Multi-Responsive Polymer Micelles as Ellipticine Delivery Carriers for Cancer Therapy

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Abstract. *In the present study, we describe the synthesis and physicochemical properties of a novel pH- and thermoresponsive micellar drug delivery system for an anticancer ellipticinium derivative based on the triblock copolymer poly(ethylene oxide)-block-[tert-butylacrylamide-co-6-(N-methacryloylamino)hexanoic acid hydrazide]-block-poly(ethylene oxide). The system was designed to meet the basic criteria required for drug carrier systems, namely, solubility in water (overcoming the insolubility of ellipticine), satisfactory drug loading, particle size suitable for an efficient enhanced permeability and retention effect and adequate stability in blood plasma (pH 7.4) followed by rapid drug release in tumors or tumor cell endosomes (pH <6.5). The copolymer in the form of a unimer can be eliminated by kidneys because the weight-average molecular weight of 21 kDa is sufficiently below the renal threshold. The half-life of drug release in a pH 5.0 buffer solution (pH of a late endosome) was ~45 h, but a negligible amount of the free ellipticine derivative was detected at pH 7.4 (pH of blood). Consequently, this supramolecular polymer conjugate is a good candidate for the delivery of ellipticine-based drugs and will therefore be subjected to more detailed studies.*

Cancer chemotherapy involves treatments with a broad variety of synthetic or natural substances that exhibit specific tumor-suppressive properties. Ellipticine, a natural alkaloid derived from the plant *Ochrosia elliptica*, is a typical representative of such compounds. Its antiproliferative activity arises mainly from its strong interaction with nuclear DNA (1), disabling the transcription of DNA during the cell cycle. Due to the high potency and moderate side-effects of ellipticine, strong efforts have been made towards additional improvement of its therapeutic profile, as well as its physicochemical characteristics, especially its poor solubility in water. There are several ways this improvement could be achieved, including modification of the drug formulation and chemical derivation of the parent compound.

A reduction in side-effects attributed to chemotherapeutics may be achieved by conjugation of the drug to natural or synthetic water-soluble polymer carriers (2, 3). Moreover, the solid tumor specificity of such polymer–drug conjugates is dramatically increased thanks to the enhanced permeation and retention (EPR) effect (4, 5). While the EPR effect is most pronounced for macromolecules with molecular weight up to 1,000 kDa, the use of water-soluble polymers is limited by their molecular weight, which must be below the renal threshold (molecular weight ~45 kDa, if the polymer is not biodegradable) to allow renal excretion after accomplishing the task. Polymer micelles belong to the self-assembly systems with apparent molecular weight usually reaching a few hundred kiloDaltons, but they retain the ability for renal excretion due to equilibrium with unimers. Moreover, micelles, in general, possess a very narrow size distribution and thus exhibit uniform behavior in an organism. In other words, polymer micelles can serve as well-defined drug delivery vehicles with a tendency to adhere to the EPR effect and to maintain all advantageous features of water-soluble polymer–drug conjugates. The unimers are, in most cases, based upon di- or triblock amphiphilic copolymers for which the hydrophilic portion can be *e.g.* poly(ethylene oxide), whereas poly(propylene oxide) or poly(methacrylates) with longer aliphatic tails are commonly used as the hydrophobic counterparts. The therapeutic cargo can be either non-covalently incorporated into the hydrophobic core of a micelle, or covalently attached to a polymer. The non-covalent approach is limited to highly hydrophobic drugs (taxanes, ellipticine, *etc.*); no chemical modification of the drug is required. In the latter case, on the contrary, almost any agent with appropriate functional groups or a suitable derivative can be involved. Moreover, a fine-tuned release profile can be achieved in comparison with the non-covalent micellar conjugates (6).

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This article presents the synthesis and physicochemical evaluation of a conceptually new pH- and thermoresponsive micelle-forming conjugate of triblock poly[ethylene oxide-*block*-(*tert*-butylacrylamide-co-6-(*N*-methacryloylamino) hexanoic acid)-block-ethylene oxide] (**2**; Figure 1) with 2-*N*-(2-oxopropyl)ellipticinium bromide (**1**; Figure 1), a tailormade cytotoxic ellipticine derivative (7) bearing a carbonyl group enabling the formation of a pH-sensitive hydrolyzable hydrazone bond with the polymer. Self-assembly behavior of the ellipticine conjugate showing crosstalk between pH- and thermoresponsivity was studied using various preparative and characterization techniques. Finally, the conjugate structure was optimized regarding drug loading and the size of micelles formed in order to obtain a drug delivery system promising for cancer therapy.

Materials and Methods

Chemicals. 2,2'-azobis(isobutyronitrile) (AIBN), bromopropanone, ellipticine, *N-tert*-butylacrylamide (NTBAM), methacryloyl chloride and methyl 6-aminohexanoate hydrochloride were purchased from Sigma-Aldrich, Prague, Czech Republic. poly(ethylene glycol)-based macro azo-iniciator ($PEOR_2N_2$) was purchased from Wako Chemicals GmbH, Neuss, Germany. Acetonitrile, methanol, *N,N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and other common solvents and chemicals were purchased from Merck, s.r.o., Prague, Czech Republic. All chemicals and solvents were of analytical grade.

Preparative procedures. Synthesis of the reactive co-monomer 6-methacrylamidohexanohydrazide (MA-HH) was carried out using two steps, as previously described in literature (8) with an overall yield of 80%.

Synthesis of 2-*N*-(2-oxopropyl)ellipticinium bromide consists of alkylation of ellipticine with bromopropanone, as described in further detail in the literature(7), with an overall yield of 75%. The general scheme of preparation of reactive copolymer **2** with consequent attachment of 2-*N*-(2-oxopropyl)ellipticinium bromide **1** is shown in Figure 1. Polymerization was carried out using a macro azo-initiator $PEOR_2N_2$ that, at elevated temperatures, forms two exactly defined PEO-based macro radicals initiating the growth of polymer chains. The final composition of copolymer **2** was then optimized by adjusting the molar ratio of the initiator and co-monomers, reaction time and concentration. Attachment of the ellipticine derivative **1** was performed by means of an acid-catalyzed condensation using a slight excess of **1** to reach a maximum drug loading.

*Preparation of copolymer of PEO, NTBAM and MA-HH [poly(PEG-block-NTBAM-co-MA-HH-block-PEO)] (**2**; Figure 1)*: $PEOR_2N_2$ (135 mg, 0.011 mmol), NTBAM (108 mg, 0.85 mmol) and MA-HH (35 mg, 0.16 mmol) were polymerized in tetrahydrofuran (THF) (8 ml) at 60˚C for 16 h under an argon atmosphere. The polymer was precipitated into diethyl ether, filtered out, dissolved in THF and precipitated again into diethyl ether. The product was dried under vacuum and characterized. Yield: 222 mg (80%); molecular weight by gel permeation chromatography (GPC): M_w=18 kDa, M_w/M_n=1.29; hydrazide group content [2,4,6-trinitrobenzenesulfonic acid assay(9)]: 0.24 mmol/g.

Attachment of 2-N-(2-oxopropyl)ellipticinium bromide to poly(PEO-block-NTBAM-co-MA-HH-block-PEO): 2-*N*-(2-Oxopropyl)ellipticinium bromide (3.1 mg, 8.1 μmol) was added to a solution of poly(PEO-block-NTBAM-co-MA-HH-block-PEO) (31 mg, 7.4 μmol of hydrazide group equivalent) and acetic acid (100 μl) in methanol (700 μl) and the reaction mixture was stirred in the dark at room temperature for 48 h. The resulting solution was diluted with methanol (5 ml) and separated by chromatography on a Sephadex LH-20 column using methanol as the eluent. The resulting conjugate (**3**; Figure 1) was isolated as a high-molecular-weight (HMW) fraction, concentrated under reduced pressure and precipitated into dried diethyl ether. The suspension was centrifuged and the precipitated yellow powder was dried under vacuum. Yield: 26 mg (76%); molecular weight (GPC): M_w=21 kDa, M_w/M_n=1.35; total 2-*N*-(2-oxopropyl)ellipticinium bromide content (determined spectrophotometrically in a methanol solution at 295 nm; ε=30500 l mol-1 cm-1): 6.3 wt%.

Evaluation of drug release rate in aqueous medium. Solutions of polymer–drug conjugate (1 mg/ml) were incubated at 37˚C in PBS (pH 7.4) and acetate buffer (pH 5.0), respectively. After specific times, the amount of released ellipticinium derivative was determined by GPC and calculated from the ratio of low-molecular-weight (LMW) and HMW peak areas at 311 nm. All experiments were carried out in duplicate.

Instrumental methods. Analyses were performed on a high performance liquid chromatography chromatograph (Schimadzu, Prague, Czech Republic) equipped with a reverse-phase column Chromolith Performance RP-18e 100×4.6 mm (Merck, s.r.o., Prague, Czech Republic) and UV/VIS detection. A mixture of water and acetonitrile was used as the eluent at a gradient of 0-100 vol% and a flow rate of 5 ml/min. Elemental composition was determined using a Perkin Elmer Elemental Analyzer 2400 CHN (Perkin Elmer, Waltham, Massachusetts, USA). Melting point temperatures were determined on a Kofler's block (VEB Analytik, Dresden, Germany). Nuclear magnetic resonance (NMR) spectra were measured on a Bruker Avance MSL 300 MHz NMR spectrometer (Bruker Daltonik, Bremen, Germany). Molecular weights of the polymers were determined by GPC using an HPLC Shimadzu system equipped with a GPC column TSKgel G3000SWxl 300×7.8 mm; 5 μm (Watrex s.r.o., Prague, Czech Republic), UV/VIS, RI Optilab®-rEX and MALS DAWN EOS (Wyatt Technology Co., Santa Barbara, California, USA) detectors using a methanol and sodium acetate buffer (0.3 M; pH 6.5) mixture (80:20 vol%; flow rate 0.5 ml/min). UV/VIS spectra were measured on a SPECORD 205 Spectrometer (Analytik Jena AG, Jena, Germany). Molecular masses were determined using mass spectrometry performed with an LCQ Fleet mass analyzer with electrospray ionization (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Dynamic light scattering measurements were carried out at scattering angle θ=173˚ on a Zetasizer Nano-ZS, Model ZEN3600 using DTS (Nano) software, version 6.20 (Malvern Instruments Ltd, Malvern, Worcestershire, UK) for data evaluation. The temperature was maintained within a narrow interval of ±0.05˚C around the set value for all the measurements. Values of hydrodynamic radius, R_H, were calculated from volume distribution functions.

Dynamic light scattering. Polymer solutions (0.1 mg/ml) were filtered through a 0.45-μm PVDF syringe filter directly into the measurement cuvettes, kept in a refrigerator at 8˚C for at least 2 h

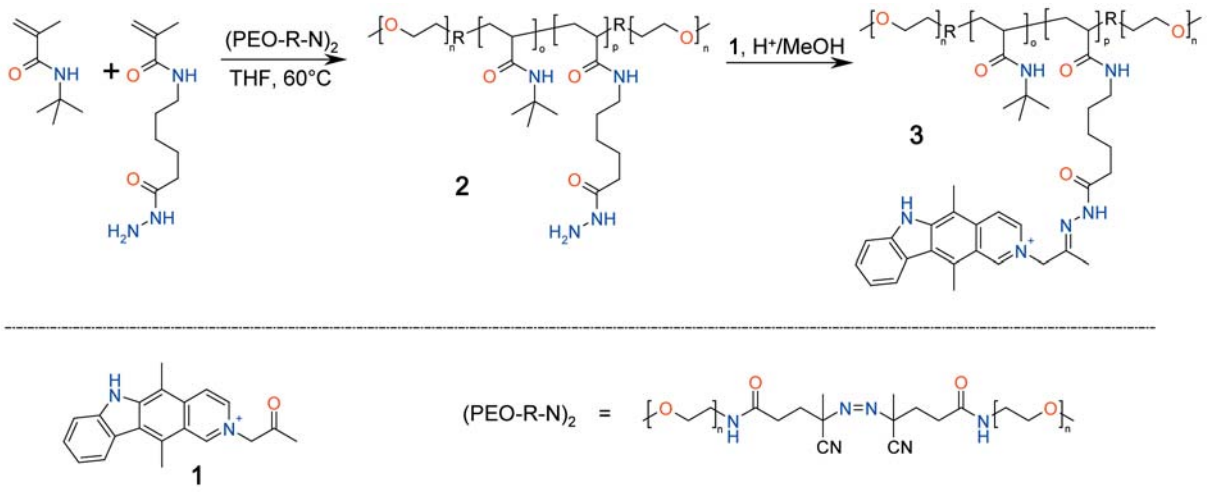

Figure 1. *Scheme of the synthesis of a polymer-ellipticine conjugate* ***3***.

and then inserted into the sample holder of the light scattering instrument. Samples were heated in given temperature steps, measurements were performed at each temperature point after reaching steady conditions (typically 20 min).

Results and Discussion

In the present report, we show the possibility of utilizing a water-soluble micelle-forming synthetic block copolymer as a carrier for ellipticine, a potent anticancer drug. The benefits of the concept of micellar drug carriers have been proven extensively, encompassing a wide range of therapeutics and amphiphilic synthetic polymers (10). Di- or triblock copolymers combining hydrophilic and hydrophobic blocks in the main chain are most suitable for forming micelles compared to other amphiphilic polymers (expressed by their sufficiently low critical micelle concentration). In this work, the PEO-based macro azo-initiator PEOR2N2 was used for incorporation of hydrophilic blocks into the block copolymer carrier, while the hydrophobic blocks consisted of statistical copolymer of NTBAM and a calculated amount of MA-HH, a reactive co-monomer needed for binding of the ellipticine derivative (Figure 1). A PEO/NTBAM ratio was established to obtain a system water soluble at RT (20˚C) but self-assembling into micellar form at body temperature (37˚C). In other words, the composition of the polymer carrier was designed for preparation of a final polymer–drug conjugate having a thermoresponsive behavior with cloud point temperature in the range of 20 37˚C. Although unmodified ellipticine is hydrophobic enough to be incorporated into the micellar hydrophobic core non-covalently, its poor solubility is the main obstacle to reaching sufficient drug loading. Therefore, a carbonyl group was introduced into the ellipticine structure and this derivative was then covalently attached to the above-mentioned reactive block copolymer at a sufficient concentration. During this step, an acid-labile hydrazone bond between the drug and polymer was formed. As shown below, the hydrazone linkage is sufficiently stable at pH ~7.4 (bloodstream) but quickly hydrolyzed at pH ~5.6 (tumor microenvironment), consequently, partial tumor specificity is achieved.

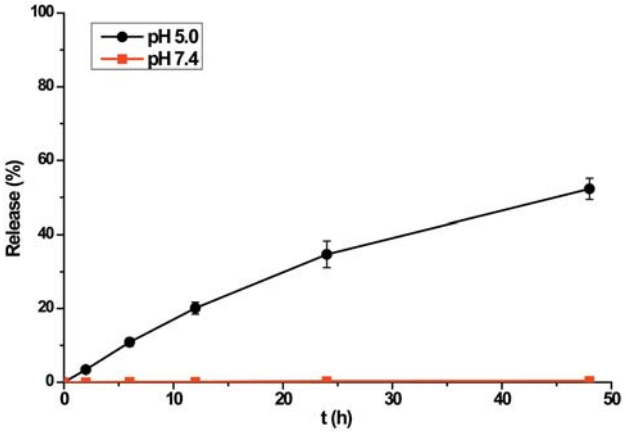

Figure 2. *Release of ellipticinium drug* ***1*** *from polymer conjugate* ***3*** *at 37˚C incubated in phosphate saline buffers of pH 5.0 and pH 7.4.*

Evaluation of drug release rate in aqueous solution. The release profile of ellipticinium drug **1** from its micellar conjugate **3** was determined (Figure 2). In the buffer of pH 7.4 (mimicking environment in blood plasma), the system

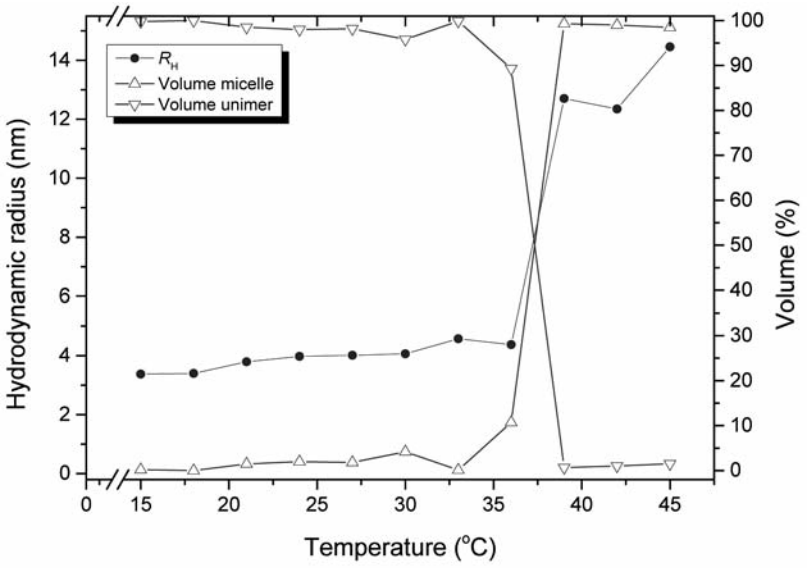

Figure 3. *Hydrodynamic radius and volume fractions of unimers and micelles, respectively, as a function of temperature for the solution of polymer **2** in phosphate-buffered saline.*

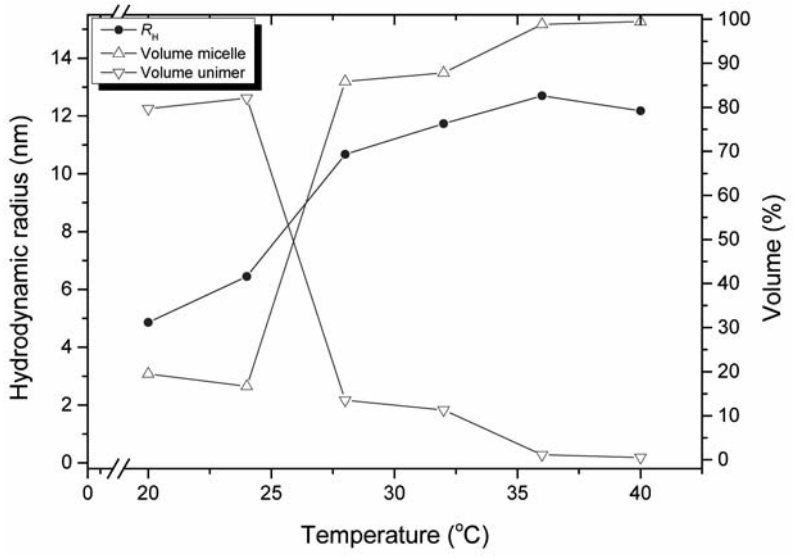

Figure 4. *Hydrodynamic radius and volume fractions of unimers and micelles as a function of temperature for the solution of polymer conjugate **3** in phosphate-buffered saline.*

remained stable (<1% of drug released within 24 h). On the other hand, in the more acidic buffer (pH 5.0, mimicking environment in late endosomes), the hydrazone linker was cleaved and the drug **1** was released (35% of drug released in 24 h). This finding was in accordance with the release rates determined in the conjugates based on hydrophilic water-soluble *N*-(2-(hydroxypropyl)methacrylamide copolymer conjugates containing similar spacer (11). For our conjugate, the rate of hydrolysis at pH 5.0 was slightly lower compared to water-soluble *N*-(2-(hydroxypropyl)methacrylamide copolymer conjugate. The most plausible explanation of this difference is the lower activity of water in the hydrophobic core of the micelle and the partition coefficient of the partly hydrophobic drug between the outer solution and micelle

core, maintaining higher concentration of the released drug in the micellar core compared to the councentration in the outer solution. Moreover, a higher drug concentration in the micellar core pushes equilibrium of the hydrazone hydrolysis/back formation towards hydrazone formation, and thus it can also contribute to the decreased net rate of hydrolysis. We observed a similar effect for doxorubicin-containing micelles with hydrazone bond-bound doxorubicin (12).

Dynamic light scattering. Formation of micelles from both a polymer precursor **2** and a polymer–ellipticine conjugate **3** was investigated to determine the temperature dependence of the hydrodynamic radius R_H and volume distribution of both, unimer and micelle fractions (Figures 3 and 4). The precursor **2** exhibited a clear transition in the temperature range between ~35 and 40˚C where the average RH value sharply increased from ~4 nm to ~12 nm. In addition, the LMW fraction was only present at temperatures below ~35˚C, completely disappearing at temperatures above ~40˚C, where the HMW fraction predominated. The polymer–ellipticine conjugate **3** behaved in a similar way, but the transition temperature range was significantly lower (~25-30˚C), which could be attributed to a hydrophobic contribution of the ellipticine derivative. The data obtained indicate that both polymers, the copolymer **2** and polymer–drug conjugate **3**, have features characteristic of thermoresponsive micelle-forming polymers, where R_H values were in the ranges of 3-5 nm and 10-15 nm for unimers and micelles, respectively.

Conclusion

The synthesis and *in vitro* physicochemical behavior of a multiresponsive, water-soluble, polymer micellar drug delivery system designed for the anticancer drug ellipticine was herein described. A novel approach, based on the covalent attachment of a previously chemically-modified ellipticine to an amphiphilic triblock copolymer *via* a hydrolytically labile hydrazone linker, was exploited. The tailor-made hydrazone linkage between the polymer carrier and ellipticine derivative was almost entirely stable at pH 7.4 (blood) but readily hydrolyzed at pH 5 (late endosome). The conjugate containing 6.3 wt% of the drug was soluble in water at room temperature in the form of a unimer solution (R_H<5 nm) but switched to the HMW micellar form (R_H>10 nm) at temperatures higher than ~30˚C. This micellar drug delivery system increases the chances of the successful application of potent chemotherapeutics of the ellipticine class to cancer treatment.

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