bound form. We observed this same kind of relationship for free and HPMA copolymer bound mesochlorin e_6 illuminated in buffer B (ref.[37]). As may be seen, the yields at the start of illumination are much greater than during the 5 – 10 min period of illumination. At 5 min of illumination, ~ 50% of the G-TAPC-Zn in the two solvents had bleached, while only ~ 20% of the P-GFLGG-TAPC-Zn had bleached. The reason for the very rapid initial photobleaching followed by a very slow rate is not clear. In any event, based on the photobleaching yields of sensitizers used successfully in the past[21], the yields observed for G-TAPC-Zn and P-GFLGG-TAPC-Zn would not interfere with their use as photosensitizers for the PDT of tumors[21].

TABLE II
Quantum yields (Φ) of the self-sensitized photobleaching of G-TAPC-Zn and P-GFLGG-TAPC-Zn as measured during different periods after the start of illumination. Concentration of reaction components (mol l^{-1}): 5 . 10^{-6} in sensitizer, 2.2 . 10^{-4} in oxygen (air saturated)

Reaction mixture	Φ	
	0 – 0.5 min illumination	5 – 10 min illumination
G-TAPC-Zn in DMSO	7.5 . 10^{-4}	5.0 . 10^{-5}
G-TAPC-Zn in CTAB-B	1.9 . 10^{-3}	3.8 . 10^{-5}
P-GFLGG-TAPC-Zn in DMSO	2.3 . 10^{-4}	2.7 . 10^{-5}
P-GFLGG-TAPC-Zn in CTAB-B	5.2 . 10^{-4}	1.2 . 10^{-5}

CONCLUSIONS

A new phthalocyanine derivative (G-TAPC-Zn) and its conjugate with HPMA copolymer (P-GFLGG-TAPC-Zn) were synthesized. G-TAPC-Zn is an efficient photosensitizers. It gives good yields of singlet oxygen, and efficiently sensitizes the photooxidation of FA via a singlet oxygen mechanism. The covalent binding of G-TAPC-Zn to HPMA copolymer reduces its singlet oxygen yield and photosensitizing efficiency, and significantly decreases its rate of photobleaching. G-TAPC-Zn is released from the polymeric carrier by the action of cysteine proteinases, papain and cathepsin B. We have shown previously[15] that the release of photosensitizers from polymeric carriers restores their photosensitizing efficiency. Consequently, polymer bound phthalocyanines have a potential in cancer photodynamic therapy, since upon the arrival in the lysosomal compartment of the target cell, their photosensitizing efficiency should be at least partially restored.

The research reported here was supported in part by NIH grant CA 51578. The photochemical measurements were also supported in part by American Cancer Society Grant DHP-73A and the Utah Laser Institute. Pat Meekins provided skilled technical assistance.

REFERENCES

1. Bayley H., Gasparro F., Edelson R.: Trends Pharmacol. Sci. *8*, 138 (1987).
2. Spikes J. D. in: *The Science of Photobiology* (K. C. Smith, Ed.), 2nd ed., p. 79. Plenum, New York 1989.
3. Krinick N. L., Kopeček J. in: *Handbook of Experimental Pharmacology* (R. L. Juliano, Ed.), Vol. 100, p. 105. Springer, Berlin 1991.
4. Gomer C. J., Rucker N., Ferrario A., Wong S.: Radiat. Res. *120*, 1 (1989).
5. Kessel D. (Ed.): *Photodynamic Therapy of Neoplastic Disease*, Vols I – III. CRC Press, Boca Raton, 1990.
6. Henderson B. W., Dougherty T. J.: Photochem. Photobiol. *55*, 145 (1992).
7. Kopeček J. in: *IUPAC Macromolecules* (H. Benoit and P. Rempp, Eds), p. 305. Pergamon, Oxford 1982.
8. Leznoff C. C., Lever A. B. P. (Eds): *Phthalocyanines. Properties and Applications.* VCH Publishers, New York 1989.
9. van Lier J. E. in: *Photodynamic Therapy of Neoplastic Disease* (D. Kessel, Ed.), Vol. I, p. 279. CRC Press, Boca Raton 1990.
10. Rosenthal I.: Photochem. Photobiol. *55*, 859 (1991).
11. Achar B. N., Fohlen G. M., Parker J. A., Keshavayya J.: Polyhedron *6*, 1463 (1987).
12. Kopeček J., Bažilová H.: Eur. Polym. J. *9*, 7 (1973).
13. Kopeček J., Rejmanová P., Strohalm J., Ulbrich K., Říhová B., Chytrý V., Lloyd J. B., Duncan R.: U.S. 5 037 883 (1991); Chem. Abstr. *107*, 141105e (1987).
14. Rejmanová P., Labský J., Kopeček J.: Makromol. Chem. *178*, 2159 (1977).
15. Krinick N. L., Sun Y., Joyner D., Spikes J. D., Straight R. C., Kopeček J.: J. Biomat. Sci., Polym. Ed., in press.
16. Rejmanová P., Pohl J., Baudyš M., Kostka V., Kopeček J.: Makromol. Chem. *184*, 2020 (1983).

17. Spikes J. D., Bommer J. C.: J. Photochem. Photobiol., B *17*, 135 (1993).
18. Verlhac J. B., Gaudemer A., Kraljic I.: Nouv. J. Chim. *8*, 401 (1984).
19. Murasecco P., Oliveros E., Braun A. M., Monnier P.: Photobiochem. Photobiophys. *9*, 193 (1985).
20. Gandin E., Lion Y., Van den Vorst A.: Photochem. Photobiol. *37*, 271 (1983).
21. Spikes J. D.: Photochem. Photobiol. *55*, 797 (1992).
22. Kopeček J., Duncan R.: J. Controlled Release *6*, 315 (1987).
23. Duncan R., Seymour L. W., O'Hare K. B., Flanagan P. A., Wedge S., Hume I. C., Ulbrich K., Strohalm J., Šubr V., Spreafico F., Grandi M., Ripamonti M., Farao M., Suarato A.: J. Controlled Release *19*, 331 (1992).
24. Krinick N. L., Říhová B., Ulbrich K., Andrade J. D., Kopeček J.: Proc. SPIE Int. Soc. Opt. Eng. *997*, 70 (1988).
25. Říhová B., Krinick N. L., Kopeček J.: J. Controlled Release *25*, 71 (1993).
26. Jiang F. N., Allison B., Liu D., Levy J. G.: J. Controlled Release *19*, 41 (1992).
27. Goff B. A., Bamberg M., Hasan T.: Cancer Res. *51*, 4762 (1991).
28. Wöhrle D., Krawczyk G.: Makromol. Chem. *187*, 2535 (1986).
29. Wöhrle D., Krawczyk G., Paliuras M.: Makromol. Chem. *189*, 1001 (1988).
30. Wöhrle D., Krawczyk G., Paliuras M.: Makromol. Chem. *189*, 1013 (1988).
31. Hall T. W., Greenberg S., McArthur C. R., Khouw B., Leznoff C. C.: Nouv. J. Chim. *6*, 653 (1982).
32. Wöhrle D., Krawczyk G.: Polym. Bull. (Berlin) *15*, 193 (1986).
33. Leznoff C. C., Lever A. B. P. (Eds): *Phthalocyanines. Properties and Applications*, p. 20. VCH Publishers, New York 1989.
34. Ulbrich K., Koňák Č., Tuzar Z., Kopeček J.: Makromol. Chem. *188*, 1261 (1987).
35. Koňák Č., Kopečková P., Kopeček J.: Macromolecules *25*, 5451 (1992).
36. Ulbrich K., Zacharieva E. I., Obereigner B., Kopeček J.: Biomaterials *1*, 199 (1980).
37. Spikes J. D., Krinick N. L., Kopeček J.: J. Photochem. Photobiol., A *70*, 163 (1993).