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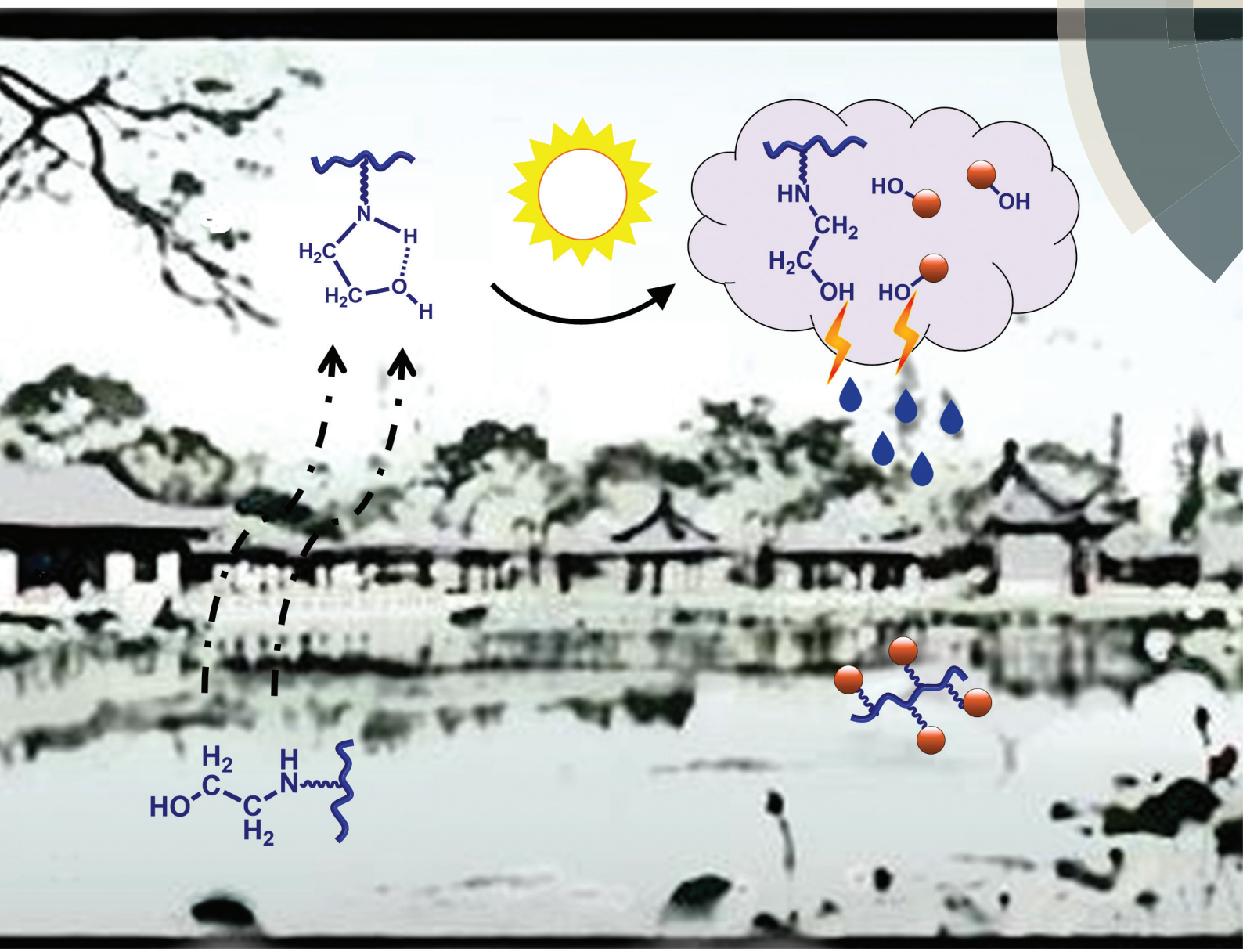

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Investigation on the controlled synthesis and post-modification of poly-[(*N*-2-hydroxyethyl)-aspartamide]-based polymers†

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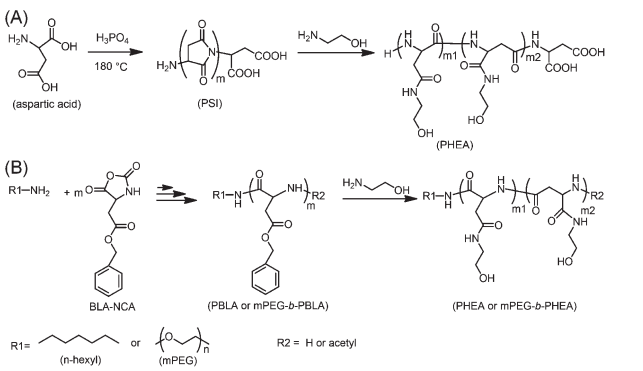
The production of poly-[(*N*-2-hydroxyethyl)-aspartamide] (PHEA)-based materials is hampered by the unsatisfactory synthesis and low reactivity in terms of post-modification. Herein, we report the controlled synthesis of PHEA-based polymers, unraveled the hydrogen bonding in PHEA, developed effective approaches to enhance the reactivity of PHEA by improving the temperature, and further demonstrated the desired aqueous solubility as well as the lyophilization stability of PHEA-based amphiphiles. This study will provide insights into the design of useful and functionalized polypeptide-based biomaterials.

Introduction

The use of synthetic polymers in experimental medicine and pharmaceutical sciences has a long history and is experiencing rapid development.[1–5] When used to fabricate drug delivery systems (DDSs), it is required that polymers afford complete solubility/dispersion in water and desired lyophilization stability, because the majority of polymeric nanomedicines are administered intravenously and stored in the lyophilized state.[6–9] Among the polymeric materials currently used, poly-[(*N*-2-hydroxyethyl)-aspartamide] (PHEA) is a representative neutral polymer with a polypeptide backbone and high water solubility. Due to its unique properties including nontoxicity, nonantigenicity, biodegradability, biocompatibility, and amenability to drug conjugation, PHEA features an ideal platform for the incorporation of hydrophobic small-molecular drugs.[10–13] In addition, PHEA modified with hydrophilic poly(ethylene glycol) (PEG) or/and hydrophobic micro/macromolecular groups has also been utilized as drug carriers.[14–16] Despite these favorable properties, PHEA-based polymers still have several drawbacks in terms of their preparation process that greatly limit their applications. Firstly, PHEA was conventionally prepared by thermal polycondensation of aspartic acid in the presence of phosphoric acid at 180 °C and subsequent aminolysis of the resulting polysuccinimide (PSI) with ethanolamine (Scheme 1A). However, this synthetic route has several disadvantages, including high reaction temperature, coloring of the obtained product, difficulties in controlling the degree of polymerization (DP), molecular weight distribution (MWD), the composition of side chain groups, and the inability to fabricate polymers with desired topological structures.[17] Nevertheless, PHEA used in most studies is still prepared using this method. Systematic investigation on the controlled synthesis of PHEA-based polymers is still lacking.[18,19] Secondly, PHEA is theoretically well-modifiable due to the plentiful reactive hydroxyl groups. However, according to many reports, the post-modification on the hydroxyl groups of PHEA often demonstrates low efficiency, which indicates the low reactivity



Scheme 1 Synthesis of PHEA-based polymers from conventional polycondensation of aspartic acid (A) or ROP of BLA-NCA (B).

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of the hydroxyl groups in PHEA.[20–24] To our knowledge, this notorious problem has not been well addressed yet.

Over the past few decades, polypeptides prepared *via* the ring-opening polymerization (ROP) of α-amino acid-*N*-carboxyanhydrides (NCAs) have attracted great attention and shown broad applications.[25–32] These polypeptides can be synthesized from varieties of NCA monomers *via* the well-controlled ROP method to feature chemical as well as functional diversity. Generally, chemical modification through the side-chain reaction is a facile synthetic route to obtain functionalized polypeptide derivatives. Nevertheless, facile and quantitative modification of side chains still remains a challenge for most polypeptides, mainly because of the limited efficiency of chemical reaction and low stability of the polymer backbone.[33–35] Alternatively, poly(β-benzyl-L-aspartate) (PBLA) is an easily accessible polypeptide which can undergo quantitative aminolysis reaction with amines *via* the formation of a succinimide intermediate.[17] The aminolysis of PBLA is highly efficient under mild conditions and it does not lead to the alteration of the DP or MWD of the original polymer, which therefore provides a useful and convenient approach for designing functional polypeptide-based biomaterials.[36–38]

Based on these understandings, we herein report our efforts in developing the facile and controlled synthesis of PHEA-based polymers *via* controlled ROP of NCA and overcoming the low post-modification reactivity of PHEA. We uncovered the possible intramolecular hydrogen bonding in PHEA-based polymers, and accordingly proposed effective approaches to enhance the reactivity of hydroxyl groups by inhibiting hydrogen bonding. We further explored the aqueous solubility, self-assembly properties, and lyophilization stability of PHEA-based materials to demonstrate their superiority over similar polymers. This systematic study may unleash the potential of this "old" polymer and enrich its applications in the design of functional biomaterials.

Results and discussion

The aminolysis chemistry of PBLA may provide the possibility to prepare PHEA-based polymers in a controlled manner (Scheme 1B). To validate this, PBLA and methoxy poly(ethylene glycol)-*b*-poly(β-benzyl-L-aspartate) (mPEG-*b*-PBLA) were synthesized as the homopolymer and block-copolymer templates through the ROP of γ-benzyl-L-aspartate-*N*-carboxyanhydride (BLA-NCA) monomer initiated by the primary amino groups of hexanamine and mPEG-NH_2 (M_n = 5000 Da), respectively. ^{1}H NMR spectra showed that PBLA and mPEG-*b*-PBLA with designed DP (which represents the DP of an amino acid segment in the text unless otherwise specified) were successfully synthesized (Fig. S1 and Table S1†). Due to the low solubility of PBLA homopolymers in *N*,*N*-dimethylformamide (DMF), we only measured the MWD of mPEG-*b*-PBLA copolymers by GPC analysis. Narrow MWD (PDI ≈ 1.1, Table S1†) was noted for all test mPEG-*b*-PBLA copolymers, which might be attributed to the living feature of the ROP of the NCA monomer.[26]

We first used PBLA (DP = 50) to investigate the aminolysis reaction with ethanolamine. As shown in Fig. S2,† the benzyl groups quickly disappeared and the characteristic peaks of ethanolamine were detected in the ^{1}H NMR spectrum after incubation of PBLA with ethanolamine (1 equivalent to the BLA unit), which was attributed to the quick formation of the succinimide intermediate.[39] By comparing the intensity ratio of CH_3 of the *n*-hexyl group with α-CH of the polymer backbone (or CH_2 of the hydroxyethyl group), we further revealed that the reaction proceeded quantitatively at 37 °C for about 12 h. At the higher molar ratio of ethanolamine/BLA (3 : 1 or 9 : 1), the aminolysis reaction could be completed much faster (approximately within 1 h) without causing any cleavage of the backbone upon incubation for up to 48 h (Fig. 1A and S3†), which further demonstrated the desired stability of the PBLA backbone during aminolysis.

Similarly, PBLA (DP = 98 and 140) and mPEG-*b*-PBLA (DP = 12, 21, and 30) were allowed to react with ethanolamine (ethanolamine/BLA molar ratio = 3 : 1) for 12 h, and ^{1}H NMR analyses further demonstrated the quantitative aminolysis reaction for all the polymers (Table 1 and Fig. S4†). GPC analyses showed the relatively narrow MWD of the resultant polymers (Fig. 1B and Table 1). Taken together, it can be confirmed that both the PHEA homopolymers and block copolymers could be conveniently synthesized through quantitative aminolysis of the PBLA-based polymers. This method also provides the feasibility to prepare PHEA-based polymers with various topological structures (*e.g.*, multi-block, multi-armed, star-shaped, and brush-like polymers) due to the abundance of NCA initiators that are readily accessible from commercial sources. In addition, polymers with similar structures, such as poly-[(*N*-3-hydroxypropyl)-aspartamide] (PHPA) and poly-[(*N*-4-hydroxybutyl)-aspartamide] (PHBA), can also be synthesized by this method (Table 1 and Fig. S5†), indicating its good generality.

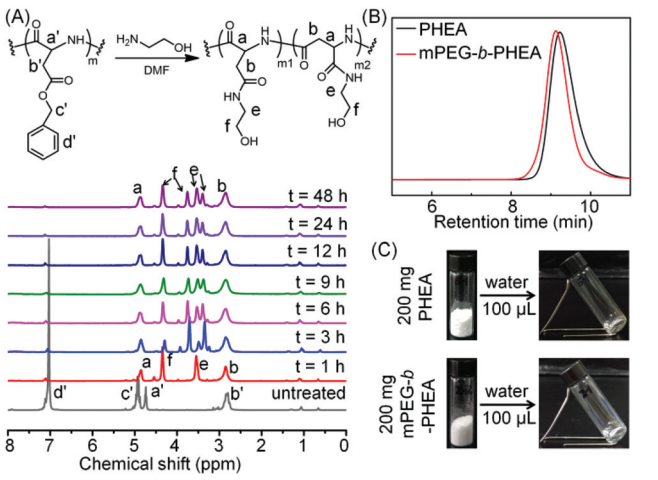

Fig. 1 (A) ^{1}H NMR spectra of PBLA in TFA-*d* after aminolysis by ethanolamine (3 equivalents to the BLA unit) for different incubation times. (B) GPC traces of PHEA (P1) and mPEG-*b*-PHEA (P5) in acetate buffer solution (0.1 M, pH 2.8). (C) Solubility of P1 and P5 in DI water.

Table 1 Polymers from aminolysis of PBLA or mPEG-*b*-PBLA

Entry	Polymer	DP[a]	DP[b]	Substitution rate[c] (%)	PDI[d]
P1	PHEA	50	50	100	1.26
P2	PHEA	98	99	100	1.24
P3	PHEA	140	142	100	1.35
P4	mPEG-*b*-PHEA	12	12	100	1.22
P5	mPEG-*b*-PHEA	21	21	100	1.28
P6	mPEG-*b*-PHEA	30	29	100	1.25
P7	PHPA	50	49	100	1.20
P8	PHBA	50	48	100	1.36

[a] Measured by ^{1}H NMR before aminolysis. [b] Measured by ^{1}H NMR after aminolysis. [c] Percentage of substitution of aspartic residues by alkamines. [d] Determined by GPC.

Aqueous solubility is a key property for polymers toward the application in drug delivery systems. Therefore, the solubility of the PHEA-based polymers was further investigated in various aqueous solutions. As shown in Fig. 1C and S6,† 200 mg of PHEA could be readily dissolved in 100 μL of water, phosphate buffered saline (PBS), 0.1 M HCl, or 0.1 M NaOH, achieving solubilities higher than 2 g mL^{-1}. The water solubility of PHEA at higher molecular weights (*e.g.*, P2 and P3) slightly decreased, which was still higher than 1 g mL^{-1}. PHPA (P7), PHBA (P8), and mPEG-*b*-PHEA copolymers (P4, P5 and P6) showed similar solubilities in aqueous solutions (Fig. 1C, S7, and S8†), and the water solubility of mPEG-*b*-PHEAs was even higher than that of mPEG-NH_2 (Fig. S9†).

The solubility of PHEA-based polymers in organic solvents was also investigated. Like most polypeptides, PHEA could be well dissolved in strong polar solvents such as DMF, dimethylsulfoxide (DMSO), formamide, *N*-methyl-2-pyrrolidinone (NMP), methanoic acid, slightly dissolved in chloroform, benzene, and methyl alcohol, while barely dissolved in *n*-hexane, ethyl acetate, tetrahydrofuran, acetonitrile, and acetone (Fig. S10†). mPEG-*b*-PHEA showed similar solubility in these solvents (Fig. S11†), indicating that strong polar solvents could be selected for the chemical reactions of PHEA-based polymers.

Besides aqueous solubility and biocompatibility, the amenability to post-modification serves as an important requirement for polymers toward biomedical applications, because functional motifs, therapeutic payloads, and diagnostic probes often need to be conjugated to the polymeric matrix. PHEA possesses large quantities of pendant hydroxyl groups on its side chains, which are theoretically amenable to a broad range of chemical agents with high activities. Many researchers have also reported the modification of PHEA through the typical ester condensation reaction or nucleophilic substitution reaction. However, these reactions display low modification efficiencies (10–30%) in various reaction solvents even at long reaction times.[21,22,40]

Based on these understandings, we thus systematically studied the reactivity of PHEA. Benzyl carbonylimidazole (Bn-CI) was synthesized (Fig. S13†) and reacted with PHEA (P1) at a 25-fold molar equivalent (half of the total hydroxyl groups) at 25 °C, and the modification efficiency *versus* the reaction time was detected by ^{1}H NMR. As shown in Fig. 2A, a low conversion ratio of Bn-CI was observed during the reaction period. When the incubation time was prolonged to 168 h, the conversion rate was still low (~25%), indicating that the low conversion ratio was not attributed to the reaction rate. Similar results were observed in the case of mPEG-*b*-PHEA (Table S2†). In addition, the reaction of mPEG-*b*-PHEA with a succinyl-podophyllotoxin derivative (PPT-SA, Fig. S14 and S15†) through the well-known coupling reaction also suffered from low efficiency (Table S2†), which was consistent with the literature reports.[41] These results thus demonstrated that only a small portion of the hydroxyl groups in PHEA can be modified while the residual hydroxyl groups were unreactive under the above reaction conditions.

Based on these results, we next studied the structure of PHEA by ^{1}H NMR. With either α or β-conformation in the polymer backbone, PHEA had four characteristics peaks: δ 2.85 (2H, –CH–C$\underline{H}_2$–CO–NH–), 3.37 (2H, –NH–C$\underline{H}_2$–CH_2–O–), 3.73 (2H, –NH–CH_2–C$\underline{H}_2$–O–), and 4.87 (1H, –NH–C$\underline{H}$(CO)–CH_2–). These four peaks were also observed in the ^{1}H NMR spectra of PHEA-based polymers (P1 and P5, Fig. S16†). However, these peaks became complicated when the NMR solvent was changed to TFA-*d*. Only newly synthesized PHEA showed four characteristics peaks in TFA-*d* (Fig. 2C). The peaks at 3.37 and 3.73 ppm of ethanolamine groups shifted to 3.54 and 4.32 ppm, respectively, due to the solvation effect of TFA-*d*. However, the original four peaks of PHEA eventually changed to six peaks at a prolonged reaction time or after placement for a certain period of time, which indicated changes of the chemical structure (Fig. 1A, 2C, S2 and S3†). Based on these findings, we hypothesized that the ^{1}H NMR variation of PHEA in TFA-*d* might be attributed to the formation of hydrogen bonds among the pendant hydroxyl groups. As newly-made materials did not have sufficient time to form hydrogen bonds, their ^{1}H NMR spectra demonstrated normal chemical

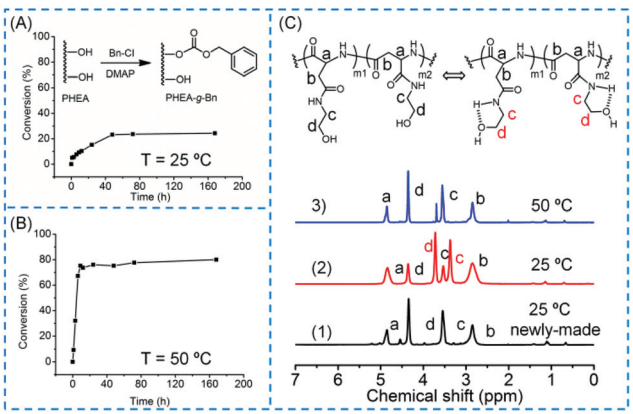

Fig. 2 (A) Conversion of Bn-CI after reaction with PHEA (P1) at 25 °C for different times. (B) Conversion of Bn-CI after reaction with PHEA (P1) at 50 °C for different times. (C) Proposed chemical structures of PHEA and the ^{1}H NMR spectra of PHEA in TFA-*d* under various conditions (1, newly made PHEA determined at 25 °C; 2, PHEA determined at 25 °C after 24 h placement; 3, PHEA determined at 50 °C after 24 h placement).

shifts. The formation of the hydrogen bonds among the pendant hydroxyl groups made their condition more similar to that in water, leading to the variation of the ^{1}H NMR shift (Fig. 2C). In addition, the electron withdrawing effect of hydrogen bonds would decrease the electron density and the nucleophilicity of the hydroxyl group, and therefore result in the low reactivity of PHEA in the anhydrous organic solvent.

Because the energy of the hydrogen bonds was lower than that of the common covalent bonds which could be easily destroyed at moderately increased temperature, we further performed the ^{1}H NMR test at a slightly higher temperature (50 °C). As expected, the ^{1}H NMR spectrum of PHEA recovered to the normal four peaks at 50 °C (Fig. 2C). Similar results were observed for the copolymer mPEG-*b*-PHEA (Fig. S17†). Based on the hydrogen bonding hypothesis, we next investigated whether increasing the temperature would enhance the reactivity of PHEA. As shown in Fig. 2B and S18,† the conversion of Bn-CI was significantly increased when the reaction temperature increased from 25 °C to 50 °C, achieving conversion over 80% after reaction with PHEA. A further increase of temperature to 70 or 90 °C led to similar efficiency (Table S3†), while it caused slight coloring of the resulting material. Therefore, the temperature of 50 °C might be optimal to recover the reactivity of the hydroxyl groups of PHEA. Similar results were observed for mPEG-*b*-PHEA (Table S3†). Furthermore, the condensation reaction using the carbodiimide/DMAP method also demonstrated high efficiency (approximately 85%) at 50 °C. Although the precise characterization of the proposed hydrogen bonding in PHEA may still be complicated, our hypothesis would provide a useful method to increase the reactivity of PHEA.

Polymeric micelles constructed from PEG-polypeptide block copolymers have shown great potential for delivering bioactive payloads to diseased tissues such as solid tumors. The core–shell nanostructure enables the encapsulation of varieties of payloads within their inner core, while the outer PEG shell features long circulation in the blood by avoiding the interactions with the surrounding biological environments.[42–44] Polymeric micelles prepared from PEG-*b*-poly(aspartic acid) and PEG-*b*-poly(glutamic acid) copolymers, incorporating antitumor drugs *via* chemical conjugation or physical entrapment, have shown high *in vivo* stability, prolonged blood circulation time, and selective tumor accumulation.[45–47] However, polymeric micelles derived from PEG-*b*-poly(aspartic acid) and PEG-*b*-poly(glutamic acid) suffer from solubility issues after lyophilization. While this problem could be partly addressed by using an excessive amount of lyoprotectants, the preparation process and the formulation composition become complicated, and the self-assembly properties (size and distribution) of the micelles may also be affected.[7–9,48,49] Therefore, lyophilization stability still remains an important yet unsolved problem for PEG-polypeptide micelles.

mPEG-*b*-PHEA had large quantities of pendant hydroxyl groups and could be readily modified by typical esterification reactions with high activities at moderately increased temperature based on this study (Fig. 3A). Therefore, it was essential to

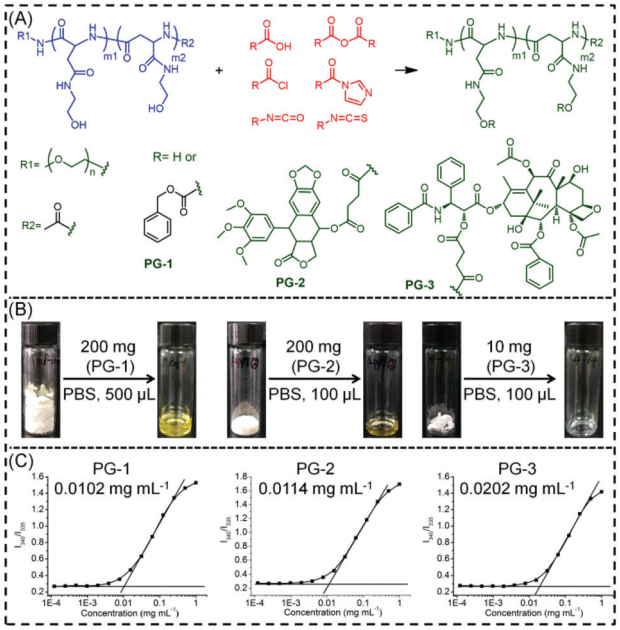

Fig. 3 (A) Reactions to modify PHEA-based polymers and the obtained amphiphiles from mPEG-*b*-PHEA. (B) Aqueous solubility of the lyophilized PG-1, PG-2, and PG-3 micelles in PBS. (C) CMC values of PG-1, PG-2, and PG-3 micelles.

investigate whether the amphiphiles from mPEG-*b*-PHEA could maintain good aqueous solubility and lyophilization stability after post-modification. To validate this, Bn-CI, the succinyl-podophyllotoxin derivative (PPT-SA), and the 2′-*O*-succinyl-paclitaxel derivative (PTX-SA) which contained hydrophobic groups or chemotherapeutic drugs were synthesized for the preparation of the mPEG-*b*-PHEA based amphiphiles (Fig. 3A). Methoxy poly(ethylene glycol)-*b*-poly(L-glutamic acid) (mPEG-*b*-PLG) with the same PEG length and DP was synthesized (Fig. S19†). mPEG-*b*-PLG reacted with benzyl alcohol (Bn-OH), podophyllotoxin (PPT) and paclitaxel (PTX) to fabricate the conjugates as the control. The detailed synthesis pathways and characterization for the modification agents and conjugates are described in the ESI (Fig. S20–S26†), and the conjugates from mPEG-*b*-PHEA and mPEG-*b*-PLG with the designed modification ratio are summarized in Table 2.

The solubility of the self-assemblies in PBS was investigated. Before lyophilization, all the micelles could be well

Table 2 Conjugates from mPEG-*b*-PHEA (P5) and mPEG-*b*-PLG

Entry	Polymer scaffold	Reaction agent	Feed ratio to polymer	Obtained ratio to polymer
PG-1	P5	Bn-CI	16	13.6[a]
PG-2	P5	PPT-SA	6	5.5[b]
PG-3	P5	PTX-SA	4	3.4[a]
PG-4	mPEG-*b*-PLG	Bn-OH	14	13[a]
PG-5	mPEG-*b*-PLG	PPT	6	5.5[b]
PG-6	mPEG-*b*-PLG	PTX	4	3.7[a]

[a] Measured by ^{1}H NMR. [b] Measured by UV-Vis spectrometry at 292 nm.

dissolved during the nanoprecipitation process, and there were no aggregations during dialysis at the concentration of 40–60 mg mL^{-1}. After lyophilization and resuspension, PG-1, PG-2, and PG-3 micelles could be readily dissolved in PBS at high concentrations, leading to hydrodynamic diameters of 20–30 nm and a narrow size distribution (Fig. 3B and S27†). Particularly, the water solubility of PG-2 micelles was nearly 10^6 fold higher than that of the original drug PPT. In addition, PG-1, PG-2, and PG-3 micelles exhibited low critical micelle concentrations (CMC, 0.01–0.02 mg mL^{-1}, Fig. 3C), indicating their desired self-assembly stabilities. However, the solubility of lyophilized mPEG-*b*-PLG micelles was much lower (Fig. S28†). In particular, PG-5 and PG-6, which had similar amounts of conjugated drugs as compared to PG-2 and PG-3, were barely dissolved. The possible reason for the huge difference between mPEG-*b*-PLG and mPEG-*b*-PHEA-based materials in terms of lyophilization stability may be attributed to that the pendant hydroxyl groups were more hydrophilic and more likely to interact with water molecules in the aqueous solution as compared to carboxyl groups.

Conclusions

In conclusion, we report the convenient synthesis and functionalization of PHEA-based polymers *via* controlled ROP of NCAs and aminolysis of PBLA. We unraveled the possible intramolecular hydrogen bonding in PHEA which accounted for its low post-modification reactivity, and accordingly greatly recovered the reaction activity by gentle heating. To the best of our knowledge, this is the first time to validate the predominant factor of temperature toward the post-modification of PHEA-based polymers. Based on these understandings, we synthesized a series of polymeric conjugates from mPEG-*b*-PHEA, and demonstrated high water solubility as well as the lyophilization stability of the self-assembled micelles from these amphiphilic materials. This systematic investigation on PHEA will provide a useful platform for the development of functional polypeptides toward their potential biomedical applications.

Acknowledgements

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