Journal of Applied Polymer Science

Polyphosphoester-based paclitaxel complexes

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ABSTRACT: We demonstrated a feasible approach for the preparation of a biodegradable, water soluble polyphosphoester based paclitaxel complex. Applying poly(hydroxyoxyethylene phosphate) which contains both a strong proton accepting P═O group and a proton donating P—OH group, paclitaxel has been physically immobilized onto the polymer via H-bonding. The water soluble complex contained 16.7 wt % paclitaxel and more than 4000 times increased drug solubility was achieved. The polymer-drug complex formed nanosized aggregates that were characterized by dynamic light scattering. Intravenous injection of poly(hydroxyoxyethylene phosphate) in rats at a dose of 1000 mg/kg did not induce any clinical signs or body weight gain reduction. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42772.

KEYWORDS: biocompatibility; biomedical applications; hydrophilic polymers; light scattering

Received 10 June 2015; accepted 24 July 2015

DOI: 10.1002/app.42772

INTRODUCTION

Paclitaxel, a natural occurring diterpene alkaloid, is a relatively new antineoplastic agent for clinical treatment of breast, lung, ovarian cancers, and head and neck cancers.[1–4] Anti-cancer agents in cancer therapy cause a large number of toxic side effects which require a reduction in the doses of the chemotherapeutic agents or occasionally interruption of therapy itself. Thus, the design of new effective agents to prevent tumor cell growth without causing non-specific side effects is clinically important. To that end the application of drug delivery systems presents a promising strategy.[5,6] The delivery systems have been designed to provide tumor tissue targeting and local drug release in order to maintain the drug concentrations within therapeutic range over long periods of time, and thus to reduce systemic side effects.[7] A number of paclitaxel conjugations at C-2' and/or C-7 position via esterification are known with peptides;[8,9] carboxylic acid-terminated polyester-b-PEG copolymers,[10,11] hyperbranched poly(ether-ester),[12] poly(L-glutamic acid),[13,14] pegylated dendrimers.[15] A novel water-soluble multifunctional polymeric prodrug paclitaxel–poly(ethyl ethylene phosphate)–folic acid (PTX–PEEP–FA), aiming to integrate three functions into one prodrug molecule, i.e. the anti-tumor drug PTX, the water-soluble and biodegradable polymer PEEP, and the targeting folic acid moiety was synthesized.[16] Zhang *et al.*[17] developed a new type of degradable, poly(ethylene oxide)-*block*-polyphosphoester-based paclitaxel conjugates [PEO-*b*-(PPE-*g*-PTX)] which formed nanoscopic assemblies with high levels of drug loading. That resulted in significant increase of drug solubility, a maximum PTX concentration of 6.2 mg mL^{-1} in water was achieved, which is 25,000-fold higher than the aqueous solubility of free PTX. The conjugation of PTX to the polyphosphoester backbone via a pH-sensitive linkage resulted in pH-triggered drug release profile and five to eightfold enhanced *in vitro* cytotoxicity.[18]

Covalent bonding of drug onto polymer carrier needs catalyst and/or elevated temperature, i.e., this is a time and cost consuming process. Another critical parameter for polymer-drug conjugates is the release of the drug from the polymeric carrier. Noncovalent interactions play key roles in many natural processes leading to the self-assembly of molecules. One of the most important driving force for self-assembly of biomacromolecules is hydrogen bonding, which also plays an important role in the self-assembly of synthetic polymers. Proton-accepting polymers can associate with proton-donating polymers via hydrogen bonding in aqueous solutions and form interpolymer complexes.[19]

Additional Supporting Information may be found in the online version of this article.

Currently a substantial amount of efforts are directed toward developing amphiphilic copolymers for physical immobilization (entrapment) of hydrophobic drugs. Paclitaxel was encapsulated into micelles derived from amphiphilic block copolymer poly(ε-caprolactone)-*block*-poly(ethyl ethylene phosphate).[20] Biodegradable polyphosphoester-based polymeric micelles and shell cross-linked knedel-like nanoparticles effectively incorporated paclitaxel achieving 10% drug payload that enhanced PTX solubility to 4.8 mg mL^{-1} in aqueous solution.[21] Hydrogen bonding of a hydrophobic drug to a polymer carrier is another type of physical immobilization. An important aspect of the physical immobilization of a drug via hydrogen bonding is the presence of strong proton acceptor groups in the polymer carrier and a hydrogen donating group in the drug. Polyphosphoesters are promising polymer carriers for physical immobilization of bioactive molecules via hydrogen bonding.[22–24] These polymers bear a strongly polar P═O group and an acidic P—OH group in the repeating unit. The phosphoryl group (P═O) is a good proton acceptor (about 2 orders of magnitude stronger than the C═O group) and forms strong hydrogen bonds with phenols and alcohols.[25–27] It is established that the P═O group of poly (hydroxyalkylene phosphate) participates in hydrogen bonding with P—OH and OH groups.[28–30] Besides, poly(hydroxyoxyethylene phosphate)s are biodegradable, biocompatible, water soluble and low toxic polymers.[31] Polyphosphoesters degraded through hydrolysis of the P—O—C ester bonds under acidic or basic conditions[31–33] and this can be particularly accelerated in the presence of phosphoesterase.[34–36] Chemotherapeutic agents possessing a phosphate unit would preferentially interact with the cancer cells.[37] Moreover, dephosphorylation often takes place more easily in cancer cells than in normal cells.[38]

Herein, considering the advantages of poly(hydroxyoxyethylene phosphate) we report a feasible approach for preparation of a delivery system based on physically immobilized (via hydrogen bonding) paclitaxel. Paclitaxel molecules possess hydroxyl and carbonyl groups both suitable for H-bond complexing. Paclitaxel hydrogen-bonded dimers with participation of the hydroxyl groups at the C7 and C2' sites and the carbonyl oxygen at the C10 and C5' atoms were decribed.[39] In the present study NMR and DLS has been used as main tools for characterization of the polyphosphoester-PTX complexes. It has been observed that poly(hydroxyoxyethylene phosphate) forms aggregates which size and stability depend on pH of the solution. Polymer-drug associates are stable under physiological conditions due to hydrogen bonding and hydrophobic interactions. This results in increased drug solubility 4000 times higher than that of the free PTX. In addition, no toxic effects were observed in rats after intravenous injection of poly(hydroxyoxyethylene phosphate) at a dose of 1000 mg kg^{-1} of body weight.

EXPERIMENTAL

Materials

Poly(ethylene glycol) with number-average molecular weight 600 g mol^{-1} (PEG 600) was purchased from Fluka. It was dried before use by a two-stage process: an azeotropic distillation with toluene and a subsequent 4 h heating at 120°C under dynamic vacuum. Dimethyl H-phosphonate (Fluka) was distilled before use. Paclitaxel (99%) was purchased from Xingcheng Chempharm Ltd., Taizhou, China.

All ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker 250 MHz instruments in $CDCl_3$ or D_2O. The average molecular mass (M_n) and molecular mass distribution (M_w/M_n) of poly(-hydroxyoxyethylene phosphate) were investigated by gel permeation chromatography applying a refractive index detector equipped with a Shodex 10 μm bead size Ohpak SB-804 HQ column (exclusion limit ~10^7) working at 40°C under a flow rate of 0.6 mL min^{-1}. An acetic acid (0.5*M*) containing sodium sulfate (0.3*M*) was used as eluent and sodium poly(vinylpyridine) standards for calibration.

Dynamic light scattering (DLS) measurements were performed using a zeta-potential and particle size analyzer ELSZ-1000, Otsuka Electronics.

Synthesis of Poly(hydroxyoxyethylene phosphate)

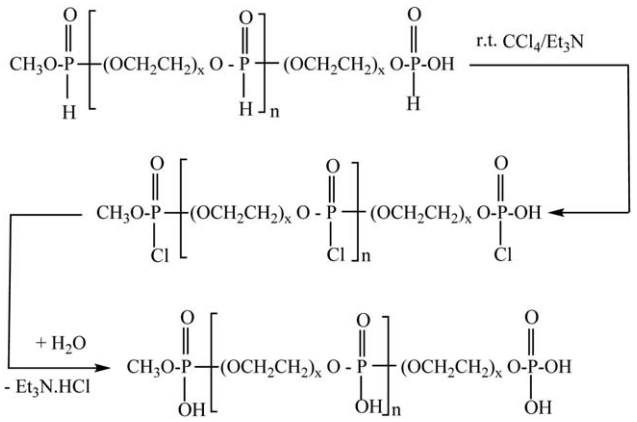
Poly(hydroxyloxyethylene phosphate) was obtained from a precursor polymer—poly(oxyethylene H-phosphonate) (for the preparation of the precursor see the Supporting Information, Scheme S1). The synthesis was carried out under inert atmosphere. Poly(oxyethylene H-phosphonate) (2.590 g, 0.004 mol building units) dissolved in acetonitrile (10 mL) and triethyl amine (0.56 mL, 0.004 mol) was added dropwise under stirring to a mixture of dichloromethane (25 mL) and tetrachloromethane (5 mL). In parallel, destilled water (0.07 mL, 0.004 mol) in 5 mL acetonitrile was dropped to the reaction mixture. The latter was stirred for 24 h at room temperature. Dichloromethane was evaporated and the solution was refrigerated at −12°C. Triethylamine hydrochloride crystals was removed by filtration. The solvents were evaporated and the residue was dissolved in water and was passed through ion exchange resin Dowex 50. After dialysis the reaction product was freeze-dried. Yield 2.12 g, 80%. The structure of poly(hydroxyoxyethylene phosphate) was proved by NMR spectroscopy.

^{1}H NMR (250 MHz, D_2O-d_2, δ): 3.93-3.99 (m, $C\underline{H}_2OP(O)(OH)OC\underline{H}_2$), 3.65–3.73 (m, $C\underline{H}_2OC\underline{H}_2$); $^{31}P\{H\}$NMR (250 MHz, D_2O-d_2, δ): 1.57 (0.03%); 0.31 (99.5%); −0.92 (0.02%); ^{31}P NMR (250 MHz, D_2O-d_2, δ): 0.31 ppm (q, CH_2O-*P*(O)(OH)OCH_2 $^{3}J(P,H)$═7.08 Hz); $^{13}C\{H\}$NMR (250 MHz, D_2O-d_2, δ): 64.68 (d, $^{2}J(P,C)$═5.65 Hz, $PO\underline{C}H_2CH_2$); 69.59 ($\underline{C}H_2O\underline{C}H_2$); 70.14 (d, $^{3}J(P,C)$═8.17 Hz, $POCH_2\underline{C}H_2$); IR: 2882 cm^{-1} *v* (CH_2); 2621, 2601 cm^{-1} *v*(P—OH); 1280, 1242 cm^{-1} *v*(P═O); 1105 cm^{-1} *v*(C—O—C); 1036 cm^{-1} *v*(P—O—C); M_n═13600 g/mol; M_w/M_n═1.17.

Physical Immobilization of Paclitaxel onto Poly(hydroxyoxyethylene phosphate)

A solution of paclitaxel (0.180 g, 0.21 × 10^{-3} mol) in ethanol was added to a solution of poly(hydroxyoxyethylene phosphate) (0.890 g, 1.34 × 10^{-3} mol) in methanol. Homogeneous solution was formed. Solvents were evaporated under vacuum (0.1 bar) at 40°C. Under the same conditions a complex from paclitaxel (0.050 g, 5.85 × 10^{-5} mol) and poly(hydroxyoxyethylene phosphate) (0.500 g, 7.55 × 10^{-4} mol) was also prepared. The products were dried to constant weight.

IR: 3475 cm^{-1} *v*(OH) broad; 2880 cm^{-1} *v*(CH_2); 2621, 2603 cm^{-1} *v*(P—OH); 1722 cm^{-1} *v*(C═O); 1653 cm^{-1} *v*(NH



Scheme 1. Synthesis of poly(hydroxyoxyethylene phosphate).

amide); 1278, 1240 cm^{-1} ν(P═O); 1105 cm^{-1} ν(C—O—C); 1036 cm^{-1} ν(P-O-C).

DLS Measurements of the Polymeric Aggregates

Synthesized poly(hydroxyoxyethylene phosphate) was dissolved in distilled water at concentrations 20 mg mL^{-1}, 5 mg mL^{-1}, 2.5 mg mL^{-1}, 1.25 mg mL^{-1}, and 1.0 mg mL^{-1}. The aqueous polymer solutions were exposed to ultrasonic irradiation for 10 min and filtered through a 0.8 μm pore size membrane filter. DLS measurements were performed with the prepared solutions at 25°C and 37°C.

The polymer–drug complex was dissolved in phosphate buffer solution at concentration 5 mg mL^{-1} for the polymer component and 1 mg mL^{-1} for the drug. The solution was exposed to ultrasonic irradiation for 10 min and filtered through a 0.8 μm pore size membrane filter. DLS measurements were performed with the prepared solution at 25°C and 37°C.

Single Dose Screening Toxicity Study

Twenty male Wistar rats (6 weeks old; body weight 170–180 g) were used in the experiments. All animals had access to laboratory food and water *ad libitum*. They were individually housed in plastic cages in a monitored environment (temperature 22 ± 2°C, humidity 50 ± 5%, 12-h light/12-h dark cycle). At the beginning of the experiment, the animals were randomly allocated to four groups of five rats each, based on their body weights measured just before starting the test chemical treatment. Three groups of the experimental animals were intravenously injected into the tail vein with poly(hydroxyoxyethylene phosphate) dissolved in 0.9% NaCl at respective single doses of 10 mg kg^{-1}, 100 mg kg^{-1}, and 1000 mg kg^{-1} of body weight. The control group of animals was injected with 0.9% NaCl. General conditions and mortality were checked daily and body weights were measured at Day 1, 6, and 13 during the experimental period. The amounts of supplied and residual diet were weighed weekly in order to calculate the average daily food intake through the entire treatment period. Two weeks after the injection the rats were subjected to necropsy after anesthesia.

RESULTS AND DISCUSSION

Poly(hydroxyoxyethylene phosphate) Characterization

Poly(hydroxyoxyethylene phosphate) (PHOEP) was obtained from the precursor poly(oxyethylene H-phosphonate) applying Atherton-Todd reaction conditions and water as reagent (Scheme 1).

The structure of the PHOEP polymer was proved by ^{1}H, $^{13}C\{H\}$, and ^{31}P NMR spectroscopy as well as by IR spectroscopy (see Experimental part). The presence of two stretching bands at 1242 cm^{-1} for hydrogen bonded P═O and at 1280 cm^{-1} for non-hydrogen bonded P═O[26, 40] and two stretching bands at around 2621 cm^{-1} and 2601 cm^{-1} for P—OH groups in the IR spectrum of PHOEP implies involvement of the P═O and P—OH groups in hydrogen bonding. In the IR spectrum of poly(oxyethylene H-phosphonate) there is only one band for P═O group at 1249 cm^{-1} (see Supporting Information) because P-H does not participate in hydrogen bonding with the P═O group.

The presence of proton donating and proton accepting groups in the PHOEP macromolecules prompts the possibility for formation of aggregates in solution. The PHOEP solutions with concentrations in the range from 20 mg mL^{-1} to 1 mg mL^{-1} were prepared in distilled water. The pH of the solutions increased from 1.5 to 3. The behavior of poly(hydroxyoxyethylene phosphate) aqueous solutions were studied by DLS at 25°C and 37°C. The size distribution curves of PHOEP aggregates measured in solutions with concentrations of 20.0, 5.0, 2.5, and 1.25 mg mL^{-1} are presented in Figure 1. It was established that depending on the concentration the polymer forms associates with mean values of the hydrodynamic radii in the range between 170 nm to 350 nm.

The results revealed that decreasing the concentration of the polymer solution resulted in decreasing of the size of the aggregates formed. At concentration 1.25 mg mL^{-1} the hydrodynamic radius of the aggregates was about 170 nm. At concentration 1 mg mL^{-1} no aggregates were detected. The light scattering intensity at 90° over incident light (I/I_0) for

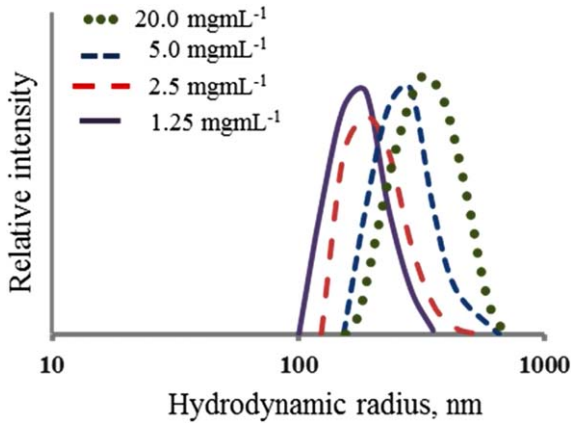


Figure 1. Size distribution curves of poly(hydroxyoxyethylene phosphate) aggregates measured at 25°C and different polymer concentrations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

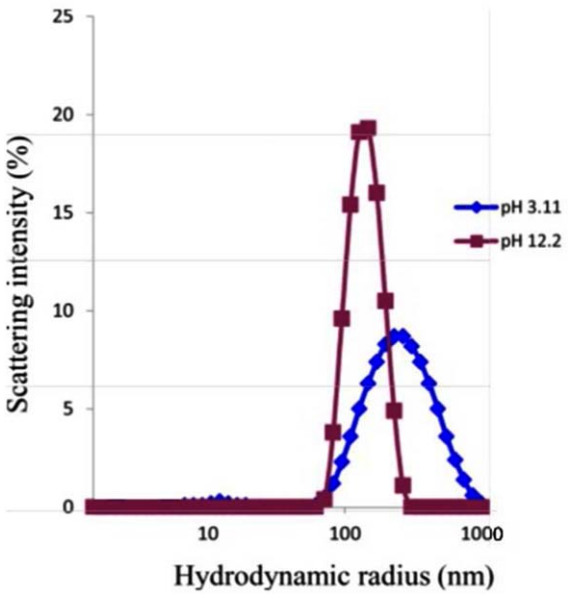


Figure 2. Hydrodynamic radii (R_h) distributions for poly(hydroxyoxyethylene phosphate) aqueous solutions (5.0 mg/mL) at 25°C: (a) pH 3.1(◆) and pH 12.2 (■). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

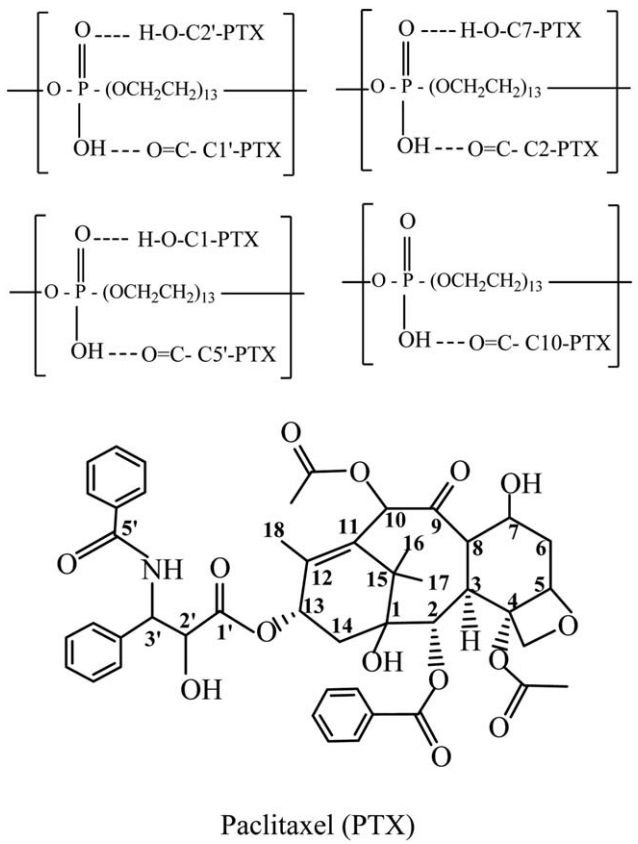
poly(hydroxyoxyethylene phosphate) aqueous solution was monitored as a function of the polymer concentration at 25°C (Supporting Information Figure S1). The plot shows that the onset of the aggregates formation is at concentration of 1.1 mg mL^{-1}. The formation of aggregates can be explained with hydrogen bonding between macromolecules. In the repeating units of poly(hydroxyoxyethylene phosphate) there are a strongly polar phosphoryl group (P═O), strong proton acceptor, and acidic P—OH group, proton donating group. Moreover, the main chain of PHOEP is built from poly(ethylene glycol) segments. Oxygen atoms of PEG units (—CH_2—O—CH_2—) may also serve as acceptor of hydrogen atoms. Two types of H-complexes can be formed. One complex can be formed between P═O and P—OH groups. The second one can arise from hydrogen bonding between P—OH group and oxygen atom of PEG units. Though P═O group is a very strong proton acceptor, most probably both complexes contribute to the self-association of the macromolecules because of the 12 times higher concentration of —CH_2OCH_2— units compared to that of P═O groups.

The hydroxyphosphate groups are moderately strong acids. The pK_a value of diethyl ester of the phosphoric acid, the low molecular model compound, is 1.39 ± 0.05.[41] Using this approximate value the degree of ionization of the hydroxyphosphate units can be calculated for the prepared solutions which are between 66 and 80%. It was also found that dissociation of the poly(carboxylic acid) was suppressed in the presence of PEO, i.e. the apparent dissociation constant, pK_a, increased upon formation of the interpolymer H-complex with PEO.[42,43] The observed aggregates in the PHOEP aqueous solutions indicates similar phenomenon—suppression of P—OH groups dissociation and participation in H-bonding with P═O or oxyethylene units. The obtained macromolecular associates are very hydrophilic and no turbidity or phase separation was observed even in the solution with concentration of 20 mg mL^{-1}. The destruction of the aggregates takes place gradually with dilution and/or increase of pH of the solution. As it is mentioned above at PHOEP concentration below 1.25 mg mL^{-1} no associates were detected. Obviously, the H-complexes dissociate due to ionization of P—OH moieties and the competitive hydrogen bonding with H_2O.

The stability of the PHOEP aggregates depends also on temperature. Supporting Information Figure S2 compares the experimental data obtained from measurements carried out at 25°C and 37°C. The size distribution curves of the aggregates in the solutions with concentrations 5.0 mg mL^{-1} and 2.5 mg mL^{-1} showed slight decrease in the mean size of the particles upon temperature increase from 25°C to 37°C. The trend of decrease in the size of the aggregates with lowering the polymer concentration was preserved at 37°C and at concentration of 1.25 mg mL^{-1} no aggregates were detected.

To study pH-dependence of poly(hydroxyoxyethylene phosphate) aggregation, DLS measurements were performed with the 5.0 mg mL^{-1} polymer solutions at pH 3.1 and pH 12.2 at 25°C (Figure 2).

The R_h distributions at pH 3.1 shows a main population of particles with R_h=286 nm. This population is assigned to intermolecular aggregation due to the hydrogen bonding interactions.



Scheme 2. Physical immobilization (via hydrogen bonding) of paclitaxel onto poly(hydroxyoxyethylene phosphate).

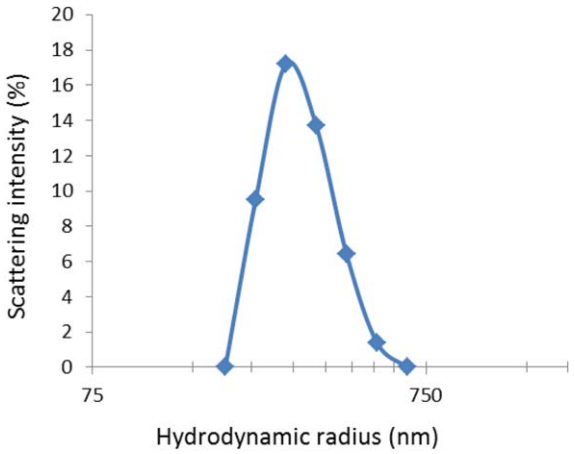


Figure 3. DLS data for the mixed poly(hydroxyoxyethylene phosphate)-paclitaxel (16.7%) aggregates in phosphate buffer solution at 25°C and concentrations: PHOEP 5.0 mg mL^{-1} and PTX 1 mg mL^{-1}. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DLS measurements were performed with the PHOEP solution at pH 12.2. The curve shows unimodal distribution with R_h=144 nm and increased scattering intensity of the polymer solution compared to that at pH 3.1. This observation indicates that the density of the polymer aggregate at pH 12.2 was larger than that at pH 3.1. It is interesting behavior which can be explained with the high concentration of sodium ions under basic conditions. The PHOEP chain is built up of oligoethylene oxide segments known for their ability to form complexes with metal cations, similar to crown ethers.[44,45] It was reported that the binding constants of PEG chains (molecular weights in the range of ca. 500–14,000) with the sodium cation showed a linear relationship with the number of binding sites available.[46] The oligoethylene oxide segments in the PHOEP present a flexible structure that can encase the metal cations which electrostatically interact with the phosphate groups that are completely ionized at pH 12. Therefore, under basic conditions the driving force and the structure of the PHOEP aggregates are different. The aggregates entrapped sodium cations, they are smaller in size and more compact than the associates formed under acidic conditions.

Physical Immobilization of Paclitaxel onto Poly(hydroxyoxyethylene phosphate) [PHOEP. PTX]

It can be accepted under physiological conditions that C2' and C7 hydroxyl groups of paclitaxel to participate in hydrogen bonding with the phosphoryl groups (P═O) in the polymer while in acidic media additionally the carbonyl oxygens at C5', C10, C4, C2 of paclitaxel to form hydrogen bond with the polymer P—OH groups (Scheme 2).

Complexes with different content of paclitaxel were prepared—16.7 wt % and 9.1 wt %. IR spectral data to support the formation of the polymer-drug complex are found in the absorptions of the groups participating in the hydrogen bonding (Supporting Information Figure S3). The very broad band in the spectral region 3500 to 3250 cm^{-1} can be assigned to OH-groups of the drug engaged in H-bonding. Some changes are observed in the stretching vibrations for the phosphoryl groups of the polymer: the intensity ratio of the bands at 1240 cm^{-1} ν(P═O...H) and 1278 cm^{-1} ν(P═O) increased, i.e. a larger fraction of P═O groups are involved in H-bonds in the drug-polymer formulation.

The size distribution curve of the mixed poly(hydroxyoxyethylene phosphate) and paclitaxel (drug mass fraction 16.7%) aqueous solution shows presence of aggregates with unimodal and narrow size distribution. The mean value of the hydrodynamic radius of the aggregates was R_h=285 nm (Figure 3). The increased aqueous solubility of the complex is a strong evidence that paclitaxel is immobilized to the poly(hydroxyoxyethylene phosphate) chain via hydrogen bonding. In addition, the polymer-drug aggregates are stabilized due to hydrophobic interactions. This is confirmed by the fact that upon double dilution the size of the aggregates changed slightly—from R_h=285 nm to R_h=260 nm (Supporting Information Figure S4).

Toxicity Study

Stringent requirements towards the components of a drug formulation pose the need for toxicological evaluation of the polymer carrier. Intravenous single dose screening toxicity study was undertaken for the following endpoint observations: body weight, food consumption, clinical signs and necropsy findings (liver and tail).

Animals were checked daily for clinical signs—edema, ulcer, scab formation, and exudate. No clinical signs were noted

Table I. Body Weight[a] and Food Intake in Rats Treated with Poly(hydroxyoxyethylene phosphate)

		Dose level (mg/kg)			
Finding	Study Day	0 (Control)	10	100	1000
Body weight (g)	Day 0	175.0 ± 3.0	174.0 ± 4.5	175.0 ± 5.4	174.0 ± 4.0
	Day 1	181.4 ± 4.0	179.2 ± 4.4	179.8 ± 5.9	179.2 ± 3.0
	Day 6	231.8 ± 9.3	226.0 ± 7.7	227.6 ± 10.2	225.8 ± 4.8
	Day 13	298.2 ± 16.7	285.2 ± 7.5	288.2 ± 15.3	291.0 ± 10.4
Food intake (g)	Day 0	20.8 ± 1.6	19.8 ± 0.8	20.6 ± 2.3	20.0 ± 1.9
	Day 6	27.8 ± 3.0	24.9 ± 1.1	24.8 ± 1.9	25.0 ± 1.6
	Day 13	29.8 ± 4.1	26.8 ± 0.8	27.2 ± 2.2	29.6 ± 4.3

[a] Dunnett's test: no significant difference.

throughout the experimental period, and all animals survived until the scheduled necropsy. At necropsy the liver, diaphragm, and tail of the animals were macroscopically examined. No changes in the liver, as well no diaphragmatic nodules were noted. Ulcer and/or scab formation at the site of injection were not observed.

Body weight gain of all animals in the three test groups was comparable to that of the control group. At the end of the experimental period no statistically significant differences in body weight and food intake were obtained in any group, i.e. even for the group treated with a dose of 1000 mg/kg (Table I).

CONCLUSIONS

In conclusion, we have developed a novel water-soluble paclitaxel-polyphosphoester complexes. Atherton–Todd reaction was employed to synthesize the polymer carrier—poly(hydroxyoxyethylene phosphate). Its backbone is built up of oligoethyleneoxide segments linked by phosphoester groups. The polymer was well tolerated by rats after intravenous injection at a dose of 1000 mg kg^{-1} body weight. Paclitaxel was physically immobilized to the polymer via multiple hydrogen bonding with participation of phosphoryl (P═O) and carbonyl (C═O) groups (proton acceptors) and P─OH and hydroxyl groups (proton donors). A drug–polymer complex was obtained which allowed more than 4000-fold increase of drug solubility in water. The results obtained revealed that poly(oxyethylene phosphate) can be regarded as multifunctional carrier of drugs. Biological evaluations of the immobilized paclitaxel onto PHOEP are in progress.

ACKNOWLEDGMENTS

Financial support of this work was provided by the National Science Fund of Bulgaria (National Center for Advanced Materials (UNION) Module 2 "New materials in medicine and pharmacy" Contract DCVP 02-2/2009.

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