Amphiphilic Poly(vinyl alcohol) Derivatives as Complexing Agents for Fenretinide

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Poly(vinyl alcohol) (PVA) substituted with oleyl chains and tetraethyleneglycol monoethyl ether chains (TEGMEE) at 1.5% and 1% degrees of substitution respectively (mol of substituent to mol of hydroxyvinyl monomer) has previously been shown to self-assemble in water, providing aggregates selectively cytotoxic toward tumor cells vs normal cells. These polymers have also been shown to increase the long-term survival of nude mice injected with both human and murine neuroblastoma cell lines. In the present work, we changed the substitution degree of the oleyl chains on the poly(vinyl alcohol) backbone and maintained constant at 1% the degree of TEGMEE substitution. We evaluated the main physicochemical characteristics of the final polymers, their cytotoxicity toward tumor cells, and their complexing ability for hydrophobic molecules. The aim was to investigate the possibility of improving intrinsic antitumor efficacy of the polymer by changing the degree of oleyl chain substitution and further increase activity by complexation with antitumor drugs. The polymers were prepared at oleyl chain substitution degrees ranging from 0.5 to 3% (mol of substituent to mol of hydroxyvinyl monomer). The most active was again the 1.5% substituted polymer. It was further characterized by exhibiting the highest complexing ability toward hydrophobic molecules allowing the formation of a complex with fenretinide (HPR). The polymer−HPR complex was stable in aqueous environment and released the free drug prevalently in the presence of fluid hydrophobic phases. It was cytotoxic toward tumor cells with minimal activity toward normal cells. Antitumor activity exceeded that of the separate complex components resulting from the concomitant effect of the polymer and the HPR solubilized by complexation.

Introduction

It is well-known that tumor cells are characterized by a series of membrane abnormalities as compared to normal cells. The most recurrent are conformational changes, enhanced lateral diffusion of membrane molecules, alteration of membrane−cytoskeleton attachments, alteration of absolute transmembrane potential,[1] and increase in membrane fluidity.[2−4] Increased membrane fluidity was proposed by our group[5] as a possible target for selective cytotoxicity, as raising the free energy of the membrane[6−8] may promote cell interactions with thermodynamically unstable environmental structures such as amphiphilic aggregates, on the basis of maximal decrease in free energy−maximal affinity for ligands.[9,10] We found that a series of amphiphilic polymers, self-assembling in an aqueous environment, could elicit cytotoxicity against tumor cells.[5] The polymers were prepared by poly(vinyl alcohol) substitution with both hydrophobic oleyl chains and hydrophilic polyethylene glycol monoethyl ethers (PEGMEE) of different molecular weights at 1.5% and 1% degrees of substitution respectively (mol of substituent to mol of hydroxyvinyl monomer).

Aggregates formed by polymer dissolution in water were characterized by different thermodynamic instability driving the polymers to interact with hydrophobic phases. Among the studied systems, the poly(vinyl alcohol)-*co*-oleylvinyl ether substituted with triethylene glycol monoethyl ether (TEGMEE), named P10(4), provided in solution the highest cytotoxicity toward tumor cell lines (neuroblastoma: SH-SY5Y, IMR-32, HTLA-230; melanoma: MZ2-MEL, RPMI7932) while it did not appreciably alter the viability of normal resting lymphocytes.[5,11] The antitumor activity of P10(4) was further confirmed in vivo, and its biodistribution did not reveal abnormal accumulation in any biological compartment.[11]

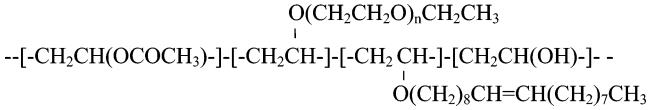

Figure 1. Poly(vinyl alcohol) substituted with oleyl chains and TEGMEE chains.

The peculiar behavior of P10(4) was correlated with the high thermodynamic instability of the aggregates formed in aqueous solution by its self-aggregation, triggering interaction with the fluid hydrophobic phases such as the tumor cell membranes, thus eliciting cytotoxicity. In these studies, we varied the oleyl chain substitution degree from 0.5% to 3% while maintaining at 1% the TEGMEE degree of substitution (Figure 1). We evaluated the main physicochemical and biological characteristics of these polymers to possibly improve their cytotoxicity toward tumor cells and further rise activity by complexation with antitumor drugs. Fenretinide, or *N*-(4-hydroxyphenyl) retinamide (HPR), a synthetic retinoid, active in a wide variety of cancer cells, was selected for complexation among other

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antitumor hydrophobic drugs due to its high tolerability and low incidence of long-term side effects.[12] However, its poor aqueous solubility strongly limits bioavailability thus reducing therapeutic efficacy. HPR complexation in the inner core of the polymeric aggregates, raising the drug aqueous solubilization, is expected to improve bioavailability. Moreover the concomitant effect of the polymer and the solubilized HPR is expected to provide a final system endowed with increased antitumor activity as compared with the separate complex components.

Materials and Methods

Preparation of the Substituted PVA. Poly(vinyl alcohol) (PVA, $M_w = 10$ kDa, 80% hydrolyzed) and polyvinylpyridine 2% crosslinked were commercial samples from Aldrich Chemical Gmbh (Steinheim, Germany). Oleyl bromide and thionyl chloride were purchased from Sigma Chemical Co. (St. Louis, MO), and all of the other reagents and solvents employed were from Fluka Chemie GmbH (Buchs, Suisse). The self-assembling polymers were prepared by partial substitution of PVA with oleyl chains at varying substitution degrees (0.5% to 3%) and triethylene glycol monoethyl ether at 1% (mol of substituent to mol of hydroxyvinyl monomer). The syntheses were carried out by dissolving PVA (3.93 g of polymer corresponding to 75 mmol of hydroxyvvinyl monomer) in 75 mL of *N*-methylpyrrolidone (NMP) in the presence of polyvinylpyridine (2 g). The solution was stirred at room temperature for 24 h, and subsequently, oleyl bromide was added in molar amounts varying from 5% (3.75 mmol) to 50% (37.5 mmol) of the hydroxyvinyl monomers present in solution. The solution was stirred again at room temperature for 24 h. Subsequently, triethylene glycol monoethyl ether monochloride was added in molar amounts corresponding to 50% (37.5 mmol) of the hydroxyvinyl monomers present in solution, and the mixture was stirred at room temperature for 24 h. Filtration was subsequently carried out to remove polyvinylpyridine, and diethyl ether was added to the solution to induce precipitation of the substituted polymer. The solid obtained was reprecipitated twice from NMP, dried under vacuum to constant weight, subsequently dissolved in water, and dialyzed against water for 15 days. After dialysis, the aqueous solution of the polymer was lyophilized. The degree of substitution of the final products was determined by elemental analysis performed using a Perkin-Elmer elemental analyzer (model 240 B) and by ^{1}H NMR using an Inova 600 spectrometer and recording the spectrum in $(CD_3)_2SO$.

Triethylene glycol monoethyl ether monochloride was prepared following a method for alogenation of alcohols.[13] Briefly, triethylene glycol monoethyl ether was dissolved in toluene in the presence of stoichiometric amounts of thionyl chloride, the mixture was stirred at room temperature for 24 h, the solvent was then evaporated under vacuum, and the residue obtained was desiccated.

Solubilization Studies. Solubilization studies were carried out by dissolving in water increasing amounts of polymers and stirring for 24 h at 37 °C. The systems were then observed by optical microscopy to evaluate the presence of solid, undissolved material indicative of water saturation by the polymer.

Dynamic Light Scattering (DLS) Measurements. DLS measurements were performed at 37 °C on polymer aqueous solutions at concentrations ranging from 0.5 to 10 mg/mL with the aim of evaluating the mean size of the polymeric aggregates and their stability over concentration changes. This concentration range was selected because, at concentrations lower than 0.5 mg/mL, the scattered intensity was under the instrument accuracy limit, whereas at concentrations higher than 10 mg/mL, a rapid increase in the scattered intensity indicated the establishment of multiple scattering.[14,15]

Measurements were performed by a Brookhaven 90-PLUS instrument equipped with a 50 mW He−Ne laser (532 nm). The scattering angle was fixed at 90°. Results were the combination of three 10 min runs for a total accumulation correlation function (ACF) time of 30 min. The mean size of the polymeric aggregates in solution was provided by their average hydrodynamic radius; results were volume-weighted.

Static Light Scattering (SLS) Measurements. SLS measurements were performed at 37 °C by a Brookhaven BI-200SM, on polymer aqueous solutions at concentrations ranging from 0.5 to 10 mg/mL, to obtain the apparent molecular weight of the polymeric aggregates by the Zimm plot.[16,17] The aggregation number (i.e., the number of the polymer chains forming the polymeric aggregate) was calculated as the ratio between the apparent molecular weight of the aggregates and that of the single polymer chains.

Electron Paramagnetic Resonance Studies. EPR spectra were recorded using a Bruker ESP300 spectrometer operating at X band (9.5 GHz). The concentration of the probe molecule (5-DSA) used in all of the studies was 10^{-4} M. For EPR measurements, the desired portions of 5-DSA in chloroform were added to a glass vial, and the solvent was evaporated. Then, the aqueous polymer solutions of desired concentrations and volumes were added to the vials under stirring. EPR spectra of the above solutions were recorded 2 h after the sample preparation using Pyrex capillary tubes (ca. 1 mm inner diameter) as sample containers.

Affinity for Hydrophobic Phases. Affinity studies of the polymers for hydrophobic phases were carried out to simulate the polymer ability to interact with cell membranes in aqueous environment. Solid and fluid hydrophobic phases (polyethylene membrane or silicone oil respectively) were used to find out an early prediction of the polymer affinity for cell membranes endowed with different fluidity such as the tumor cell membranes (more fluid) or the normal cell membranes (less fluid). The polymer aqueous solutions (5 mL, containing 5 mg/mL polymer) were introduced in glass tubes containing 12.7 μL of silicone oil (density 1.09 g/mL, Fluka) or a polyethylene membrane (Celgard 2500, Hoechst, 25.4 μm thickness, 5 cm^2 area) fixed at the bottom by a stopping rubbery disk (the silicone oil and polyethylene membrane volumes were the same). The systems were allowed to equilibrate for 24 h at 37 °C and then analyzed by HPLC to evaluate the decrease in polymer concentration ($-\Delta C$), due to the establishment of polymer−hydrophobic phase interactions, considered representative of the polymer affinity for the cell membranes. The equilibration was fully established after 24 h as at longer time periods no further variation in the polymer concentration was detected in all the analyzed systems. The HPLC assays of the aqueous phase were carried out by a size exclusion silica diol column (Chromegapore MSE Diol 5 μm, 1000 A, Superchrom, Milan, Italy) using water at 0.2 mL/min as the mobile phase and a UV detector at 240 nm (Dionex, Milan I). The system was maintained at 37 °C.

Complexation Ability toward Hydrophobic Molecules. The complexation ability of the polymers toward hydrophobic molecules was studied to evaluate their suitability as complexing agents for hydrophobic drugs in water. The complexation studies were carried out by dispersing 1 mg of naphtalene (Carlo Erba, Italy) in 5 mL of water containing polymer concentrations increasing from 0.5 to 10 mg/mL. The polymer solutions were allowed to equilibrate with the hydrophobic moiety for 24 h at 37 °C and, after filtration through a 0.2 μm pore size filter, analyzed by a spectrofluorimeter FP 750 Jasco at the waveleght of excitation 270 nm and emission 350 nm.

Polymer−HPR Complexation. The polymer characterized by the highest affinity for naphthalene was selected as a complexing agent for HPR. The polymer−HPR complex was prepared by adding 20 mg of HPR to 10 mL of water saturated with the polymer. The system was stirred at 37 °C for 24 h and subsequently filtered through a 0.2 μm pore size filter. The filtered solution was analyzed by HPLC to evaluate the concentration of HPR solubilized by the polymer. As a comparison, the solubility of pure HPR in water was also evaluated by stirring 20 mg of HPR in 10 mL of water at 37 °C for 24 h and subsequently filtering through a 0.2 μm pore size filter and analyzing the solution by HPLC. The HPLC assays were carried out by a Nova-Pak C18 (150 × 3.9 mm, 4 mm, Waters) column, UV detector at 345

nm. The mobile phase was a mixture of water (20%) and acetonitrile (80%). The injecting volume was 20 μL, and the flow rate was 1.0 mL/min. The system was thermostated at 37 °C. In these conditions, the retention time of HPR was 11.00 min.

Evaluation of HPR Release from the Complex. The HPR release from the complex in aqueous phase was studied by placing the filtered aqueous solution of the complex (5 mL) in a releasing cell separated by a dialysis membrane (3 cm^2 area) from a receiving compartment containing water (20 mL) and chloroform (5 mL) stratified at its bottom. The presence of chloroform assured the maintenance of sink conditions throughout the experiment due to a continuous extraction from the aqueous phase of the free HPR dissociated from the complex and diffused through the dialysis membrane. At defined time intervals, the organic phase was removed and evaporated, and the residue was added with acetonitrile and analyzed by HPLC for its HPR content. The HPLC conditions were the same used for the polymer−HPR complexation studies. The HPR release from the complex was also evaluated in the presence of solid (polyethylene membrane) or fluid (silicone oil) hydrophobic phases. As it concerns the study in the presence of solid hydrophobic phases, the polyethylene membrane was used instead of the dialysis membrane, to separate the releasing from the receiving compartment, and all of the other conditions were the same as the release in the aqueous phase. The release in the presence of the fluid hydrophobic phase was carried out by introducing 0.5 mL of silicone oil into the releasing cell containing the aqueous solution of the complex, separated from the receiving compartment by the dialysis membrane. All of the other conditions were the same described for the release in the aqueous phase.

Biological Studies. Antitumor activity of the polymers and the complex has been evaluated on HTLA-230 neuroblastoma cell lines. The polymers and the complex have also been tested on normal resting lymphocytes.

Cell lines were maintained in DMEM growth medium containing 10% fetal bovine serum and 100 ng/mL each penicillin and streptomycin (all from Sigma) at 37 °C in 5% CO_2. Experiments were performed during the logarithmic phase of cell growth. Cells were seeded in 6-well plates (Corning Incorporated, NY) (10^5 cells/well) in triplicate. After 48 h cells were treated with growth medium containing varying amounts of the different polymers or the complex. As a comparison, pure HPR was tested at the same concentrations present in the complex. Due to its hydrophobicity, HPR was initially dissolved in ethanol and subsequently diluted with the growth medium. The effects of the polymers and the complex on cell growth and death were determined by cell count and the trypan blue exclusion method. The cells were also analyzed by FACS to evaluate the presence of necrosis or apoptosis. Cells maintained in medium alone and treated with PVA were used as controls.

Statistical Analysis. All values in the figures and text are expressed as mean ± SD of N experiments (with $N \geq 3$). Statistical data analysis was performed using Student's t-test. Data sets were examined by ANOVA. The P values less than 0.05 were considered statistically significant.

Results

Characterization of the Modified Polymers. Using ^{1}H NMR analysis, the oleyl chain substitution degree was obtained by comparing the integral of the peak at 5.32 δ assigned to the vinyl protons (CH═CH) of the oleyl chain with the integral of the peak at 1.95 δ assigned to the methyl protons ($COCH_3$) of the acetyl moiety present at 20% in the PVA backbone. The TEGMEE substitution degree was obtained by comparing the integral of the peak at 1.17 δ assigned to the terminal methyl protons (CH_3) of TEGMEE with the integral of the peak at 1.95 δ assigned to the methyl protons ($COCH_3$) of the acetyl moiety. Both ^{1}H NMR and elemental analysis revealed that the substitution degrees (mol of substituent to mol of hydroxyvinyl monomer) of the TEGMEE chain was about 1% and ranged from 0.5% to 3% for the oleyl chain depending on the different amounts of oleyl bromide used in the synthetic procedure (Table 1). The ratio between oleyl and TEGMEE chain substitution degree in the different derivatives may be considered representative of the hydrophobic/hydrophilic balance of each substituted polymer and has been used to identify the different polymers thereafter named as P_n: $P_{0.5}$, $P_{1.0}$, $P_{1.5}$, $P_{2.0}$, $P_{2.5}$, and $P_{3.0}$, with n corresponding to the ratio between oleyl and TEGMEE substitution degree.

Table 1. Substitution Degree Percentage of Oleyl Chains and TEGMEE Chains on the Polyvinyl Backbone Expressed as mol of Substituent Per 100 mol of Hydroxyvinyl Monomer

% substituent in the preparative mixture[a]		% substituent in the final polymers[b]				oleyl/TEGMEE ratio[c]
		oleyl chain		TEGMEE		
oleyl chain	TEGMEE	^{1}H NMR	EA	^{1}H NMR	EA	n
5	50	0.5	0.5	1.1	0.9	0.5
10	50	1.0	0.9	1.0	1.0	1.0
20	50	1.4	1.6	1.1	1.0	1.5
30	50	2.0	1.9	0.9	1.1	2.0
40	50	2.4	2.6	1.0	1.2	2.5
50	50	3.1	2.8	0.9	1.2	3.0

[a] Percentage of substituent introduced in the preparative mixture. [b] Percentage of substitution in the final polymers determined by ^{1}H NMR and elementhal analysis (EA). [c] Ratio between oleyl and TEGMEE substitution degrees on the final polymers (n).

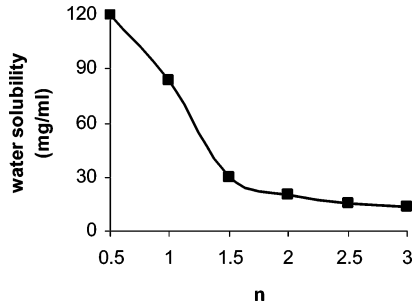

Figure 2. Water solubility of the P_n polymers at 37 °C.

Solubilization Studies. The water solubility of the different polymers decreased as the n values increased (Figure 2) in accordance with the hydrophobicity enhancement due to the increase in the oleyl chain substitution on the polymeric backbone.

DLS and SLS Measurements. DLS measurements of the polymer aqueous solutions revealed the presence of aggregates characterized by low polydispersity (minimum 0.20; maximum 0.31) and mean size not appreciably influenced by the polymer concentration (Table 2). This indicated that the polymers aggregate in nearly monodisperse systems following a closed association model.[18] A decrease in the mean diameter was observed from $P_{0.5}$ to $P_{1.5}$ and an increase from $P_{1.5}$ to P_3 (Table 2, Figure 3). The aggregation number, obtained by the SLS measurements, increased from $P_{0.5}$ to $P_{1.5}$ and decreased from $P_{1.5}$ to P_3 (Figure 4).

EPR Studies. EPR spectroscopy has been demonstrated to be a powerful technique to probe microenvironments of supramolecular structure involving macromolecules.[19] We selected 5-doxyl stearic acid (5-DSA), with the doxyl (4,4-dimethyl-*N*-oxyl-oxazolidinyl) group located five carbons away from the carboxyl headgroup (Figure 5), as the spin probe. Figure 6 shows the EPR spectra of 5-DSA in water and in the presence of

Table 2. Mean Size of the Polymeric Aggregates (nm) Formed by Dissolution in Water of Different Polymer Concentrations at 37 °C[a]

polymer conc. (mg/mL)	mean size of aggregates (nm)					
	$P_{0.5}$	$P_{1.0}$	$P_{1.5}$	$P_{2.0}$	$P_{2.5}$	$P_{3.0}$
0.5	586 ± 0.13	491 ± 0.09	285 ± 0.10	341 ± 0.13	620 ± 0.14	710 ± 0.14
1	575 ± 0.11	483 ± 0.11	280 ± 0.11	330 ± 015	612 ± 0.12	700 ± 0.12
2	570 ± 0.11	472 ± 0.12	269 ± 0.12	328 ± 0.08	603 ± 0.10	694 ± 0.10
4	564 ± 0.10	465 ± 0.10	253 ± 0.08	317 ± 0.11	594 ± 0.11	690 ± 0.13
6	560 ± 0.11	457 ± 0.08	240 ± 0.08	305 ± 0.08	586 ± 0.09	688 ± 0.12
8	556 ± 0.10	450 ± 0.12	236 ± 0.10	296 ± 0.13	580 ± 0.13	682 ± 0.11
10	550 ± 0.12	445 ± 0.13	230 ± 0.12	290 ± 0.11	572 ± 0.10	680 ± 0.09

[a] Each value represents the mean ± SD of three independent experiments.

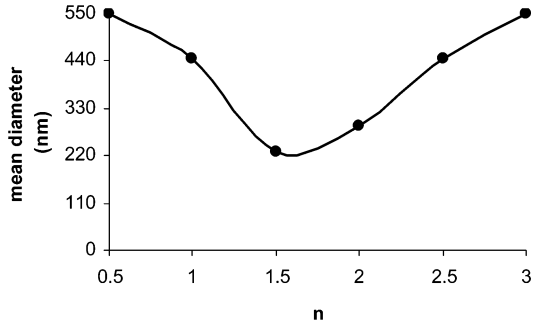

Figure 3. Mean diameter (nm) of the polymeric aggregates in water formed by 10 mg/mL Pn at 37 °C.

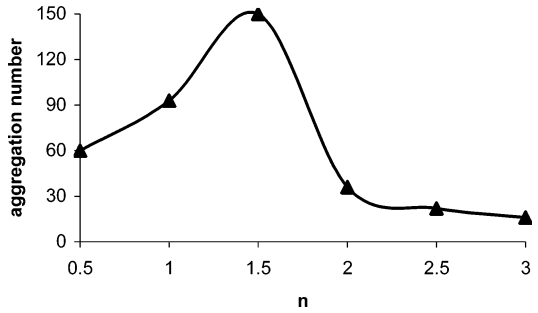

Figure 4. Aggregation number of the P_n polymers in water at 37 °C.

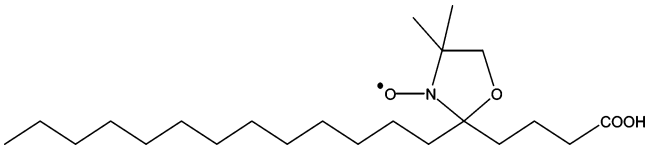

Figure 5. 5-Doxyl stearic acid (5-DSA) structure.

polymeric aggregates. The EPR spectrum of 5-DSA in water solution consists of a sharp three-peak signal characteristic of free probe molecules in fast motion in a polar environment (Figure 5). In the presence of the different substituted polymers, a significantly different EPR spectrum was observed. The spectrum is consistent with the probe in slow-motion condition, causing a partial resolution of the anisotropic components of the magnetic tensors.[20] Because of the significant differences in the spectra recorded in water and in the presence of polymeric aggregates, it can be concluded that that 5-DSA interact strongly with the polymeric aggregates. To confirm that 5-DSA is incorporated into the organic phase of the aggregates, we measure the hyperfine splitting constant, a_N, which is a measure of the polarity of the medium in which the radical resides.[21]

In the present case, a_N values are given by

$$a_N = 1/3(A_{||} + 2A_{\perp})$$

where $A_{||}$ is the time-averaged electron−nuclear hyperfine tensor

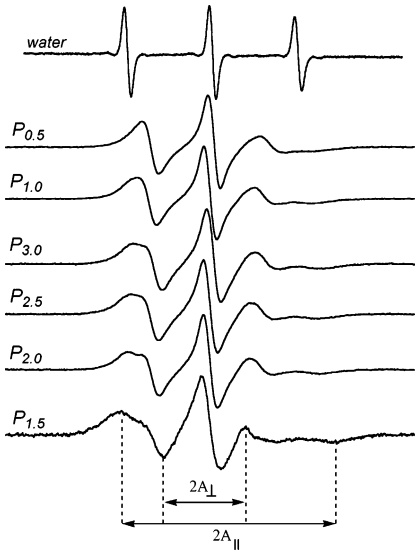

Figure 6. Experimental ESR spectra of 5-DSA recorded in water and in the presence of the different polimers at 298 K.

(parallel) and $2A_{\perp}$ is the time-averaged electron−nuclear hyperfine tensor (perpendicular).

Free 5-DSA in water shows a high hyperfine-coupling constant of a_N 15.8 G, because of the highly polar environment. In the presence of the different substituted polymer, 5-DSA is incorporated into the aggregates, and this causes a decrease in the hyperfine-coupling constant to a values in the range of 14.4−14.6 G, because of the decreased polarity of the aggregates.

The order parameter, S, is a measure of the spin probe arrangement in a supramolecular assembly and varies from 0 to 1 with $S = 1$ being in a completely ordered (crystalline) state and $S = 0$ in a completely random state. The order parameter of fatty acid spin labels can be estimated directly from the spectrum using the approximate relation[22]

$$S = 1.66[A_{||} - (A_{\perp} + C)]/[A_{||} + 2(A_{\perp} + C)]$$

where $C = 4.06 - 0.053\ (A_{||} - A_{\perp})$ MHz. $A_{||}$ and $A_{\perp}$ are equal to half the separation of the outer and inner extrema as indicated in Figure 6.

The investigated polymeric aggregates were found to have S values increasing rapidly to 0.42 when n goes from $P_{0.5}$ to $P_{1.5}$ and then decreasing slowly with the P_2, $P_{2.5}$, and P_3 derivatives (Figure 7).

Affinity for Hydrophobic Phases. All of the polymers displayed affinity for hydrophobic phases as all of the analyzed solutions were characterized by a decrease in the polymer concentration after equilibration both in the presence of the polyethylene membrane (Figure 8) and silicone oil (Figure 9). $P_{1.5}$ provided the highest decrease between the analyzed

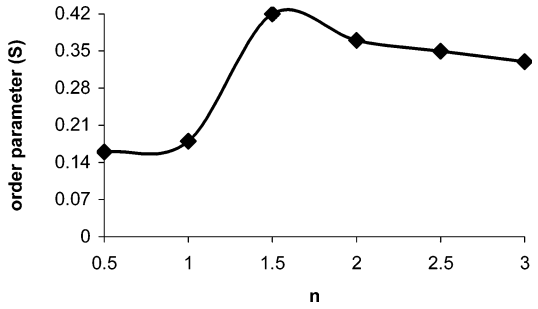

Figure 7. Order parameter of the different P_n polymers.

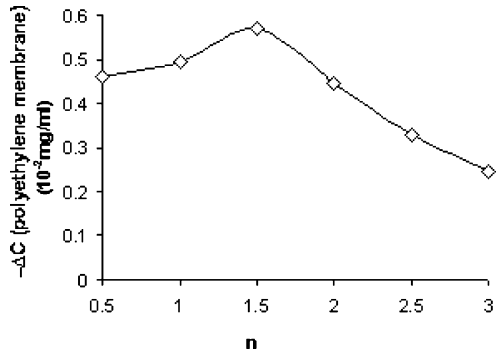

Figure 8. Concentration decrease ($-\Delta C$) of the Pn polymers in water, 37 °C, in the presence of polyethylene membrane.

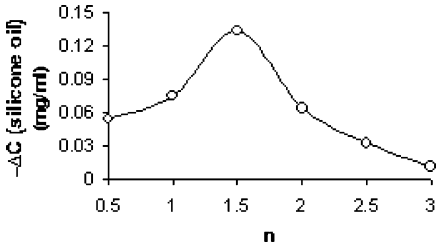

Figure 9. Concentration decrease ($-\Delta C$) of the Pn polymers in water, 37 °C, in the presence of a fluid hydrophobic phase (silicone oil).

polymers; the decrease was more evident in the presence of silicone oil than polyethylene membrane.

Complexation Ability of the Polymers toward Hydrophobic Molecules. The fluorescence increase of the polymer−naphtalene aqueous solutions with respect to the pure naphthalene solution, calculated by the ratio between the naphthalene emission fluorescence/excitation fluorescence in the presence of the polymer and that without polymer, indicated that all of the analyzed polymers were able to complex hydrophobic molecules. Figure 10, reporting the fluorescence increase in the presence of 2 mg/mL polymer, disclosed that $P_{1.5}$ held the best complexing ability among the analyzed polymers providing the highest increase in fluorescence intensity. The same trend was obtained with the other polymer concentrations analyzed. Based on these results, $P_{1.5}$ was selected as the complexing agent for HPR. The HPR complexation was carried out in water containing 30 mg/mL polymer, corresponding to the aqueous solubility of $P_{1.5}$ at 37 °C. Complexation greatly improved the intrinsic solubility of HPR from 1.71 μg/mL (pure HPR) to 111 μg/mL (complexed HPR). The HPR content in the complex was 0.37% (w:w).

HPR Release from the Complex. The release studies indicated that HPR remains prevalently associated with the polymer in an aqueous environment in the absence of hydrophobic phases as about 20% is released in 48 h. In the presence of hydrophobic phases, in comparison, release of HPR is

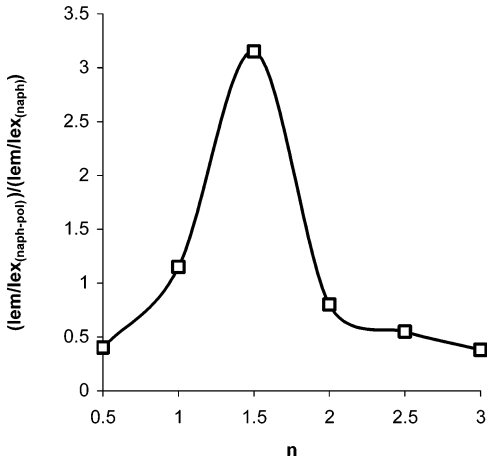

Figure 10. Fluorescence increase of naphthalene in water ($I_{em}/I_{ex(naph-pol)}$)/($I_{em}/I_{ex(naph)}$), in the presence of 2 mg/mL of the different P_n polymers at 37 °C.

enhanced. The increase is minimal in the presence of polyethylene membrane (about 26% released in 48 h) and considerable in the presence silicone oil (about 40% released at 48 h; Figure 11).

Biological Analysis. All of the polymers analyzed displayed dose-dependent cytotoxicity toward neuroblastoma cell lines. The cytotoxicity of $P_{1.5}$ was stronger than the other analyzed polymers (Table 3). Pure HPR did not provide any cytotoxic effect at the concentrations used in the present study (0.37% of the complex weight). The $P_{1.5}$−HPR complex displayed increased cytotoxicity than the separate complex components. In the presence of both $P_{1.5}$ and $P_{1.5-HPR}$, the tumor cells underwent massive and rapid detachment from the culture flask, became smaller, and showed nuclear condensation. The cytofluorimetric studies indicated that the cytotoxic effect was due to apoptosis. In the presence of resting peripheral blood mononuclear cells, the toxicity of all of the analyzed polymers and the complex resulted very low as assessed by flow cytometric assay of DNA content.

Discussion

In previous studies,[5] we have shown that the substitution of PVA with both oleyl and polyethylene glycol monoethyl ether (PEGMEE) chains provided a series of amphiphilic polymers endowed with dose- and time-dependent cytotoxicity toward tumor cells. The most active of the series was the polymer substituted with 1.5% oleyl chain and 1% tetraethylene glycol monoethyl ether (TEGMEE) (mol of substituent to mol of hydroxyvinyl monomer). In the current study, we have varied the oleyl chain substitution degree from 0.5% to 3%, maintaining at 1% the degree of TEGMEE substitution. All of the polymers obtained displayed high aqueous solubility in accordance with their self-assembling ability allowing shielding of the hydrophobic chains from the aqueous environment. Differences in solubility correlated with the oleyl chain substitution degree whose increase from $P_{0.5}$ to P_3 progressively hindered the shielding process, decreasing the aggregates affinity for water and thus the polymer solubility (Figure 2). The increase in degree of oleyl chain substitution from $P_{0.5}$ to $P_{1.5}$ increased moreover the packing density of the aggregates, as indicated by the progressive mean diameter decrease (Figure 3) and aggregation number increase (Figure 4). It also raised the order parameter (Figure 7). This trend occurred only from $P_{0.5}$ to $P_{1.5}$, with a further increase in the oleyl chain substitution degree,

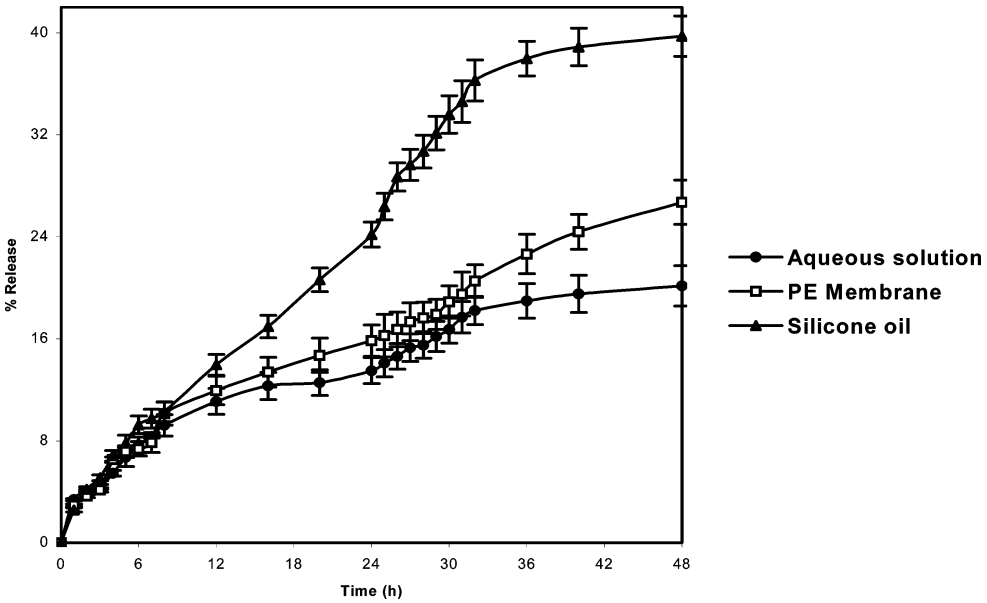

Figure 11. Fenretinide release from the $P_{1.5}$−fenretinide complex in aqueous solution at 37 °C (●) and in the presence of polyethylene membrane (□) or silicone oil (▲).

Table 3. Dose-Dependent Cytotoxic Activity toward HTLA-230 Neuroblastoma Cell Line and Resting Lymphocytes[a]

	HTLA-230 mortality %					resting lymphocytes mortality %
	no treatment	5 μg/mL	10 μg/mL	20 μg/mL	50 μg/mL	50 μg/mL
$P_{0.5}$	1 ± 0.65	10 ± 1.21	20 ± 0.63	33 ± 1.87	42 ± 3.51	7 ± 3.61
$P_{1.0}$	3 ± 1.13	16 ± 1.56	32 ± 4.78[b]	49 ± 1.47[b]	65 ± 6.10[b]	6 ± 2.53
$P_{1.5}$	5 ± 0.50	33 ± 1.56[b]	85 ± 2.54[b]	97 ± 2.86[b]	100 ± 0.00[b]	16 ± 3.27
$P_{2.0}$	2 ± 1.28	14 ± 2.39	21 ± 2.43[b]	27 ± 5.35[b]	32 ± 5.96[b]	9 ± 3.16
$P_{2.5}$	2 ± 0.95	11 ± 1.25	16 ± 3.21	19 ± 3.42[b]	24 ± 2.76[b]	7 ± 2.90
$P_{3.0}$	3 ± 2.61	5 ± 2.23	11 ± 1.89	15 ± 3.76	19 ± 2.92[b]	8 ± 1.51
$P_{1.5-HPR}$	2 ± 2.69	46 ± 3.29[b]	97 ± 1.75[b]	100 ± 0.00[b]	100 ± 0.00[b]	15 ± 3.11

[a] Values are expressed as a mean ± SEM of four independent experiments. [b] $p < 0.05$ vs HTLA-230 control.

from $P_{1.5}$ to P_3, inverting the trend, decreasing the packing density of the aggregates as indicated by the progressive mean diameter increase (Figure 3) and aggregation number decrease (Figure 4). A decrease in the order parameter also occurred (Figure 7). It is likely that the progressive increase in packing density from $P_{0.5}$ to $P_{1.5}$, driven by the hydrophobicity increase, reached a limiting step in $P_{1.5}$. Further increases in degree of oleyl chain substitution along P_2, $P_{2.5}$, and P_3 promoted a new, looser aggregation modality, due to the achievement of steric hindrance levels interfering with the polymer chain flexibility required for densely packed structures. The packing density increase from $P_{0.5}$ to $P_{1.5}$ also enhanced the structural order of the aggregates, as indicated by the EPR studies. The increased order parameter of the spin probe suggests that the aggregates made of $P_{1.5}$ are the more organized and more structured among the analyzed polymers. The decrease of entropy, presumably associated with the increased order, provides thermodynamic instability endowing the polymeric aggregates, and $P_{1.5}$ in particular, with a tendency to interact with environmental structures such as hydrophobic molecules (naphtlalene, fenretinide) or hydrophobic phases (polyethylene membrane, silicone oil in vitro; cell membranes in vivo). The interaction with hydrophobic phases such as the cell membranes may explain the cytotoxicity of these polymers, and that of $P_{1.5}$ in particular, toward the tumor cells. The higher cytotoxicity toward tumor cells than resting lymphocytes (Table 3) may be explained in accordance with the higher membrane fluidity, and thus higher thermodynamic instability, of the tumor cell membranes, favoring their interaction with the thermodynamically unstable polymeric aggregates, in an attempt to decrease the total free energy of the cell membrane-polymer system.[5] Also the interaction of the polymeric aggregates with hydrophobic molecules may be considered an attempt to decrease the total free energy of the polymer and the hydrophobic molecule in water by formation of a polymer−molecule complex. The highest thermodynamic instability of the $P_{1.5}$ polymeric aggregates accounting for their highest tendency toward complexation. The HPR complexation with $P_{1.5}$ significantly increased the drug aqueous solubility so improving HPR antitumor activity. Indeed, pure HPR did not display any antitumor activity at the amounts corresponding to the complex concentrations used in the present study while the complex elicited the drug antitumor activity as indicated by the increased cytotoxicity of $P_{1.5}$−HPR with respect to $P_{1.5}$ at concentrations ranging from 5 to 20 μg /mL (Table 3). The lack of activity of pure HPR, in common with many other hydrophobic drugs, is related to its insolubility in water causing drug association in molecular aggregates when the starting solution of the drug in organic solvent (ethanol in the present study), is diluted with the aqueous medium (cell cultures growth medium in vitro, blood in vivo).[23] Drug dissolution from the molecular aggregates is a rather inefficient process in providing the molecularly dispersed (solubilized) drug in aqueous environment which is required for interaction with the biological structures and is thus responsible for activity.[23] Complexation, in comparison, maintains a dynamic equilibrium between the complexed and the free drug, providing a rapid

supply of free drug in solution as its concentration decreases following interaction with the biological structures. Complexation with $P_{1.5}$ provided, moreover, increased drug release in the presence of fluid hydrophobic phases, suggesting that also in vivo the complex might release the drug prevalently in the presence of tumor cells (more fluid membranes) than normal cells or in blood during circulation. This hypothesis is supported by the observation that the $P_{1.5}$−HPR complex is no more cytotoxic than the pure $P_{1.5}$ polymer toward resting lymphocytes, whereas it shows greater activity toward the tumor cells (Table 3), in accordance with a prevalent HPR release in the tumor versus normal cells. This makes $P_{1.5}$ an interesting candidate for complexation of hydrophobic drugs whose antitumor activity is restrained by difficulties in achieving suitable solubilization levels in an aqueous environment. The resulting complex, moreover, is endowed with novel characteristics such as high antitumor effect due to both the intrinsic antitumor activity of the polymer and the drug, and higher cytotoxicity toward tumor cells than normal cells due to the prevalent polymer affinity for tumor cell membranes, enhancing the complexed drug release in the presence of tumors.

Conclusions

PVA substituted with 1.5% oleyl and 1% TEGMEE chains (mol of substituent to mol of hydroxyvinyl monomer) had previously been shown to self-assemble in water in aggregates highly cytotoxic toward tumor cells with minimal activity toward normal cells. It had also been shown to increase the long-term survival of nude mice injected with both human and murine neuroblastoma cell lines. Variation in the degree of oleyl chain substitution decreased the polymer antitumor activity. Its complexing ability toward hydrophobic molecules allowed complexation with fenretinide, significantly improving the drug aqueous solubility. The complex was stable in an aqueous environment, releasing the free drug prevalently in the presence of tumor cells. The complex antitumor activity was higher than the separate complex components due to the concomitant effect of the polymer intrinsic activity and the HPR solubilized by complexation. HPR was thus able to elicit its therapeutic potential at concentrations otherwise inactive as a pure drug.

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References and Notes

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