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Why synthesize protein–polymer conjugates? The stability and activity of chymotrypsin-polymer bioconjugates synthesized by RAFT

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ABSTRACT

α-Chymotrypsin, a commonly used protease, was modified with well-defined oligomers synthesized by RAFT. The well defined polymers were synthesized based on the monomers N,N-dimethylacrylamide (DMAm) or oligo(ethylene oxide) methyl ether acrylate (OEOA). The polymers were conjugated to free amine groups on chymotrypsin through an *in-situ* 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) coupling approach. The protein–polymer conjugates retained enzymatic activity, and the higher molecular weight DMAm and OEOA polymer, created protein–polymer conjugates with significantly enhanced stability, presumably due to the high molecular weight polymer preventing autolysis of the α-chymotrypsin.

1. Introduction

The ability to precisely synthesize a macromolecule with controlled functionality and molecular architecture for a given application is an ongoing target in the field of polymer chemistry. One area where this synthetic ability is particularly important is in the preparation of bioconjugates, or materials that combine a biologically relevant molecule with a synthetic compound [1]. Bioconjugates are an emerging class of materials that offer the benefits of activity and function in biological applications with the flexibility of chemical functionality and structure possible through synthetic chemistry [1,2]. A particularly interesting group of bioconjugates are protein-polymer hybrids, wherein the attached polymer can provide a synthetic handle to modulate the performance of the biomaterial [3–6]. The polymer attached to the protein can serve multiple roles including, stabilizing the protein–polymer conjugate [3], shifting the optimal pH and temperature for the enzyme [7], leading to responsive or "smart" biomaterials [7,8].

The synthesis of bioconjugates, including protein–polymer conjugates, typically involves one of two strategies, the "grafting-to" and the "grafting-from" approaches [1,9]. In grafting-to, a polymer is first synthesized, and subsequently attached to the protein, or other biomolecule, using an efficient organic reaction [9]. In contrast, the grafting-from approach first attaches a small molecule initiator or chain transfer agent (CTA) to the protein, or biomolecule of interest, and then directly grows the polymer from the protein in an aqueous solution [10]. The advantages of grafting-from include simple purification, and in many cases a higher grafting-density [9–11]. However, the difficulties with grafting-from include potential loss of protein stability upon attaching the initiator or CTA [12], and choosing reaction conditions that preserve protein stability while giving well controlled polymers [11]. In contrast, grafting-to offers the advantages of simple synthesis and characterization of the polymer and protein before conjugation, and that the polymerization conditions do not affect protein stability [9,13,14]. The disadvantages of grafting-to include difficulty achieving high graft density, particularly with high molecular weight polymers, and difficulty purifying the polymer from the conjugate after synthesis [10–12]. A representation of the grafting-from and grafting-to strategies is given in Scheme 1.

Reversible deactivation radical polymerization (RDRP) methods have revolutionized the fields of polymer chemistry and material science [15]. Nitroxide mediated polymerization (NMP) [16], atom

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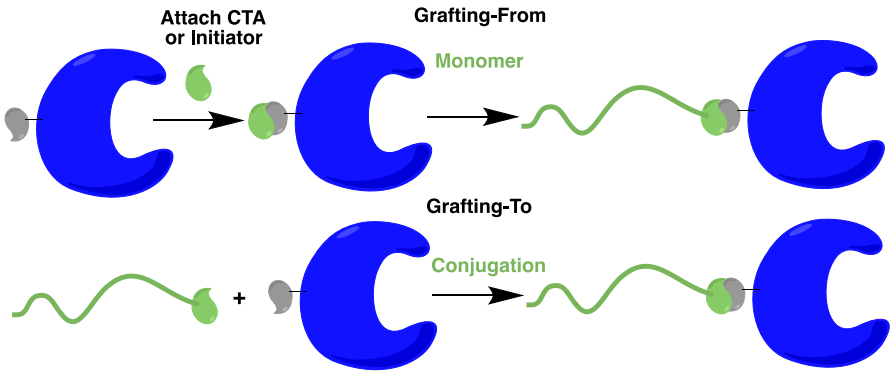


Scheme 1. Top shows a grafting-from strategy for synthesizing a protein-polymer bioconjugate, and bottom shows a grafting-to strategy for bioconjugate synthesis.

transfer radical polymerization (ATRP) [17,18], and reversible addition-fragmentation chain transfer polymerization (RAFT) [19], are the three most commonly used RDRP methods. Each of these three RDRP methods has been used to create well controlled protein–polymer conjugates [8,20–29]. RDRP methods are particularly well suited to protein–polymer conjugate synthesis since both the grafting-from and grafting-to methods can be used to create well defined biohybrids [1]. This manuscript focuses on RAFT polymerization, as a tool to synthesize well-defined protein–polymer conjugates. RAFT is well suited to the synthesis of bioconjugates [30,31], including protein–polymer conjugates [8,12,23,25,32,33], since it creates living polymers from a wide variety of functional groups, and offers excellent control over short chains [34,35].

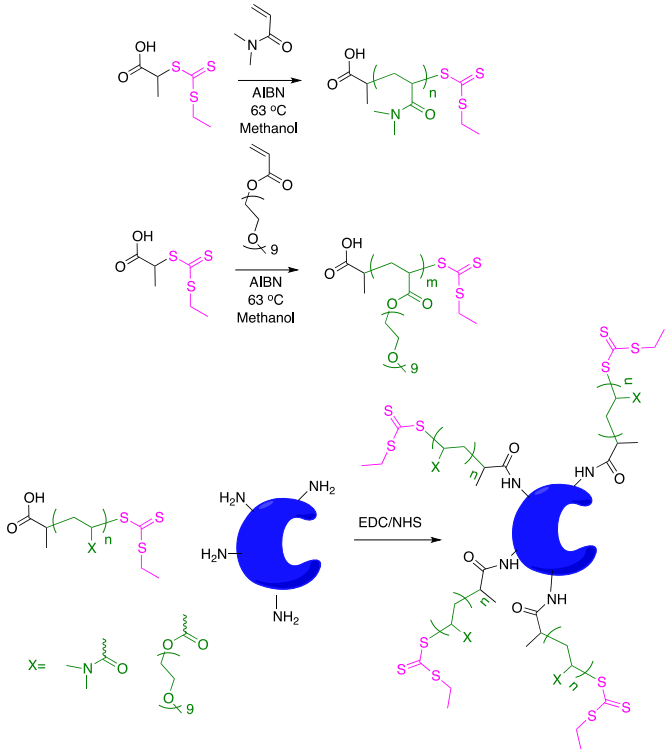
This paper uses α-chymotrypsin as the enzyme to be conjugated with synthetic polymers made by RAFT. Chymotrypsin is a protease, an enzyme that digests other proteins, including other α-chymotrypsin molecules (autolysis), by catalyzing peptide bond hydrolysis [36,37]. Due to promiscuous activities, conjugation with synthetic polymers can dramatically improve the stability and useful lifetime of proteases such as trypsin and chymotrypsin [7,38–40]. Although chymotrypsin polymer bioconjugates have been synthesized by ATRP [7,20], to the best of our knowledge there are no examples of chymotrypsin–polymer conjugates with the polymer synthesized by RAFT.

2. Results and discussion

In this paper RAFT polymerization was used to synthesize polymers containing a single carboxylic acid group, from the R group of the CTA. RAFT was used to synthesize the polymers of N,N-dimethylacrylamide (DMAm) and oligo(ethylene oxide)methyl ether acrylate (OEOA) of average molecular weight = 480. Polymers with number average molecular weight below ~5000 were chosen since the short chain facilitates grafting-to processes. Subsequently, each polymer was conjugated to free amine groups on chymotrypsin to create amide bonds through an *in-situ* 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) coupling strategy. Subsequently the activity and stability of these bioconjugates was determined and compared to that of the unmodified chymotrypsin. A summary of this approach is given in Scheme 2.

2.1. Synthesis and characterization of polymers made by RAFT

In this approach, three polymers were synthesized, and subsequently attached to chymotrypsin. 2-(((ethylthio)carbonothioyl)thio)propanoic acid (PAETC) was used as the chain transfer agent. RAFT polymerization was used to create the poly(DMAm) (pDMAm) and the poly(OEOA) (pOEOA) based chains, using AIBN (0.2 mol equivalents to CTA) as the initiator, at 63 °C, with methanol being the solvent. The temperature of 63 °C was chosen to be below methanol's boiling point of 64.65 °C [41], and at 63 °C AIBN has a half life of approximately 12.4 h [42]. The three polymers synthesized are labeled pDMAm-low MW for a polymer with a target of 10 repeat units of DMAm giving a targeted molecular weight of ~1200, pDMAm-high MW for a polymer with a target of 48 repeat units of DMAm giving a targeted molecular weight of ~5000, and pOEOA for a polymer with a target of 10 repeat units of OEOA giving a targeted molecular weight of ~5000. In all cases the monomer conversion after 24 h of reaction time was over 95%, the limit of NMR measurement.



Scheme 2. Synthesis of pDMAm and pOEOA chains containing a single carboxylic acid by RAFT polymerization, followed by the subsequent conjugation of the oligomers to the protein through EDC/NHS coupling.

As indicated in Fig. 1, the pDMAm-low MW oligomer was very well controlled, with a narrow molecular weight distribution, centered around 9 repeat units of DMAm, as measured by electrospray ionization mass-spectrometry (ESI-MS). This is in good agreement with the targeted degree of polymerization of 10 units. For the pDMAm-high MW, matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS), was used to characterize the polymer. As shown in Fig. S1(a), the pDMAm-high MW polymer gave a well-defined peak centered at a molecular weight of 4500. This corresponds to an average degree of polymerization of approximately 43 units, in acceptable agreement with the targeted degree of polymerization of 48 units. Similarly, MALDI-MS for the pOEOA oligoCTA, shown in Fig. S1(b), identified a molecular weight distribution centered around 10 repeat units of OEOA.

Each of the MS data sets were fitted with Gaussian functions to determine the number averaged molecular weight (M_n), corresponding number averaged degree of polymerization (DP_n), weight averaged molecular weight (M_w), and the dispersity (M_w/M_n). These values are given in Table 1 and the raw MS data and fitted Gaussian functions are shown in Fig. S2.

2.2. Conjugation of the oligoCTAs to chymotrypsin

Once synthesized and characterized, all polymers were conjugated to chymotrypsin to produce well-defined protein–polymer conjugates. Polymer conjugation may afford hybrids that avoid autodigestion of chymotrypsin. The DMAm and the OEOA based polymers were conjugated to free amine residues on chymotrypsin using an *in-situ* EDC/NHS coupling strategy. This is possible since the pDMAm and pOEOA based polymers contain a single carboxylic acid group from the R group of the CTA, which is a useful reactive handle for EDC/NHS coupling. A 30–60-fold excess of polymer to amine groups (both terminal amine and lysine residues) was used.

Successful grafting of the pDMAm-low MW oligomer to the protein is shown in Fig. 2. Fig. 2A shows the MALDI-MS data for the unmodified chymotrypsin, which has a single sharp peak at 25,600 *m/z*, which agrees well with the molecular weight of chymotrypsin [43]. There are no other major or broad peaks in the native chymotrypsin sample. Fig. 2B gives the MALDI-MS data for the chymotrypsin-pDMAm-low MW conjugate. As seen in Fig. 2B, there is complete modification of the native chymotrypsin by the pDMAm oligomer, as indicated by a shift of the MALDI-MS spectrum to a peak centered at approximately 30,000 *m/z*. This corresponds to an average of 4 pDMAm oligomers attached per chymotrypsin molecule. MALDI-MS is used for the analysis, since it directly shows the molecular weight of the singly charged complex. Although sodium dodecyl sulfate poly(acrylamide) gel electrophoresis (SDS-PAGE) could be used to characterize these complexes, we have not done so here as SDS-PAGE measures apparent molecular weights which can be skewed by differential interactions of protein-conjugated polymers with the polyacrylamide gel matrix relative to those interactions for protein-based molecular weight

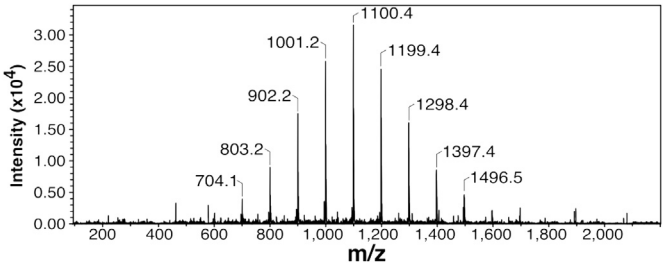


Fig. 1. ESI-MS data for the low MW oligoCTA based on DMAm.

Table 1
Number averaged molecular weight (M_n), corresponding number averaged degree of polymerization (DP_n), weight averaged molecular weight (M_w), and the dispersity (M_w/M_n) for both the pDMAm-low MW, pDMAm-high MW and pOEOA based polymers.

	M_n	DP_n	M_w	M_w/M_n
pDMAm-low MW	1100	8.95	1140	1.04
pDMAm-high MW	4500	43.3	4630	1.03
pOEOA	4970	9.90	5330	1.07

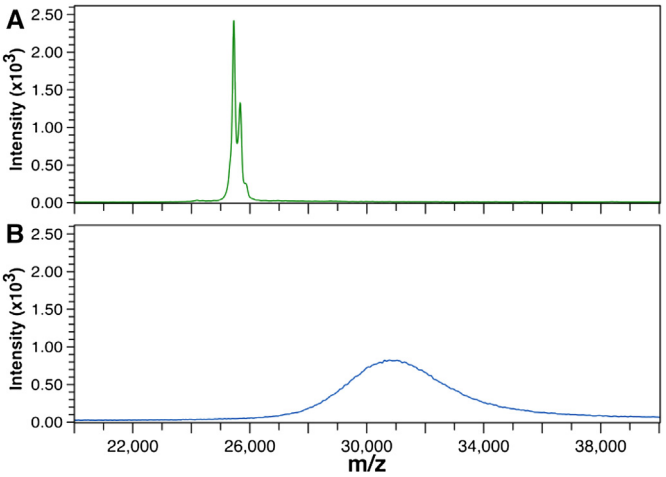


Fig. 2. A. MALDI-MS data for native chymotrypsin. B. MALDI-MS data for the chymotrypsin–pDMAm conjugate, indicating a clear shift towards higher molecular weight.

standards lacking conjugated polymers. Fig. S3 gives the aqueous size exclusion chromatography data for native chymotrypsin, and the chymotrypsin-pDMAm-low MW conjugate. Although the SEC data in Fig. S3 indicate a broader distribution than that obtained from the MALDI-MS, the SEC data and the MALDI-MS data both indicate efficient conjugation of the pDMAm-low MW oligomer to the protein, with the MALDI-MS indicating negligible unmodified protein. Similarly Fig. S4a indicates by SEC the coelution of the pDMAM-low MW based oligomer containing the RAFT end group at 309 nm, and the protein at 280 nm. This suggests conjugation of the polymer and the protein, and negligible free polymer since the unconjugated would elute at lower molecular weight.

A similar EDC/NHS conjugation protocol was used to attach the pDMAM-high MW and the pOEOA polymers to chymotrypsin. Fig. S5 shows the MALDI-MS data for the chymotrypsin-pDMAm-high MW conjugate. The chymotrypsin-pDMAm-high MW conjugate had significantly poorer signal to noise than chymotrypsin-pDMAm-low MW conjugate, however, no native protein and the peaks corresponding to 1, 2 and 3 attachments could clearly be seen in the MALDI-MS data. Unlike the pDMAm based conjugates, ionization efforts with a library of MALDI-MS matrices proved to be a challenge for the pOEOA based conjugate and failed to produce enough ions to obtain any discernable signal. However, as indicated in Fig. 3 aqueous SEC clearly indicated successful conjugation between chymotrypsin and the pOEOA oligomers. There was a significant shift of the molecular weight distribution to higher molecular weight, after chymotrypsin was conjugated with the pOEOA based oligomers. Additionally the SEC data indicate only a small amount of native chymotrypsin, indicating conjugation between the polymer and the protein. Similarly Fig. S4b indicates by SEC the coelution of the pOEOA based CTA at 309 nm, and the protein at 280 nm. This indicates conjugation of the polymer and

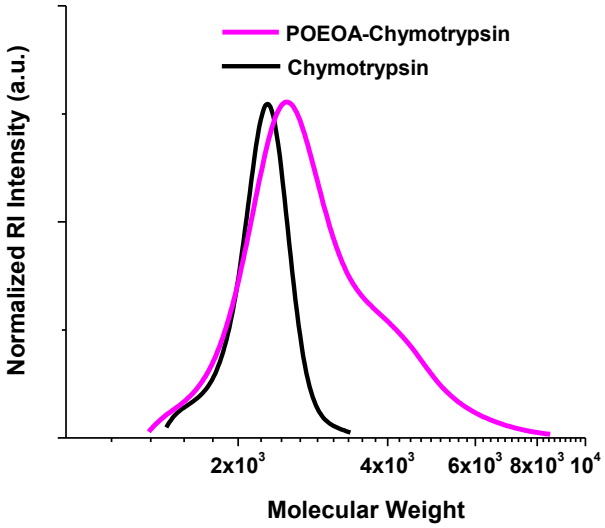


Fig. 3. Aqueous SEC molecular weight distributions for native chymotrypsin and chymotrypsin conjugated with the pOEOA oligomer.

the protein and negligible free polymer since the unconjugated would elute at lower molecular weight.

2.3. Enzymatic activity and stability of the protein–polymer conjugates

Finally, the enzymatic activity of the chymotrypsin–polymer conjugates was assessed, and the stability was determined by measuring the activity as a function of time. Since chymotrypsin is a protease, the enzymatic activity of chymotrypsin can decrease over time, due to proteolytic digestion of one chymotrypsin by a second chymotrypsin molecule [36]. However, conjugating a polymer to chymotrypsin offers the potential to stabilize the enzyme by decreasing the rate of proteolytic degradation while maintaining enzymatic activity against smaller substrates. To determine the activity and stability of chymotrypsin and the chymotrypsin–polymer conjugates, a colorimetric N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Suc-AAPF-pNA) hydrolysis assay was used. The activity of the native chymotrypsin was compared to the chymotrypsin-pOEOA and the chymotrypsin-pDMAm-low MW and pDMAm-high MW conjugates in Fig. 4A. As indicated in Fig. 4A, the pOEOA based conjugate had essentially the same activity as the native chymotrypsin, while the pDMAm-low MW based conjugate had approximately 2/3 the activity of the native chymotrypsin, and the pDMAm-high MW based conjugate had approximately 1/3 the activity of the native chymotrypsin all measured against the Suc-AAPF-pNA substrate. The decrease in activity for the pDMAm based bioconjugate is most likely due to the functional group potentially interfering with the active site, steric effects, or a less efficient stabilization of chymotrypsin against enzymatic degradation. To discriminate between these effects, the normalized activity of each conjugate was measured as a function of time, as displayed in Fig. 4B and C.

Fig. 4B displays the activity of native chymotrypsin, and both the pDMAm based conjugates measured at several time points over a 2.5 h period. The native enzyme lost almost 90% of its activity over the 2.5 h measurement. This is likely due to the digestion of one chymotrypsin molecule by a second chymotrypsin. Similarly the chymotrypsin-pDMAM-low MW conjugate exhibited a near identical loss of enzymatic activity over time. In contrast, the chymotrypsin-pDMAM-high MW conjugate showed improved

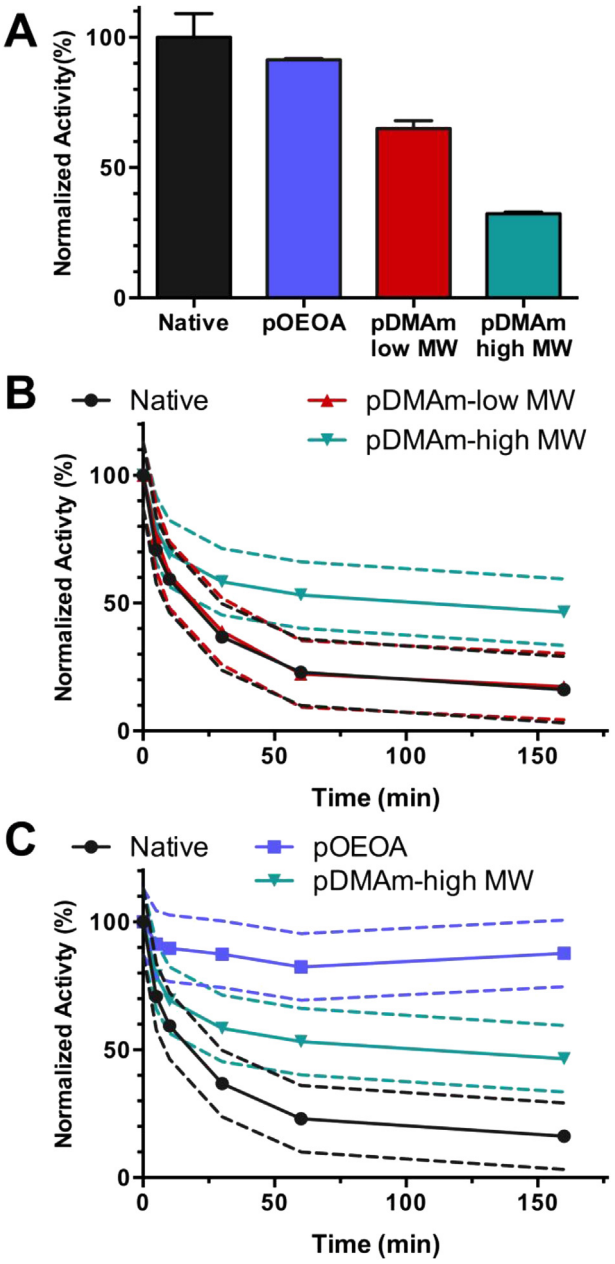


Fig. 4. A. Activity of native, pOEOA-conjugated and pDMAm-conjugated chymotrypsin for hydrolyzing the N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide substrate. B. Stability of the native, pDMAm-low MW conjugated and pDMAm-high MW conjugated chymotrypsin as a function of time, measured from the activity as a function of time for hydrolysis of N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide. Data points represent the average of three independent trials ± standard deviation (dashed lines). C. Stability of the native, pOEOA-conjugated and pDMAm-high MW conjugated chymotrypsin as a function of time, measured from the activity as a function of time for hydrolysis of N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide. Data points represent the average of three independent trials ± standard deviation (dashed lines).

stability over time. The pDMAM-high MW conjugate lost 50% of its original activity as a function of time, compared to a loss of 80–90% activity for the native protein and the pDMAM-low MW based conjugate. This indicates that higher molecular weight polymers allow for better stabilization of the protein against digestion by a protease.

Finally, Fig. 4C compares the enzymatic activity of the native enzyme, the pDMAm-high MW conjugate and the pOEOA conjugate as a function of time. As shown in Fig. 4C the pOEOA conjugate

is significantly better stabilized against autolytic digestion than the pDMAm-high MW conjugate. In both cases the average molecular weight of the attached polymer is approximately 5000, and the difference in stability suggests that the OEOA based polymer is better at stabilizing the enzyme than the DMAm based polymer.

3. Conclusions

RAFT polymerization was used as a tool to synthesize well-defined protein–polymer conjugates. Two monomers, N,N-dimethylacrylamide (DMAm) and oligo(ethylene oxide)methyl ether acrylate (OEOA) of average molecular weight = 480 were used to create narrowly distributed polymers containing one carboxylic acid group per chain. The carboxylic acid group was used as a reactive handle, to conjugate the polymer to amine groups on chymotrypsin using EDC/NHS coupling. Well-defined conjugates were synthesized, with negligible unmodified protein. Finally, the activity of chymotrypsin was determined using Suc-AAPF-pNA. The results of these activity assays indicate that the pOEOA conjugate has essentially the same activity as the native enzyme, while a third of the activity is lost in the low molecular weight pDMAm conjugate, and two thirds of the activity were lost in the high molecular weight pDMAm conjugate. Additionally, the pOEOA conjugate is stable and shows negligible loss of activity over time. In contrast, the high molecular weight DMAm conjugate displayed a relatively low loss of stability over time, while the native enzyme and the pDMAm low molecular weight conjugate show significant losses of activity over time, with a half-life of approximately 20 min. These results indicate that in certain cases, such as the pOEOA based conjugate, the presence of the polymer can significantly improve the stability of the enzyme without compromising the enzymatic activity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.polymer.2015.04.010.

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