Published in final edited form as:

Polym Chem. 2017 September 14; 8(34): 4983–4987. doi:10.1039/C7PY00196G.

Poly(*N*-(2-Hydroxypropyl) Methacrylamide)-Valproic Acid Conjugates as Block Copolymer Nanocarriers

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Abstract

We report nanoassemblies based on block copolymers of *N*-(2-hydroxypropyl) methacrylamide (HPMA) in which drug cleavage enhances the biological compatibility of the original polymer carrier by regeneration of HPMA units. Drug release via ester hydrolysis suggests this approach offers potential for stimuli-responsive drug delivery under acidic conditions.

The lack of site-specificity and inefficient drug administration in traditional cancer treatment has resulted in a search for suitable chemotherapeutic delivery systems. Polymeric micelles, colloidal nanomaterials formed *via* self-assembly of amphiphilic block copolymers, have great potential in this application.¹ The hydrophobic block serves as the micelle core, allowing incorporation of hydrophobic drugs *via* noncovalent interactions in the nanocarrier and enhancing drug viability through increased circulation time *in vivo*.² Moreover, the hydrophilic block forms the micellar shell or corona, providing a stabilizing interface between the hydrophobic core and the aqueous environment and preventing adsorption to proteins and slowing renal filtration.

Chemical conjugation or solubilization of anti-cancer drugs has resulted in various stimuli-responsive polymeric delivery vehicles in which release can be induced by changes in temperature³ or pH,⁴ by irradiation with light,⁵ or by introduction of specific enzymes.⁶ Taking advantage of the mildly acidic environment of lysosomes, endosomes, and tumor tissues,⁷ pH-responsive polymeric drug delivery conjugates present a strategy for passive targeting and treatment of cancerous tissues through the enhanced permeation and retention effect and subsequent hydrolytic drug release.⁸

Judicious choice of the hydrophilic shell-forming block is vital for micelle stabilization and proper circulation in the blood stream. Poly(*N*-(2-hydroxypropyl) methacrylamide) (PHPMA) has been extensively investigated as the hydrophilic component, as it is a water-soluble, non-charged, nontoxic, non-immunogenic, and biocompatible polymer.^{9, 10} Several

acid-labile PHPMA drug conjugates have been reported utilizing hydrazone linkages between PHPMA and DOX¹¹ or other antitumor drugs.¹² For example, an acid-sensitive *cis*-aconityl bond between DOX and HPMA copolymer precursor was described by Kopeček and coworkers.^{9, 13}

We are particularly interested in materials based on polymers of HPMA because they contain hydroxyl moieties that can be utilized as handles for installation of various drugs via ester linkages, affording potentially-responsive delivery systems.^{14, 15} Hennink and coworkers previously reported block copolymers of HPMA with HPMA-dilactate, where hydrolysis under basic conditions (pH 10) led to hydrolysis and liberation of HPMA units to achieve a completely hydrophilic polymer.¹⁶ Our group previously synthesized core-crosslinked star polymers which conjugated the hydroxyl group of PHPMA to the carboxyl functionality of the anticancer drug methotrexate.¹⁴ Here, we demonstrate that modifying HPMA with valproic acid (VPA) will render the resulting polymer sufficiently hydrophobic to form amphiphilic block copolymers where both blocks are based on PHPMA. In this way, the functionalized block can serve as the micellar core loaded with a covalently bound drug for enhanced stability. VPA is an established antiepileptic drug that has recently been shown to effectively inhibit histone deacetylases (HDACs).^{17, 18} HDAC inhibitors promote hyperacetylation of lysine residues on histones, often leading to a variety of antitumor effects (*e.g.*, cell cycle arrest, apoptosis, disruption of angiogenesis, etc.).¹⁹ VPA has demonstrated antitumor activity towards many tumor types, including cancers of glioma, breast, colon, and cervical tissues.¹⁸ However, it is not known whether these effects are selective for cancerous tissue, therefore there is a direct need for targeted systems. Along these lines, Stefan *et al.* have investigated polymeric systems for delivery of VPA.²⁰ Poly(ethylene glycol)-*b*-poly(γ -2-propylpentanoate- ϵ -caprolactone) amphiphilic block copolymers were designed such that hydrolysis of the polyester block led to release of VPA and eventual backbone degradation.

In this work, VPA was conjugated to the hydroxyl group of HPMA, yielding a methacrylamide monomer (VPMA) that was used to prepare the hydrophobic segment of an amphiphilic block copolymer with PHPMA. The resulting block copolymers of PHPMA-*b*-P(HPMA-*co*-VPMA) underwent self-assembly in aqueous solutions to yield micelles with a hydrophilic and biocompatible corona of PHPMA and a hydrophobic and drug-conjugated core of P(HPMA-*co*-VPMA). Acidic hydrolysis of the VPMA units within the micelle cores liberated the VPA drug to result in additional HPMA units. Therefore, drug release from this block copolymer was expected to induce a transformation of PHPMA-*b*-P(HPMA-*co*-VPMA) to a product containing more HPMA units, enhancing the biocompatibility as compared to the original polymeric drug carrier.

HPMA was polymerized by RAFT polymerization²¹ using 4-cyano-4[(dodecylsulfanylthiocarbonyl)-sulfanyl]-pentanoic acid (CDTPA) as the chain transfer agent (CTA) to garner control over the molecular weight and molecular weight distribution (Fig. 1(a)). This CTA is known to control the polymerization of methacrylamide monomers and has been widely used to prepare well-defined PHPMA homopolymers and copolymers.^{14, 16} A PHPMA homopolymer with a molecular weight of 8.0 kg/mol and a

narrow molecular weight distribution ($M_w/M_n = 1.1$) was obtained, as evidenced by gel permeation chromatography (Fig. S1).

Following the synthesis of water-soluble PHPMA, we reasoned that chain extension with a hydrophobic HPMA-drug conjugate would afford amphiphilic block copolymers with covalently bound drugs, also resulting in enhanced drug stability. Therefore, VPA was used as a model anticancer drug to conjugate to HPMA to obtain HPMA-valproic acid (VPMA) (Scheme S1). ^1H and ^{13}C NMR spectra and mass spectrometry confirmed the successful synthesis of this compound (Figs. S2–S4).

To prepare the block copolymers, PHPMA was chain extended with VPMA under the same conditions (*i.e.*, initial monomer concentrations and temperature) used for the synthesis of PHPMA. However, the chain extension was sluggish, with only 10% monomer conversion being achieved after 48 h. We postulated that slow polymerization was potentially due to steric hindrance imparted by the lengthy and bulky alkyl chain of VPMA. Moreover, chain transfer reactions of the methine group of VPMA (*b* in Fig. 1) to form a stable tertiary radical were possible during the radical process, leading to high molecular weight shoulders indicative of branched polymer species in the gel permeation chromatograms. To address these issues (*vide supra*), we used HPMA as a comonomer with VPMA for the synthesis of the second block. The incorporation of HPMA was envisioned to reduce the steric hindrance from the bulky alkyl chains of the VPMA monomer units and decreased the possibility of chain transfer of the methine hydrogen of VPMA. Various comonomer ratios were tested, starting with a 1:1 ratio, then increasing the concentration of VPMA to incorporate more drug (Table 1). It is clear that the drug loading in the block copolymer increases with increasing the feed ratio of VPMA to HPMA (entries 3 and 4). The molecular weight distributions of all block copolymers were narrow, suggestive of a controlled polymerization process. The absolute molecular weights of the diblock copolymers ranged from 16.6 to 19.9 kg/mol (Table 1). A sample GPC trace of the block copolymer **4** showed a shift toward lower retention time after chain extension, confirming successful block copolymerization (Fig. 2).

Micelles were prepared by a solvent exchange method, with a solution of the block copolymer in *N,N*-dimethylformamide (DMF) being dialyzed against deionized water for three days. During dialysis, the turbidity of the solution increased, suggesting aggregate formation *via* the self-assembly of the hydrophobic VPMA- containing block. The drug loading can be controlled by simply tuning the amount of VPMA in the monomer feed during the polymerization to form the hydrophobic segment. In this way, we can achieve high drug loading in a facile manner, as compared to traditional methods of physical drug encapsulation that often suffer from low efficiency. Dynamic light scattering (DLS) was used to determine the size of the self-assembled block copolymers from Entry 2 (Fig. S5). The self-assembly of block copolymers **2–4** (Table 1) resulted in sizes ranging from 24–28 nm, consistent with the formation of micelles. Figure S5 shows a representative DLS size distributions for the block copolymer detailed in entry **2** from Table 1. The size ($D_h \approx 24$ nm) and spherical nature of the micellar aggregates was further confirmed by transmission electron microscopy (TEM) (Fig. 3a).

The formation of these spherical micelles in an aqueous environment is driven by association of the hydrophobic VPMA-containing segments that leads to block copolymer self-assembly. The critical micelle concentration (CMC) indicates stability of the polymeric nanostructures under dilute conditions, which could be an important consideration for potential biological applications. An insensitivity of the micelles to dilution could improve viability as drug carriers due to increased nanocarrier stability. The CMC of PHPMA-*b*-P(HPMA-*co*-VPMA) conjugate was determined using pyrene as a model fluorescent probe. The concentrations of copolymer **4** were varied from 3.00 mg/mL to 3.18×10^{-6} mg/mL, with a fixed pyrene concentration of 6×10^{-6} M. The fluorescence emission spectra were recorded from 360 to 600 nm (Fig. S6 a, $\lambda_{\text{ex}} = 340$ nm). The CMC was estimated to be 0.026 mg/mL as the intersection of the extrapolated linear regions at low and high concentrations (Fig. S6b). The obtained CMC is in good agreement with other reported values for PHPMA copolymer-based micelles and suggests good micelle stability in aqueous media.²²

Release of the model drug VPA was envisioned to occur *via* ester hydrolysis, the rate of which was expected to be pH-dependent. Drug release studies of the block copolymer micelles were conducted at 37 °C at both acidic (to mimic conditions in tumor tissues) and physiological pH. For example, the block copolymer micelle from entry **4** (Table 1) was dissolved in phosphate buffer solutions (pH = 5 and pH = 7.4). A series of samples were removed at predetermined time points and were characterized using ¹H NMR spectroscopy. Ester hydrolysis in the acidic buffer was followed by the reduction of the signals at 2.3 ppm attributed to the methine proton of the VPMA conjugate. The kinetics of drug release show a gradual hydrolysis of VPMA over time, with about 47% release being achieved after 5 d at pH 5 (Fig. S7). This result is consistent with previous reports of drug delivery system *via* ester hydrolysis.²³ Drug cleavage at pH 7.4 was considerably slower, with no significant release over time in neutral PBS. Dynamic light scattering was used to confirm the observations from the ¹H NMR spectroscopy analysis. Micellar solutions from entry **4** (Table 1) were incubated at acidic and neutral pH. The size of the particles in these solutions were measured by DLS and compared to the original polymer nanostructures (Fig. 3b). As expected, a reduction in size from 24 nm to ~5 nm was observed at pH = 5. This reduction in size can be explained by release of the hydrophobic drug rendering the core block hydrophilic and eliminating the amphiphilicity necessary for polymer self-assembly, resulting in the presence of unimers in solution. In contrast, the block copolymer micelles incubated at physiological pH did not decrease in size ($D_h = 38$ nm after 9 d), suggesting the persistence of self-assembled micelles under these conditions.

To gain additional insight of the drug release process, the presence of the released valproic acid was examined by gas chromatography (GC). The sample incubated in acidic buffer for 5 days at 37 °C exhibited a peak at 8.3 min, corresponding to the elution time of pristine valproic acid (Fig. S8). However, GC of the original block copolymer showed no peak at the elution time for VPA. Additionally, the polymer samples treated in physiological buffer for 10 days at 37 °C were characterized by GC, with no free valproic acid being detected (Fig. S8). These results clearly indicate the drug was specifically detached from the block copolymers upon ester hydrolysis at reduced pH, which is consistent with other reports of

valproate ester hydrolysis under mildly basic conditions.²⁰ It is also noteworthy that the block copolymer was stable in physiological buffer at extended times, indicating the potential of this polymer-drug conjugate as a pH-specific drug release system.

To further examine the cytotoxicity, HCT 116 colon cancer cells were incubated with different concentrations of PHPMA homopolymer 1 and VPA-conjugated PHPMA copolymer 4 at physiological pH (pH = 7.4). More than 90% of cells survived when cultured for 72 h with 200 µg/mL of either polymer 1 or 4 (Fig. S9). This result suggests that both PHPMA homopolymer and polymer-drug conjugate have low cytotoxicity, even at high concentrations. In the case of treating cancer cells with the polymer-drug conjugate, the high cell survival rate can be attributed to the low anti-cancer efficiency and high IC₅₀ value of the model drug valproic acid.²⁴ Therefore, we are currently investigating conjugation with alternative drugs that possess more potent anti-cancer activity.

In this report, we present the synthesis of pH-responsive block copolymer micellar drug delivery platform based on PHPMA. Chemical conjugation of the anticancer drug VPA to PHPMA polymer enhanced the loading and bioavailability of the drug. The low CMC of the block copolymer micelle suggests their stability in the blood stream. Drug release studies indicated that ester hydrolysis occurred at pH ~ 5. However, the micelles were stable over long periods at physiological pH, indicating that the release process is selective to acidic media. Taking advantage of the acidic environment of cancerous tissues relative to healthy cellular environments, we believe this approach to drug delivery may provide advantages in cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This material is based upon work supported by the National Science Foundation (DMR-1606410) (B.S.S.). Research reported in this publication was partially supported by the National Institutes of Health under award number R01CA172310.

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Fig. 1.

a) RAFT homopolymerization of *N*-(2-hydroxypropyl) methacrylamide). b) Chain extension of a PHPMA macroCTA with HPMA and VPMA to yield an amphiphilic block copolymer-drug conjugate, which assembles into micelles in water. Acidic hydrolysis of ester linkages induces release of VPA.

Fig. 2. GPC traces of the block copolymer $\text{PHPMA}_{53}\text{-}b\text{-P(HPMA}_{15}\text{-}co\text{-VPMA}_{28})$ (entry **4**) and the corresponding PHPMA_{53} macroCTA.

Fig. 3. (a) Transmission electron micrograph of the micelles (21 nm) from entry 2; (b) Dynamic light scattering size distributions (hydrodynamic diameter) of the block copolymer-based micelles from Entry 4 (Table 1) at acidic and physiological pH.

Table 1
Results of Polymerizations of *N*-(2-Hydroxypropyl) Methacrylamide (HPMA) and Its Valproate Ester (VPMA).

Entry	Polymer	Time (h)	Feed ratio ^a	Conversion (%)	M_n^b (g/mol)	PDI^b	Micelle size ^c (nm)
1	PHPMA ₅₃	5	100:0:1	51	8 000	1.10	N/A
2	PHPMA ₅₃ - <i>b</i> -P(HPMA ₂₆ - <i>co</i> -VPMA ₁₉)	3	50:50:1	43	19 900	1.19	24
3	PHPMA ₅₃ - <i>b</i> -P(HPMA ₂₁ - <i>co</i> -VPMA ₂₅)	3	50:75:1	40	18 700	1.25	28
4	PHPMA ₅₃ - <i>b</i> -P(HPMA ₁₅ - <i>co</i> -VPMA ₂₈)	3	25:75:1	45	16 600	1.13	25

^aFeed ratio represents [HPMA]:[VPMA]:[CTA].

^bDetermined using GPC equipped with a multi-angle light scattering (MALS) detector.

^cMeasured by dynamic light scattering (DLS)