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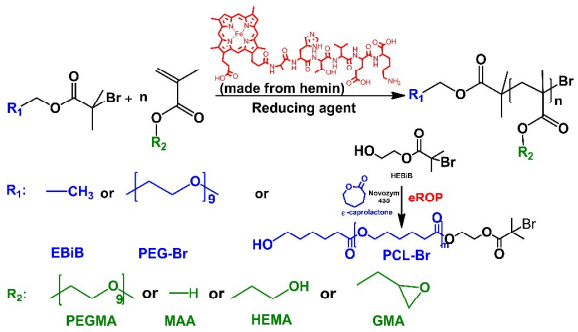

Use enzyme mimetic (DhHP-6) as an ATRP catalyst for the synthesis of a series of functional polymers.

Journal Name

ARTICLE

Enzyme Mimetic-Catalyzed ATRP and Its Application in Block Copolymer Synthesis Combining with Enzymatic Ring-Opening Polymerization

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Deuterohemin-*β*-Ala-His-Thr-Val-Glu-Lys (DhHP-6) as a peroxidase mimic shows good catalytic capability towards the polymerization of functional vinyl monomers in an aqueous buffer solution of pH 3.0-11.0 or a mixed solvent of DMF-H_2O under moderate conditions of activators regenerated by electron transfer atom transfer radical polymerization (ARGET ATRP). More importantly, by combining the above ATRP process with enzymatic ring-opening polymerization (eROP), three types of block copolymers with biodegradable segment, that is, PCL-PGMA, PCL-PMAA, and PCL-PHEMA, were synthesized, demonstrating that the combination of enzymatic ATRP and eROP using renewable biocatalysts is a versatile approach for the construction of potentially biocompatible block copolymers.

Introduction

As one of the most widely used controlled/living radical polymerization techniques, atom transfer radical polymerization (ATRP) allows for the polymerization of a wide range of vinyl monomers with different functional groups including amino, epoxy, hydroxyl and so on.[1,2] Polymers with predetermined molecular weight and narrow polydispersity (PDI) could be easily perpared using ATRP.[3-5] Copolymers with complex chain architectures, in terms of topology, composition and functionality, could also be synthesized *via* changing the structure of ATRP initiators.[6-10] Generally, ATRP is conducted with transition metal-based catalysts and most widely-used nitrogen-containing ligands, thus has obvious limitations for the preparation of some special polymer products, for example, polymers for biomedical applications, owing to the presence of small amount of metal residues that are toxic to living tissues.[11-12]

Enzymes are environmentally friendly, non-toxic and renewable biocatalysts.[13-14] They can generally operate under mild reaction conditions.[15] Among the six main groups of enzymes classified by the Enzyme Commission, oxidoreductases, transferases and hydrolases have been employed for the synthesis of different kinds of polymers.[14,16] However, only oxidoreductases could realize enzymatic radical polymerization,[17] where horseradish peroxidase (HRP), catalase and laccase, being the classic oxidoreductases, have demonstrated their wide application in enzyme catalyzed radical polymerization.[15,17-19] In traditional enzyme catalyzed radical polymerization systems, a ternary system composed of enzyme, H_2O_2 and *β*-diketone, are always used.[4] The molecular weight and PDI could not be controlled, which will limit the products' application in special use.

Controlled/living radical polymerization catalyzed by peroxidase or other oxidoreductases has never been reported until the publication of Bruns' and di Lena's works,[20-22] where they demonstrated that protein/enzymes from renewable resources, such as HRP, laccase, catalase, could act as catalysts in an analogous ATRP process. Bruns and his coworkers named the enzymes with ATRP catalysis activity as ATRPase.[22] In addition, Bruns and coworkers found that even hemoglobin from bovine blood (Hb) or human erythrocytes could catalyze the polymerization of vinyl monomers under ATRP conditions.[23] Recently, as an application of ATRPase, peroxidase catalyze surface-initiated ATRP (SI-ATRP) from lignin nanofibres, or filling polymersomes with polymers have been reported.[24-25] As the common structure of hemoglobin, hematin, HRP, and other perosidases, iron porphyrin is indeed the real active catalysis part of ATRPase, proved by Kadokawa and Matyjaszewski.[26-27] For example, Kadokawa and coworkers utilized hematin to catalytically polymerize N-isopropylacrylamide in a mixed solvent of DMF-H_2O based on the ATRP mechanism.[26] Meanwhile, Matyjaszewsiki and coworkers decorated hemin by PEG chains and reduced the vinyl moieties on iron porphyrins. The catalytic performance of the simple hemin was significantly improved in the preparation of well-defined polymers, and can be used, but not limited, in water.[27]

Deuterohemin-*β*-Ala-His-Thr-Val-Glu-Lys (DhHP-6, Scheme 1, a) is a synthesized heme-containing peroxidase mimic composed of six amino acid residues and one iron porphyrin,

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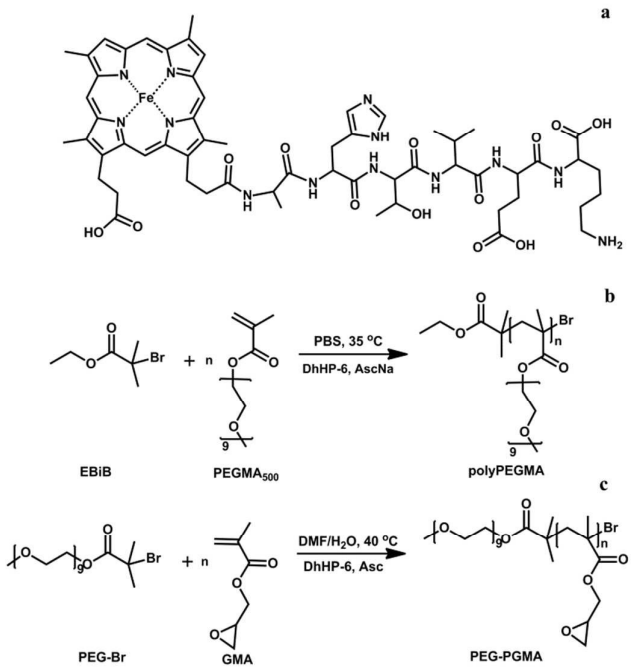
showing high peroxidase enzyme activity.[28-29] According to previous studies, DhHP-6 presents a lot of biological activities in improving cell survivals and inhibiting apoptosis against reactive oxygen species (ROS).[30-32] Structure and properties of DhHP-6 suggest that it might serve as a good candidate of ATRPase, although no relevant work has been reported to the best of our knowledge.

In the present work, we study the catalytic ability of DhHP-6 in controlled radical polymerization. DhHP-6 shows a powerful catalytic ability and a good tolerance to a wide range of pH values (from 3.0 to 11.0), demonstrating a great potential in the production of diverse polymers with different functional groups including epoxy, –COOH and -OH *via* ARGET ATRP. As a typical enzymatic polymerization, there are numerous reports on hydrolases catalyzed enzymatic ring opening polymerization (eROP) and its combining with metal catalyzed ATRP to synthesize copolymers.[33-35] While, for the first time, the combination of enzymatic ATRP with other chemical or enzymatic polymerization techniques will be reported herein. We demonstrate the double-enzymatic synthesis of block copolymers by the combination of eROP and enzymatic ATRP, showing the first example on the synthesis of copolymers by the joint of different kinds of enzymatic polymerizations and proving DhHP-6 a new promising environment benign ATRPase.



Scheme 1. (a) Structure of DhHP-6. (b) DhHP-6 catalyzed ARGET ATRP of $PEGMA_{500}$. (c) DhHP-6 catalyzed ARGET ATRP of GMA use PEG-Br as initiator.

Experimental

Materials and methods

All chemicals were of analytical grade and used without further purification unless otherwise noted. Poly(ethylene glycol)methyl ether methacrylate ($PEGMA_{500}$), glycidyl methacrylate (GMA), methacrylic acid (MAA) and hydroxyethyl methacrylate (HEMA) were purchased from Sigma-Aldrich and passed through a column of basic alumina to remove inhibitors before use. Ethyl 2-bromoisobutyrate (EBiB) and ε-caprolactone were purchased from Sigma-Aldrich and used as received. DhHP-6 was obtained as a gift sample from College of Life Science, Jilin University (Changchun , China). Novozym 435 (Candida antarctica lipase B immobilized on acrylic resin, CALB, Novozymes) was dried and kept under nitrogen according to literature report.[36] Toluene was dried by refluxing with Na/benzophenone ketyl for 24 h. 2-Hydroxyethyle 2-bromoisobutyrate (HEBiB)[37] and macroinitiaor consisted of polyethylene glycol fragment (PEG-Br)[44] were synthesized according to literature report, respectively, whose structures were identified *via* ^{1}H NMR spectroscopy (see Figure S2 for HEBiB, Figure S3 for PEG-Br**,** ESI†). Phosphate buffer solutions (PBS) were prepared according to the Chinese Pharmacopoeia.

Monomer conversion was determined by ^{1}H NMR spectroscopy on a Bruker Avance III (400 MHz) instrument, using D_2O, $CDCl_3$, or DMSO-d_6 as solvents. Conversion of PEGMA was calculated by comparing the integrals of two vinly protons of residual monomer (δ 5.71 and δ 6.13 ppm), and the overlapping signal (δ 4.00 to δ 4.40 ppm), which were corresponding to two protons of PEGMA and two protons of the polyPEGMA.[38] All measurements were performed at 25 °C.

Number average molecular weight (M_n) and polydispersity (PDI) of polymers were recorded on gel permeation chromatography (GPC) using a Malvern instrument (Viscotek T5000 and Viscotek T1000 org GPC/SEC column thermostated to 35 °C and calibrated by linear polystyrene standards), equipped with a Malvern refractive index detector, maintained at 25 °C and using tetrahydrofuran (THF) as mobile phase with a flow rate of 1.0 ml min^{-1}. For copolymer PCL-PHEMA and PCL-PMAA, M_n and PDI were recorded on GPC *via* Malvern instrument (Viscotek I-MBHMW-3078 300 mm × 7.8 mm, exclusion limit > 10×10^6 g mol^{-1} column thermostated to 45 °C, calibration by linear polystyrene standards), equipped with a refractive index detector, thermostated to 35 °C. and using DMF containing 20 mM lithium bromide as mobile phase with flow rate of 0.7 ml min^{-1}.

General procedure for the synthesis of poly($PEGMA_{500}$) by ARGET ATRP

DhHP-6 (2.8 mg, 2.3 μmol), KBr (65.0 mg, 0.55 mmol), and $PEGMA_{500}$ (1.1 g, 2.2 mmol) were dissolved in PBS (pH 7.0, 2.0 mL) in 10 mL branch-necked flask, followed by immersed in a 35 °C oil bath and purging with nitrogen for 0.5 h. Then L-ascorbic acid sodium solution (AscNa, 0.50 mL, 0.068 mmol in PBS buffer) was added into the reaction mixture. The flask was sealed under nitrogen atmosphere. EBiB (10 μL, 0.07 mmol) was added to start the reaction. Samples were taken from the flask at timed intervals for ^{1}H NMR and GPC analysis.

Synthesis of PGMA by DhHP-6 catalyzed ARGET ATRP

GMA (1.08 g, 7.61 mmol) was dissolved in N,N'-dimethylformamide (DMF, 3 mL) in 10 mL branch-necked flask followed by the addition of DhHP-6 aqueous solution (0.2 mL, 11.5 mM). The reaction mixture was maintained at 40 °C and purged with nitrogen for 0.5 h. Then, L-ascorbic acid solution (0.2 mL, 0.75 M) was added into the reaction mixture, and the reaction flask was sealed under nitrogen atmosphere. EBiB (11 μL, 0.075 mmol) or PEG-Br (85 μL, 0.13 mmol) was added to start the reaction. Samples were taken out of the flask at certain time intervals for ^{1}H NMR and GPC analysis.

eROP of ε-caprolactone from bifunctional ATRP initiator

Novozym 435 (56 mg) was weighed into a 10 mL round-bottom flask, which was then purged with nitrogen for 10 min. The flask was kept under nitrogen. ε-Caprolactone (1.10 mL, 9.57 mmol) was added and dissolved by freshly dried toluene (2.20 mL), then HEBiB (90 μL, 0.57 mmol) were added into the flask *via* syringe immediately. The flask was immersed in an oil bath and maintained at 80 °C under magnetic stirring for 12 h. The reaction was terminated by removing the enzymes by filtration. The product (PCL-Br) was added into cold methanol dropwise, and dried under vacuum to give a white solid in a yield of 80.58%. The structure was determined by ^{1}H NMR spectroscopy, and the molecular weight was analyzed *via* GPC using THF as mobile phase.

Synthesis of copolymers (PCL-PGMA) by employing PCL-Br as aroinitiator *via* DhHP-6 catalyzed ARGET ATRP

Macroinitiator PCL-Br (50 mg, M_n = 2130 by GPC, 0.023 mmol) was dissolved in DMF (3 mL) in a branch-necked flask and DhHP-6 (2.5 mg, 2.03 μmol dissoved in 0.2 mL water) was added. Then the reaction system was purged with nitrogen for 0.5 h and maintained at 50 °C. L-ascorbic acid solution (0.2 mL, 0.62 M) was added into the reaction mixture, this system was sealed under nitrogen atmosphere. GMA (0.2 mL, 0.22 g, 1.52 mmol) was added *via* syringe to start the polymerization. After 12 h, the reaction was quenched by exposure to air, and the crude product was precipitated out by adding the reaction solution into ether dropwise and separated by centrifuging at 5000 rpm for 5 min. The precipitate was redissolved in chloroform, and then precipitated in ether followed by centrifugation at 5000 rpm for 5 min. This process was repeated three times. The resulted solid was dried under vacuum at room temperature. The structure was determined by ^{1}H NMR spectroscopy, and the molecular weight was analyzed *via* GPC using THF as mobile phase.

Synthesis of amphiphilic copolymer (PCL-PHEMA) by employing PCL-Br as maroinitiator *via* DhHP-6 catalyzed ARGET ATRP

Macroinitiator PCL-Br (100 mg, M_n = 2130, 0.047 mmol) by GPC (THF as mobile phase) was dissolved in DMF (3 mL) in 10 mL branch-necked flask and DhHP-6 (4.6 mg, 3.74 μmol, dissolved in 0.2 mL water) was added. Then the system was purged with nitrogen for 0.5 h and maintained at 50 °C. L-

Table 1. ARGET ATRP of $PEGMA_{500}$ reactions and conditions (35 °C)

Entries	[$PEGMA_{500}$]/[EBiB]/[DhHP-6]/[AscNa]/[KBr]	pH	Conv (%)[a]	M_{th}[b]	M_n[c]	PDI[c]	Time (h)
1	32/1/0.033/1/8	6.5	80.65	12900	6020	1.08	2.0
2	32/1/0.033/1/8	7.0	88.49	14160	6080	1.24	3.0
3	32/1/0.033/1/8	7.5	78.74	12600	5740	1.12	1.5
4	64/1/0.033/1/8	7.0	68.03	21770	6840	1.18	1.5
5	64/1/0.033/1/4	7.0	54.35	17390	5420	1.13	1.0
6	64/1/0.033/1/2	7.0	66.06	21140	7779	1.17	2.0
7	32/1/0.033/1/0	7.0	86.21	13790	22480	1.19	4.0
8	32/1/0.033/0/8	7.0	7.86	-	-	-	24
9	32/1/0/1/8	7.0	6.12	-	-	-	24
10	32/0/0.033/1/8	7.0	-	-	-	-	24

[a]Measured by ^{1}H NMR.
[b]M_{th} = *([$PEGMA_{500}$]/[EBiB]) × conversion × 500*.
[c]Determined by GPC.

Table 2. ARGET ATRP of PGMA reactions and conditions

Entries	[GMA]/[initiator]/[DhHP-6]/[Asc]	Conv(%)[c]	M_{th}[d]	M_n[e]	PDI[e]	Time(h)
1[a]	100:1:0.03:2	55.25	8940	7830	2.01	1.75
2[a]	63:1:0.005:1.25	33.30	2830	3100	2.07	4.0
3[b]	66:1:0.087:5.6	38.46	4650	8430	1.38	6.0

[a]Reaction temperature = 40 °C in a mixed solvent of DMF-H_2O(DMF:H_2O=7.5:1);
[b]Reaction temperature = 50 °C use PCL-Br as macroinitiator, in a mixed solvent of DMF-H_2O(DMF:H_2O=7.5:1).
[c]Measured by ^{1}H NMR. [d]M_{th} = *([GMA]/[initiator])×conversion×142+$M_{n(initiator)}$*.
[e]Determined by GPC.

ascorbic acid solution (0.2 mL, 0.76 M) was added into the reaction mixture, this system was sealed under nitrogen atmosphere. Hydroxyethyl methacrylate (HEMA, 0.5 mL, 4.11 mmol) was added to start the reaction. The reaction stopped after 12 h, then precipitated the DMF solution into cold ether and centrifuged at 5000 rpm for 5 min. The precipitate was redissolved in acetone，then precipitated in cold ether followed by centrifuging at 5000 rpm for 5 min. This process was repeated for three times, the solid was dried under vacuum at room temperature over 12 h (HEMA monomer conversion was tested by ^{1}H NMR (in DMSO-d_6) to give a conversion yield of 50.52%). The resulted copolymer of PCL-PHEMA could not be dissolved in THF, ethanol, $CHCl_3$ or dichloromethane, but is slightly soluble in acetone and completely soluble in DMF, DMSO and 1,4-dioxane. The structure was identified by ^{1}H NMR spectroscopy(in DMSO-*d*6, Figure S15, ESI†) and the molecular weight was analyzed via GPC using DMF as mobile phase.

Synthesis of amphiphilic copolymer (PCL-PMAA) by employing PCL-Br as maroinitiator *via* DhHP-6 catalyzed ARGET ATRP

Macroinitiator PCL-Br (115 mg, M_n = 2130, 0.054 mmol) by GPC (THF as mobile phase) was dissolved in DMF (3 mL) in 10 mL branch-necked flask and DhHP-6 (4.5 mg, 3.67 μmol, dissolve in 0.2 mL water) was added. Then the reaction system was purged with nitrogen for 0.5 h and maintained at 50 °C. L-ascorbic acid solution (1 M, 0.2 mL) was added into the reaction mixture, we sealed this system under nitrogen atmosphere. Methacrylic acid (MAA, 0.5 mL, 5.81 mmol) was added to start the reaction. The reaction stopped after 12 h, then precipitated the DMF solution into cold ether, separate the products by centrifuged and get oily liquid at the bottom of the flask. The oily liquid was dispersed in ethanol,then precipitated in cold ether followed by centrifugation at 5000 rpm for 5 min. Repeat this process three times, the solid was dried under vacuum at room temperature over 12 h to give a yield of 8.28%). The resulted copolymer of PCL-PMAA is not soluble in THF, ethanol, acetone, CHCl3 or dichloromethane, but could completely dissove in DMF, DMSO and 1,4-dioxane. The structure was identified by ^{1}H NMR spectroscopy(in DMSO-d_6, Figure S16, , ESI†).and the molecular weight was analyzed *via* GPC using DMF as mobile phase.

Results and discussion

DhHP-6 has potential applications in the prevention and treatment of cellular disfunction related diseases.[29-31] Meanwhile, DhHP-6 can act as an environmental benign ATRP catalyst in water or DMF-H_2O mixed solvent, avoiding the use of toxic transition-metal-based catalysts. As DhHP-6 is highly soluble in water,[30] we first chose PEGMA as a monomer and sodium ascorbate as a reducing agent (scheme 1, b), and conducted the polymerization in PBS. As can be seen from the results (Table 1, Figure 1 and S4-S8, , ESI†)the molecular

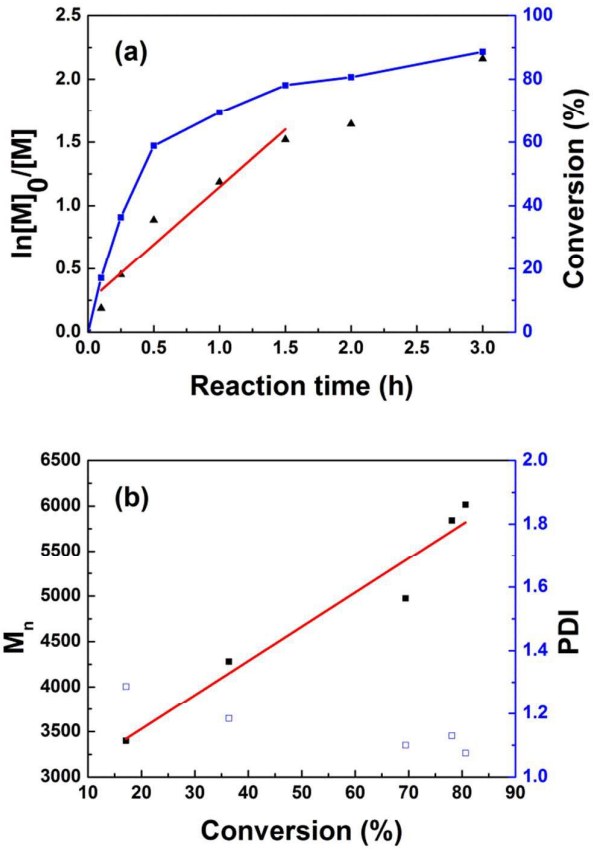


Figure 1. (a) First-order kinetic plot (▲) for DhHP-6 catalyzed ARGET ATRP of $PEGMA_{500}$ in PBS buffer at 35°C (entry 1, pH = 6.5) and plot of monomer conversion *vs*. reaction time (■). (b) Number-average molecular weight(■) and PDI(□) of polyPEGMA *vs*. monomer conversion.

Note:[$PEGMA_{500}$]/[EBiB]/[DhHP-6]/[AscNa]/[KBr]=32/1/0.033/1/8.

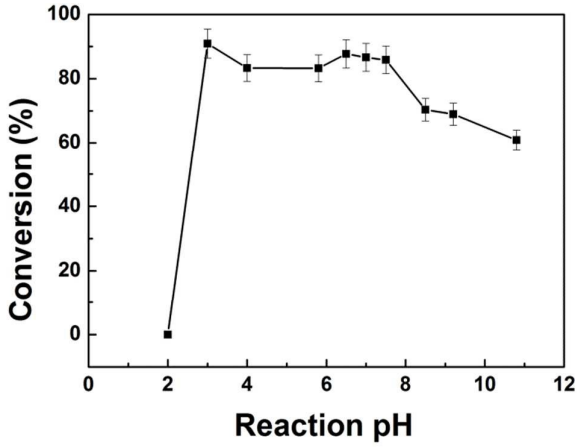


Figure 2. Influence of pH on DhHP-6 catalyzed ARGET ATRP of PEGMA conversion.

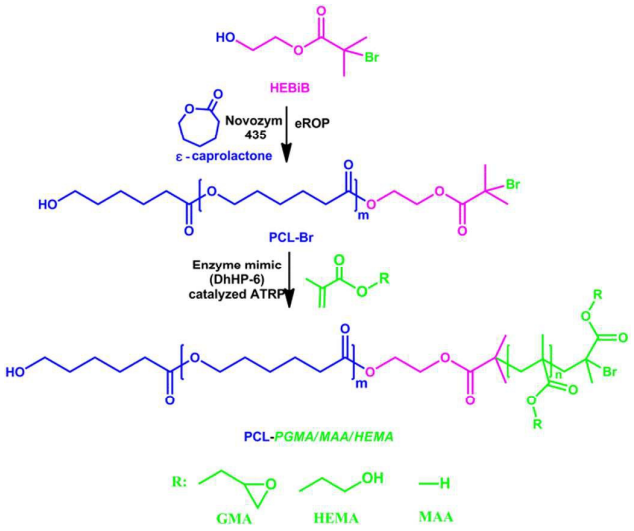
Note: [$PEGMA_{500}$]/[EBiB]/[DhHP-6]/[AscNa]/[KBr]=32/1/0.033/1/8.

weights of the resulting polymers increased linearly with monomer conversion and the PDI were relatively low (1.03-1.3). The polymerization rate was fast, and semilogarithmic kinetic plot of $\ln([M]_0/[M])$ *vs* time changed linearly during the first 1.5 or 2.0 hours of the reaction, indicating that the concentration of the growing radicals was constant.

In order to confirm that the initiation was induced from ATRP initiator, we utilized polyethylene glycol (PEG, Mw = 550) containing bromoisobutyrate (PEG-Br) to synthesize copolymers. Synthetic PEG-PGMA copolymers with lower molecular weight were identified by ^{1}H NMR and GPC (see Figure S11, S12, , ESI†), which confirmed that the reaction was started from PEG-Br. Meanwhile, in order to explore the application of DhHP-6 catalyzed enzymatic polymerization, macroinitiator PCL-Br was prepared *via* eROP of ε-caprolactone with bifunctional ATRP initiator such as HEBiB, then using PCL-Br as enzymatic ATRP macroinitiator to build copolymers with different functional vinyl monomers(see scheme 2). The copolymer samples were characterized by GPC and ^{1}H NMR, which indicated that the copolymers were successfully synthesized.

At first, we attempted to conduct DhHP-6 catalytic ATRP reaction without halide salts (see Table 1, Entry 7). The resulted semilogarithmic kinetic plot of $\ln([M]_0/[M])$ *vs* reaction time changed linearly during the first 2.0 hours of the reaction. The monomer conversions were up to 86.21% in 4 h. However, the M_n of polymers did not change linearly with the increase of monomer conversion, indicating that the molecular weight of this reaction is not controllable. This may be because DhHP-6 does not carry halogen atoms in the iron porphyrin center, and the halogen atom was dropped out during the DhHP-6 preparation process (see mass spectrum data in Figure S1**,** ESI†). Matyjaszewski and coworkers reported that ATRPase systems had poor halidophilicity, leading to the decrease of the deactivation efficiency.[27] This may directly result in the molecular weight uncontrolled. While, DhHP-6 catalyzed polymerization could not start without the organic halide initiators, indicating that the reaction proceeds according to the ATRP mechanism. Based on literature report,[27] additional halide salts can make the deactivation of ATRPase faster and thus realize controlled polymerization. So, we study the effect of KBr on DhHP-6 catalytic polymerization by adding a certain amount of KBr to the reaction system. After addition of KBr, linear increase of the polymers' M_n *vs* the increase of monomer conversions were obtained (Figure 1, S4 and S5, ESI†), further demonstrating the above mechanism. So, other reactions were conducted by the addition of KBr. Meanwhile, we further investigated the effect of KBr content on the reaction system by systematically varying the proportion of KBr from twice to four and eight times of initiator (Table 1, entries 4-6 and Figure S6-S9**,** , ESI†). At whatever KBr contents, we all get low PDI of polymer products and the M_n increase with the conversion linearly, but twice excess amount of KBr makes the PDI little broader. Therefore, we use eight times excess of KBr in the rest of experiments.

The initial molar ratio of monomer to initiator also increased with a decreasing amount of EBiB in this set of experiments.



Scheme 2. Copolymers synthesis procedure by the combination of enzymatic ATRP and eROP.

This is another characteristic of living polymerization. The conversion reached plateau at about 70.0%, but the molecular weight increased accordingly. This indicated that the M_n of the polymers was also tunable by changing the ratio of monomer to initiator. The limited conversion may be caused by the lower concentrations of initiator in the reaction system.

As a typical peroxidase, HRP is known to be sensitive to pH when acting as an ATRPase.[22] DhHP-6, an enzyme mimic, may also be pH dependent as nature products. Therefore, we start to explore its pH dependency by changing the pH values of the reaction solutions with PBS buffers (Figure 2). No matter the reaction environment is acidic (pH 3.0) or basic (pH 11.0), semilogarithmic kinetic plot of $\ln([M]_0/[M])$ *vs* time changed linearly (Figure S18, ESI†). The monomer conversions are all higher than 60% in 3 h. These results suggest that DhHP-6 as an ATRPase is quite stable over a large pH range, which may be attributing to its special structure, that is, a mimetic peptide with only six amino acid residues. Protonation and deprotonation of amino acid chains did not affect the central iron porphyrin's activation and deactivation with alkyl bromides. PBS buffer of nearly neutral pH was chosen as reaction medium for further experiments (see Table 1. Entries 1-3). Results show semilogarithmic kinetic plot of $\ln([M]_0/[M])$ *vs* reaction time changed linearly during the first 1.5 or 2.0 hours of the reaction, after that the reaction stopped and M_n of polyPEGMAs increased linearly with monomer conversion and the PDI were ranged from 1.07 to 1.24(see Figure 1, S4 and S5, , ESI†). These results demonstrated the controlled fashion of the DhHP-6 catalyzed system. The M_n and PDI of the products in different pH values are within the same level, which also means that pH has no effect on this enzyme catalyzed ATRP. At the same time, a series of control

experiments were arranged to further validate that the reaction was really induced by DhHP-6/EBiB/AscNa system (Table 1, Entries 8-10). In the absence of the initiator, EBiB, no signal of PEGMA polymer formation was shown after 24 h incubation under ATRP conditions. In the absence of enzyme mimic – DhHP-6, or reducing agent – sodium ascorbate, the monomer conversion is only 6.12% or 7.86% in 6 h and maintain steady until 24 h. When any of the lacked reagents was added into the incubated system, the reaction started immediately and monomer conversion increased linearly with reaction time (Figure S17, , ESI†).

It's worthy to note that, when conducting polymerization in a buffer of pH 3.0 directly, the monomer conversion can reach 97% in 4 h (Figure S18**,** , ESI†).While, when the system was operated in PBS of pH 2.0, the reaction did not take place even in 72 h. But, when the system's pH was adjusted to 3.0 by the addition of 0.1 M NaOH solution, the polymerization starts immediately and monomer conversion can reach 66.67% in 10 h. These results demonstrated that the ATRPase activity of DhHP-6 was suppressed temporarily and it can be reactivated by simply changing the solution pH.

As a versatile polymer carrying a lot of functionalized epoxy groups, PGMA has drawn much attention and could be used in the fields of polymer chemistry, biomedical engineering and materials science.[39-43] An ARGET ATRP of GMA in a mixed solvent of DMF-H_2O (7.5:1) by using DhHP-6 as catalyst was conducted (Table 2, entry 1). The semilogarithmic kinetic plot of $\ln([M]_0/[M])$ *vs* reaction time changed linearly and M_n of PGMA increased linearly with monomer conversion, which indicates that the polymerization is based on ATRP mechanism (Figure S10**,** ESI†). The PDI of DhHP-6 catalyzed PGMA products was a little broad (all above 2.0). In this part, the absence of KBr may lead to the decrease of the deactivation efficiency, which cause the active species unevenly distributed. To further determine that the reaction was induced from the ATRP initiator, we synthesized an ATRP initiator consisted of methoxy PEG (PEG-Br) according to the literature report.[44] This macroinitiator was used to perform polymerization (Table 2, entry 2, (react procedure showing in scheme 1, c). The structure of the resulting polymer was verified by ^{1}H NMR spectroscopy (Figure S11**,** , ESI†). The polymer was successfully connected with PEG-Br as confirmed by end group analysis *via* ^{1}H NMR spectroscopy, whose M_n is 3100 and PDI is 2.07by GPC (Figure S12**,** ESI†). It must be noted that it was necessary to dissolve DhHP-6 in water first, and neat DMF as solvent made the reaction lose control and the monomer conversion quite low, likely due to the low solubility of DhHP-6 in DMF. DhHP-6 also exhibited good ATRPase activity in organic media. End group analysis indicated that the reaction was initiated from the ATRP initiator, further demonstrating that the reaction was based on ATRP mechanism.

eROP is a well-established biocatalytic and clean process for the synthesis of degradable biomaterials.[45-47] Herein, we investigated the double-enzymatic synthesis of block copolymers by the combination of enzymatic ATRP and eROP.

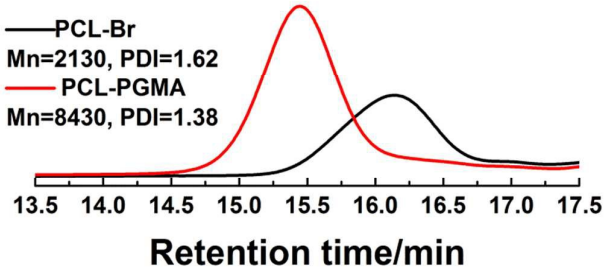


Figure 3. GPC traces of PCL-Br macroinitiator (black) and copolymer of PCL-PGMA (red).

Poly(ε-caprolactone) (PCL) is a kind of degradable polymer that can be used in drug delivery and tissue engineering,[48-49] therefore we choose PCL as one useful fragment of the target copolymer for potential biological applications. Firstly, we synthesized hydroxyl group-containing 2-hydroxyethyl 2-bromoisobutyrate (HEBiB), and used this bifunctional initiator to initiate ε-caprolactone polymerization by eROP to obtain PCL with ATRP initiator on the end of the polymer chain (PCL-Br, structure see Figure S13, , ESI†), whose M_n was analyzed by GPC (Figure 3, black trace). After purification, we conducted DhHP-6 catalyzed ATRP of GMA using PCL-Br as initiator (see Table 2, entry 3 for detailed reaction conditions, scheme 2 for react procedure). Analysis the M_n and PDI of PCL-PGMA by GPC (Figure 3, red trace). revealed monomodal distribution with a clear shift to higher molecular weight after enzymatic ATRP of GMA. This suggests the absence of PCL-Br macroinitiator or homopolymer of GMA in the final copolymer products. Compared to PCL-Br, the molecular weight increased, and PDI was narrower than PCL-Br. Structure was confirmed by ^{1}H NMR spectroscopy (Figure S13, S14, ESI†), which also indicates the successful preparation of PCL-PGMA copolymers through a combination of these two enzymatic polymerization methods. Control experiments showed that no PGMA polymer was generated using neat PCL as initiator (data not show), further proving that the DhHP-6 catalyzed polymerization was based on ATRP mechanism. In order to inspect the versatility of this combination, we also replaced the vinyl monomer with other monomers bearing other functional groups, that is, hydroxyethyl methacrylate (HEMA) and methacrylic acid (MAA).^{1}H NMR and GPC analysis of the obtained copolymers confirmed the predominatly block structures. From the ^{1}H NMR spectrum, we can see that the functional groups in the vinyl monomer are retained during the process. As PCL homopolymer could not dissolve in DMSO-d6, but the ^{1}H NMR spectrum of PCL-PHEMA and PCL-PMAA all clearly show the characteristic peaks of PCL segment (see Figure S15 for PCL-PHEMA and Figure S16 for PCL-PMAA, ESI†), indicating the successful linkage of PCL with the corresponding vinyl polymer. This also demonstrates that DhHP-6 as an ATRPase possess good tolerance to functional groups on HEMA(–OH) and in particular excellent compatibility with

carboxyl groups of MMA. As reported, acrylic and methacrylic acid could react with metal ATRP catalyst and the direct polymerization of acrylic or methacrylic acid based on ATRP mechanism is not available.[9] To the best of our knowledge, this is the first example of enzyme catalyzed MAA polymerization directly based on ATRP mechanism. Compare to copolymer of PCL-PGMA, these two amphiphilic copolymers' M_n and PDIs are more higher, while M_n values of copolymer PCL-HEMA and PCL-PMAA are higher and the PDIs are much broader. This discrepancy could be attributed to GPC analysis, as neat PCL could not dissolve in DMF at the test temperature, but PHEMA and PMAA all dissolve easily in DMF at that temperature. Self-assembly of these two amphiphilic copolymers in the GPC mobile phase may lead to this phenomenon. All the GPC trace show only one peak and increased M_n, which also indicates that there is no homopolymer in the final products. In an individual experiment of DhHP-6 catalyzed MAA polymerization under ARGET ATRP, the monomer conversion was 50.0% in 6.5 h, with a M_n of 3270 and PDI of 1.40 (Figure S19, ESI†). The product's property, GPC trace and ^{1}H NMR spectrum all demonstrated the successful syntheses of these copolymers.

The combination of two different enzymatic polymerizations, i.e., enzymatic ATRP and eROP, to synthesize copolymers is a new concept of biocatalysis that can be used in multistep chemical routes. Our work demonstrates a good approach to synthesize various copolymers with biomedical applications.

Conclusions

In conclusion, we found that, in DhHP-6 catalyzed ATRP process, molecular weights of the resulting polymers increased linearly with monomer conversion and the PDI were relatively low, which indicate DhHP-6 showed good activity of ATRPase and can tolerate with different kinds of functional groups on vinyl monomers. This catalytic system can easily combine with eROP to synthesize useful copolymers with different functional groups (epoxy, –COOH and –OH). The integration of eROP and enzymatic ATRP avoided the use of toxic transition metal catalysts, and was proved to be a promising environmentally benign process for the production of biomaterials. As an artificial structure, DhHP-6 is easier to make and less expensive to regenerate or change the enzyme's structure for certain purposes. DhHP-6 with functional groups can be further modified by other chemical structures or immobilized onto different substrates to expand its applications. All in all, enzyme or enzyme mimetic-catalyzed ATRP will be a valuable method in the field of biomedical polymer chemistry.

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