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Activity Improvement and Thermal Stability Enhancement of D-Aminoacylase Using Protein-Polymer Conjugates

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Research Article

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ABSTRACT

In this study, the synthesis of new polymer-protein conjugates using a grafting-from strategy was performed by employing photo-induced electron transfer reversible addition-fragmentation chain transfer (PET-RAFT) polymerization. D-aminoacylase is an industrially significant enzyme for the preparation of chiral amino acids and it is coupled with reversible addition-fragmentation (RAFT) chain transfer agent (CTA) using-activated ester chemistry. The effects of polymeric side chain compositions on the activity of D-aminoacylase were studied with two different polymeric side chain lengths. For this reason, two monomers, a hydrophilic N-(2-aminoethyl acrylamide) and a hydrophobic and N- (iso-butoxymethyl) acrylamide were used, respectively. It was found that modification by grafting from strategy increased the thermal stability of the D-aminoacylase enzyme. Additionally, the hydrophobic monomer conjugate has been reported to increase the activity of the enzyme more than the hydrophilic monomer.

Keywords: PET-RAFT polymerization, Grafting from method, Bioconjugation, D-aminoacylase, Acrylamide

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Introduction

Enzymes have drawn attention in industrial applications such as food processing [1], detergents, textiles, and, organic synthesis [2] in recent years. However, environmental conditions such as high temperature and pH value are significant drawbacks to their use in these applications requiring enzymes that are very active and stable. Polymers are extensively used to overcome these drawbacks by ligating onto proteins. [3, 4]. The protein-polymer hybrids, known as bio-conjugates, have modulated various properties of the protein, such as stability, activity, and reusability, so they could be used as potential materials in industrial usage, drug delivery, biocatalysts, and biosensors [3]. Research in the previously reported studies in the literature demonstrated that the polymer-protein conjugation greatly modified the properties of the attached enzyme, especially its activity and stability [4]. As an example, Griebenow and coworkers studied with α- Chymotrypsin that hydrolyses site-specific peptide bonds of proteins and they increased the long-term stability of α- chymotrypsin using pegylation since α-chymotrypsin is autolyzed while hydrolyzing peptide bonds [5].

Bioconjugates are formed using 'grafting from' and 'grafting to' methods. While in grafting to method, polymer are initially synthesized before ligation, in grafting from method, the polymer grows on the protein attached to the chain transfer agent by addition of monomer [6]. The grafting to method has some disadvantages, such as requiring the use of a low-weight polymer. The 'grafting-from' method may bring about considerably high grafting density and therefore simpler purification [7]. Surely, there are some challenges in this method like poor control of polymer growth and a limited monomer selection [8]. The present methods like atom transfer radical polymerization (ATRP) [9], and reversible addition-fragmentation chain transfer (RAFT) [10,11] have been validated in the polymerization of acrylates and acrylamides. Also, ATRP with low copper contend and some photo-catalyzed reactions have led to grafting-from metal sensitive proteins [12,13]. RAFT has been one of the families of reversible deactivation radical polymerization (RDRP) methods and has become one of the most preferred polymerization techniques due to its versatility [14,15]. Synthesizing polymers with RDRP techniques has been interesting because of its advantages such as predictability of the chain length of the polymer, low dispersion, and constantly growing chain length, which has been defined as 'livingness' [16-18]. One of RDPR techniques RAFT has been the focus of attention due to its facilitation of use and functional group tolerance. However, conventional RAFT, in particular, has advantages as it relies on the thermal radical produced, as well as similar handicaps with other radical polymerization techniques, such as termination reactions

that restrict chain growth. In addition, the completion time of the RAFT polymerization should not be too long to provide control over the chain length distribution, but it can sometimes take a long time [19]. Photo-chemical RAFT polymerization could be carried out at low temperatures such as room conditions and with a light source, which can provide sustainable and environmentally friendly green chemistry applications [20-22]. Therefore, photochemistry has opened new horizons in polymer chemistry [23,24]. In this context, photo-induced electron/energy transfer reversible addition-fragmentation chain transfer polymerization (PET-RAFT) has been put forth as one of the interesting photochemical polymerization techniques. PET-RAFT technique differs from the conventional RAFT method in that the chain transfer agent (CTA) used also acts as an initiator type. PET-RAFT method provides convenience such as using the inductive energy of light at low temperatures and recognizing functional group tolerance [25]. Recently, studies on the handicaps of RAFT polymerization have developed methods that perform RAFT polymerization photochemically rather than thermally. PET-RAFT method has been preferred in protein-polymer conjugations, especially because the polymerization occurs at ambient temperature, visible light has been used to induce, the reaction has been fast and the chain length could be easily controlled [26]. Sumerlin and coworkers have recently reported that polymer-protein hybrids were generated using grafting from method and photo-induced electron transfer (PET) RAFT polymerization method was used for the synthesis of polymer [27]. They declared that a chain transfer agent (CTA) and a photocatalyst agent Eosin pair were used to synthesize of lysozyme polymer hybrid by grafted-from method. These conditions were used to grow polymers from D-aminoacylase enzyme in this study.

D-amino acids and their derivatives are widely used in the industries such as medicine and cosmetics [28]. D-amino acids are produced using enzymatic transformation in the recent manufacturing processes [29]. Recombinant D-aminoacylase enzyme (EcD) was produced using *E. coli* DH5α host strain. EcD was modified with a chain transfer agent and the subsequent grafting of monomers of N-(iso-butoxymethyl) acrylamide (NIBMA) and N-(2-aminoethyl acrylamide) (AEA) using the photo RAFT method was reported here in this work. N-(iso-butoxymethyl) acrylamide (NIBMA) is water-miscible monomer and also significantly hydrophobic since it does not dissolve in aqueous media when polymerized [30]. It is a fact that, there are a few reporting on the polymerization of NIBMA via reversible deactivation radical polymerization (RDRP) in the literature. NIBMA has been incorporated to block copolymers for the formation of nanoassemblies of coatings and some polymer films [31]. AEA is a hydrophilic monomer and the polymer synthesized from it is known as a hydrogel, its hydrophilicity offers several advantages such as being used as a drug delivery system regardless of pH [32]. These enzyme grafted polymers (pNIBMA and pAEA) are expected to increase thermal stability of the enzyme and improve its enzymatic activity.

Here, we aimed to report the synthesis of D-aminoacylase-polymer conjugates using grafting from method. Two different monomers were used for the enzyme conjugation in this work and the effects of these two monomers on the D-aminoacylase enzyme were investigated and compared.

Experimental Section

Production of Recombinant D-aminoacylase Enzyme

D-aminoacylase coding nucleic acid sequence was purchased from BIOMATIK. amn gene (GenBank: CP033635.1) was amplified by PCR using two primers;

P1 (5'TTTTTCCATGGATATGCAGGTTGACTGGCTGATC3')
and
P2 (5'TTTTTCTCGAGGTGAGAGGTCTGACGACGCAG3')

contained unique NcoI and XhoI (Promega Corporation) restriction sites. The PCR product was cloned into the pET 22b (+) expression vector with an N-terminal six-histidine tag. Chemically and electrocompetent *E. coli* DH5α cells were transformed by heat shock at 42 ℃ with the pET22bAmn plasmid. Transformation mix was spread on LB agar culture plates including ampicillin (100 µg /mL) and they were incubated at 37°C overnight. After transformation, plasmids were isolated from positive clones and confirmed by DNA sequencing. Rosetta-gamiTM (DE3) cells were transformed with pET22bAmn plasmid for recombinant D-aminoacylase expression. Rosetta-gamiTM (DE3) transformants were subcultured at 37 ℃ for 16 h in Luria–Bertani (LB) medium containing 100 µg/mL ampicillin, 50 µg/mL streptomycin, 50 µg/mL chloramphenicol, 10 µg/mL tetracycline. The subculture was cultured until the OD_{600} reached 0.6 after transfer to a fresh medium., and IPTG (final concentration 0.1 mM) was used for induction. Rosetta-gamiTM (DE3) cells were grown with vigorous shaking at 37 ℃ for 4 h after induction and these cells were harvested by centrifugation at 8000 rpm for 10 min at 4 ℃.

All operations in D-aminoacylase purification were performed at 4°C unless otherwise mentioned. Harvested cells were re-suspended in buffer A (100 mM NaCl, 100 mM NaH_2PO_4 buffer, pH 8.00) and disrupted by ultrasonic treatment. The supernatant was acquired as raw enzyme solution by centrifugation at 30 000 rpm for 20 minutes at 4 °C. Cleared cell lysate having crude enzyme solution was loaded onto a column filled with a Ni-NTA agarose affinity matrix (Qiagen, USA) pre-equilibrated with buffer A. The weakly bound histidine-rich proteins were washed away from the column after extensive washing of the resin with buffer A. Then the recombinant D-aminoacylase enzyme was eluted with buffer B (300 mM imidazole; 100 mM NaH_2PO_4, 100 mM NaCl, pH 8.00) at a flow rate of 0.5 mL/min. The fractions were collected and the d-aminoacylase activity was analyzed. Fractions containing higher enzyme activity were pooled [33,34].

Synthesis of Chain Transfer Agent (CTA)

Chain transfer agent (CTA) was synthesized as previously reported [35]. Briefly, potassium hydroxide (14.6 g, 0.26 mol) was dissolved in distilled water (15.0 mL) and ethanethiol (18.6 mL, 0.26 mol) was dissolved in acetone (150 mL) then the potassium hydroxide solution was added slowly to the prepared ethanethiol solution by stirring on an ice bath. After adding carbon disulphide (16.1 mL, 0.27 mol) to the reaction mixture, the mixture was stirred for 30 min on ice bath. End of the time, the mixture was left to heat up to room temperature and 2-bromopropionic acid (22.0 mL, 0.25 mol) was supplemented dropwise and the reaction mixture was left to stir overnight at room temperature. Solution was evaporated to remove solvent using rotary evaporator.

The residue was dissolved in ether (200 mL) first in a separator funnel, and then water (200 mL) was added. The yellow ether layer was collected and washed with 200 mL water six times subsequently, and then once with saturated sodium chloride solution. CTA, a viscos yellow liquid solidifying upon freezing, was obtained by removing solvent using rotary evaporation. CTA was defined by proton NMR spectroscopy.

Synthesis of N-hydroxysuccinimide- Based CTA (NHS-CTA)

NHS-CTA was synthesized similar to the previously reported literature [36]. N-hydroxysuccinimide (NHS, 3.3 g, 29.7 mmol) was suspended in dry dichloromethane (100 mL) and then added dropwise into the solution consisting of 2-(1-isobutyl) sulfanylthiocarbonylsulfanyl-2-methyl propionic acid (CTA, 5.0 g, 19.8 mmol) and dicyclohexylcarbodiimide (DCC, 6.5 g, 29.7 mmol) in dichloromethane (150 mL) under nitrogen atmosphere on ice bath and the mixture was left to stir overnight at room temperature. After solvent evaporation and column chromatography (SiO_2: ethyl acetate/ petroleum ether 3:1) were carried out, the bright yellow solid product at high yield product was obtained and it was characterized by H-NMR.

Synthesis of D-aminoacylase- CTA

Grafting from method was modified to previously reported study [37]. Briefly, the concentration of D-aminoacylase (EcD) (3 mg/ml) was adjusted using 1×PBS solution (pH 7.5) in a total volume (10 mL). After that NHS-CTA (30 mg) was dissolved in 2 mL dimethyl sulfoxide (DMSO) and added dropwise to the stirring D-aminoacylase solution and the mixture was stirred for 2 h. Finally, the modified enzymes were dialyzed against 1× PBS containing 10% glycerine using a 25 k MWCO dialysis membrane (Spectrum Labs).

Grafting from D-aminoacylase- CTA

Grafting from the method reported in previous studies was modified for this study [37]. EcD was conjugated with two different acrylamide monomers, hydrophilic and hydrophobic. These are AEA (N-(2-aminoethyl acrylamide)) and NIBMA (N-(iso-butoxymethyl) acrylamide), respectively and the conjugated enzymes were called EcD-pAEA and EcD-pNIBMA. For the synthesis of EcD-pAEA, N-(2-aminoethyl acrylamide) (AEA) (31 mg, 0.2 mmol) was firstly added to EcD¬-CTA solution and then Eosin Y (0.015 mg, 0.023 mmol), TEMED (N,N,N′,N′-Tetramethylethylenediamine, 0.5 mL, 0.38 mg, 0.0023 mmol) and 75 µL of DMF were added to the 10 mL round bottom flask with a stir bar then the reaction mixture was exposed to nitrogen for degassing and sealing process for 30 min. Polymerization process was started with blue light while stirring. The blue light was extinguished to quench polymerization after 15 min, 30 min and 60 min and the obtained products were named as EcD-pAEA -S, EcD-pAEA -M and EcD-pAEA-L respectively. The synthesized products (EcD-pAEA-S, EcD-pAEA -M and EcD-pAEA-L) were characterized using 1H NMR spectroscopy to confirm that polymerization had occurred. The products were lastly purified using dialysis against 100 mM NaH2PO4 (pH 8.00) solution.

For the synthesis of EcD-pNIBMA, this time NIBMA (34 mg, 0.2 mmol) was placed into the round bottom flask containing the EcD-CTA solution. After that the same reagents (Eosin Y, TEMED and DMF) which were mentioned above were added to reaction mixture while stirring then the reaction mixture was exposed to nitrogen for degassing and sealing process for 30 min. Polymerization was irradiated with blue light while stirring after that the products were named EcD-pNIBMA -S after 15 min, EcD-pNIBMA -M after 30 min and EcD-pNIBMA -L after 60 min. To confirm the polymerization, the obtained crude reaction products were characterized by using 1H NMR spectroscopy. The crude reaction mixture was purified by dialysis against 100 mM NaH_2PO_4 (pH 8.00) solution. Conjugates were analyzed by SDS-PAGE.

To the best of our knowledge, functional group concentration decreases with molecular weight as shown in Fig.1, drawn theoretically. The best-fitting equation could also be used to calculate average molecular weight from the ratio of the weight of H of OH functional groups to the weight of H in CH_2 groups.

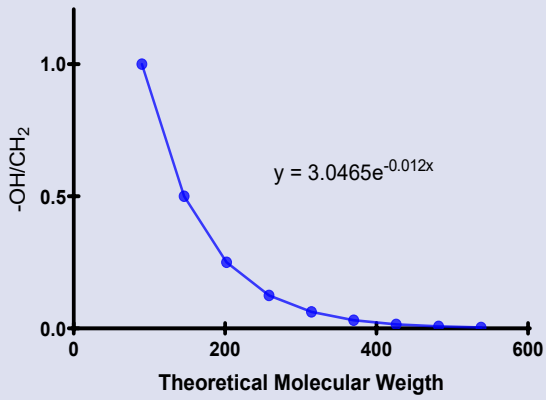


Figure 1. Mathematical expression of the increase in the theoretical molecular weight based on the decreasing ratio of side groups.

Hydrolysis Activity and Thermal Stability of D-Aminoacylase and its Polymer Conjugates

D-aminoacylase and their conjugates activities were determined with the 2,4,6-trinitrobenzenesulfonic acid method (TNBS) [38] and used N-acetyl-D-leucine as a substrate. The standard reaction mixture was prepared using 100 mM sodium phosphate buffer (pH 8), 50 mM N-acyl-d-leucine and an appropriate amount of the enzyme for the determination of enzymatic activity and incubated at optimum temperature 45ºC for 30 min. End of the incubation time, the absorbance value of the reaction mixtures containing D-aminoacylase and their conjugates were measured at 335 nm using a Nanodrop DeNoVIX spectrophotometer (Wilmington, USA). The relative enzymatic activities of EcD-pNIBMA and EcD-AEA conjugates were calculated based on D-aminoacylase activity and drawn using Graphpad Prism 8.1 software.

To determine the thermal stability of D-aminoacylase and their conjugates, they were pre-incubated at 45ºC and pH 8 for different times (0, 30, 60, 90, 120 minute) after pre-incubation, the substrate was added to these enzymes' solutions and the observed orange colour was indicated that the enzymes were active and also the absorbance values were determined using a spectroscopy.

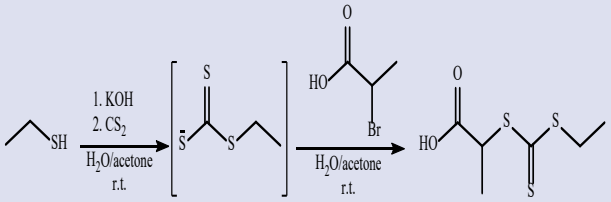
Thermal stability graph was plotted using Graphpad Prism 8.1 software of the enzyme solutions in absence of substrate for various at optimum temperature 45ºC, respectively. Residual activities were determined under D-aminoacylase activity assay conditions.

Results and Discussion

The ligation of polymers to proteins is recently used to improve proteins' biological activities [39]. The polymer-protein conjugates are usually occurred using grafting-to method, but this approach has disadvantages such as requiring polymer with low molecular weight. On the other hand, the grafting from approach allows high molecular weight polymers to be used, easy to purify and grow directly on proteins [40,41]. Consequently, grafting from method is commonly preferred instead grafting to method in the bio-conjugation process.

PET-RAFT, a polymerization method, occurs at room temperature by inducing visible light in the presence of a photocatalyst. PET-RAFT method has remarkable advantages such as low temperature requirement, visible light induction and regulation, and it is also environmentally friendly [42]. Therefore, in this study, we decided to use PET-RAFT method to acquire grafting from polymerization of D-aminoacylase-conjugates. Eosin Y was preferred as a photocatalyst to inspect synthesis of acrylates and ethlyacrylates in DMSO. To catalyze the polymerization process, Eosin Y was used with TEMED.

CTA, 2-(((ethylthio)- carbonothioyl) thio) propanoic facilitation aid was synthesized to use in production of polymers by RAFT polymerization as shown in Scheme 1. [43].



Scheme 1. Synthesis of chain transfer agent (CTA) by using RAFT polymerization

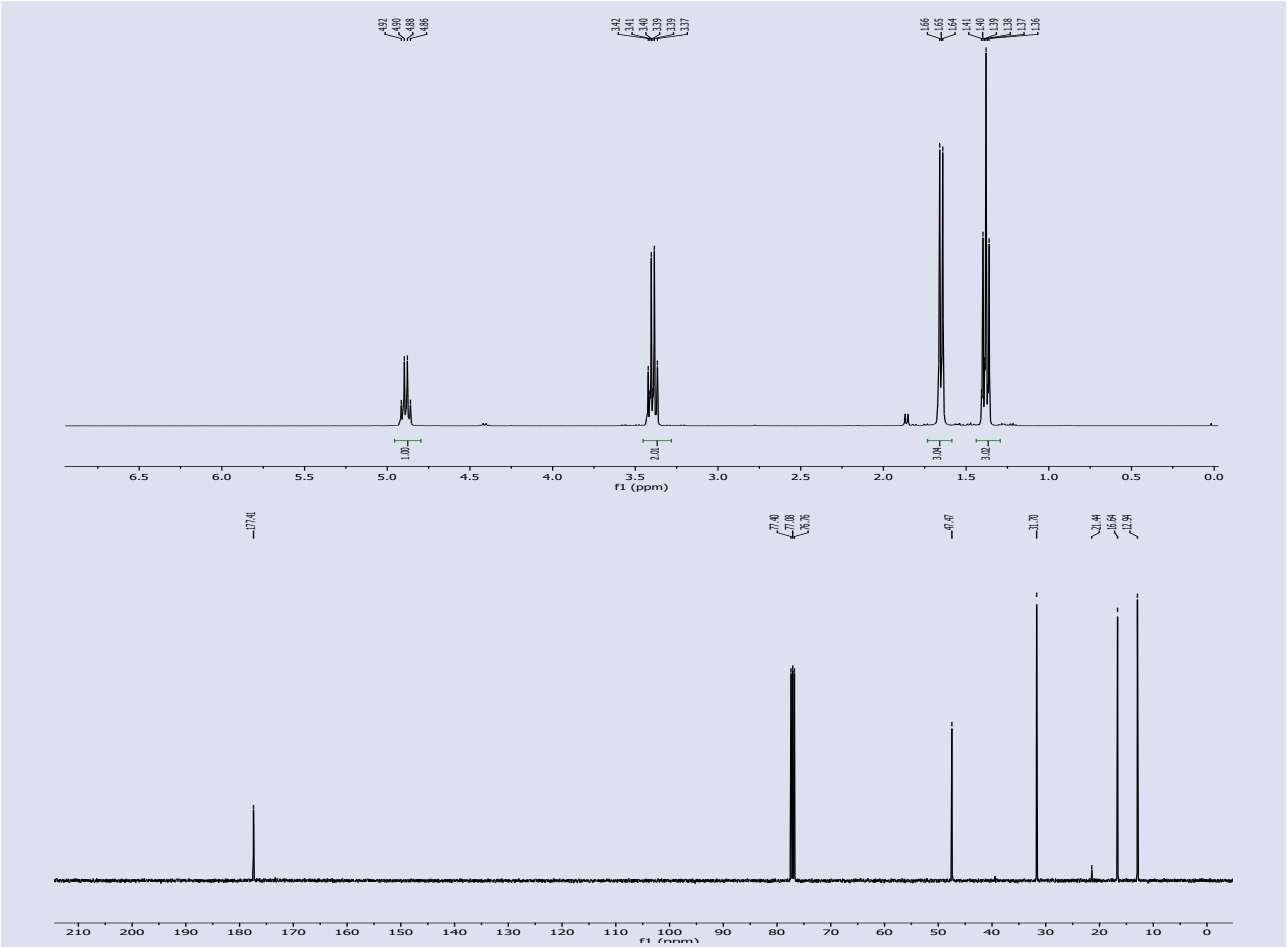
^{1}H-NMR and ^{13}C-NMR data of CTA were given below, and these data were also presented in Scheme 2.

^{1}H-NMR (400 MHz, $CDCl_3$, ppm): δ = 4.92-4.86 (q, *J*= 8 Hz, 1H), 3.42-3.37 (q, *J*= 8 Hz, 2H), 1.66-1.64 (d, *J*= 8 Hz, 3H), 1.40-1.36 (t, *J*= 8 Hz, 3H).

^{13}C-NMR (100 MHz, $CDCl_3$, ppm): δ = 177.4, 47.5, 31.7, 16.6, 150.4, 12.9.

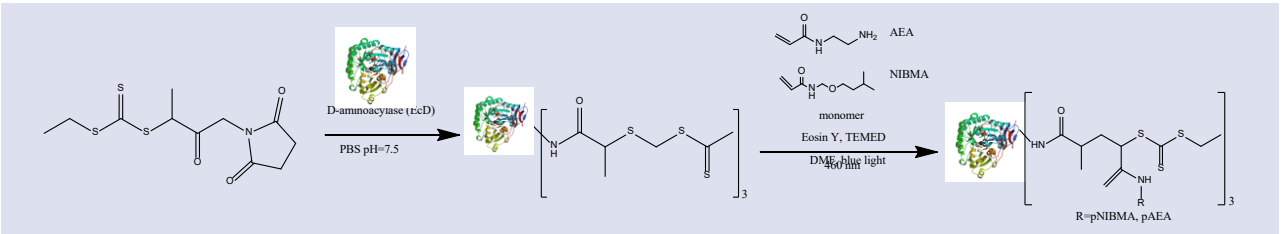
N-hydroxysuccinimide esters (NHS) are significant vehicles used in bio-conjugate chemistry and provide a handle for growth polymers [44]. NHS-CTA was generated by adding CTA on N-hydroxysuccinimide ester. Recombinantly produced EcD was prepared 1×PBS solution in at a concentration of 3 mg/ml, it was added slowly dropwise on the enzyme solution after NHS-CTA was dissolved in DMSO. DMSO, as a solvent was required to dissolve CTA and slowly feeding, was prevented the enzyme precipitation that might result from high concentration of DMSO in reaction medium [45].

Subsequently, the obtained EcD-CTA was dialyzed to remove DMSO against 1× PBS with 10% glycerine using 25 k MWCO dialysis membrane tubing bag. The grafting from the polymerization was carried out using a polymerization catalyst TEMED together with Eosin Y as a photocatalyst in the presence of monomer. During the reaction period, it was observed that the color of the reaction medium changed from red to yellowish due to photodegradation of Eosin Y.



Scheme 2. H NMR and C NMR spectra of CTA

Throughout the grafting-from polymerization with D-aminoacylase-CTA using NIBMA and AEA monomers, bio-conjugates with different molecular weights were synthesized at different time periods and produced bio-conjugates were called dependent on their lengths (short: EcD-pAEA-S, EcD-pNIBMA-S; medium: EcD-pAEA-M, EcD-pNIBMA-M; and long EcD-pAEA-L, EcD-pNIBMA-L) (Scheme 3.)



Scheme. 3 Synthesis of D-aminoacylase-polymer conjugates by using grafting-from method and performing the polymerization using photo-induced electron/energy transfer reversible addition-fragmentation

The polymerization ended quenching blue light after 60 min and the milky appearance of reaction media confirmed the suspension formation. After the polymerization completed, bio-conjugates were purified using dialysis membrane tubing.

The molecular weights of conjugates were calculated by using H-NMR data of the grafted side polymers of pNIBMA and pAEA from D-aminoacylase. Theoretically drawn molecular weight versus integral weight ratio curve of hydroxyl to aliphatic hydrogen (Fig 1) and best fitting equation were used for calculation. Molecular weights data were tabulated in Table 1.

Table. 1 Molecular weight data for grafted side polymers of pNIBMA and pAEA from D-aminoacylase – -graft-pNIBMA and D-aminoacylase -graft- pAEA produced using PET-RAFT polymerization

D-aminoacylase polymer conjugates	Molecular Weights of Bio-conjugates (g/mol) Reaction Time (min)		
	15	30	60
EcD-pNIBMA	465	620	632
EcD-pAEA	532	544	560

According to the molecular weight results, it is observed that the molecular weight of the side groups could be controlled by the reaction time. Molecular weights of bio-conjugates were increased dependent on reaction time and these data were plotted using Graphpad Prisms 8.1 Software as shown in Fig 2.

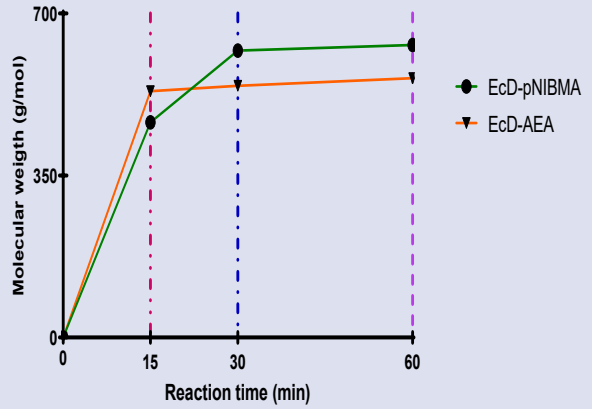


Figure 2. Variation of molecular weights of EcD-pNIBMA and EcD-AEA dependent on reaction time points.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of EcD-pNIBMA and EcD-pAEA demonstrated the polymers were ligated on the D-aminoacylase (Fig. 3). Besides that, SDS-PAGE results of the EcD- pNIBMA and EcD-pAEA confirmed that there was no free D-aminoacylase pointing out accomplished grafting from reactions.

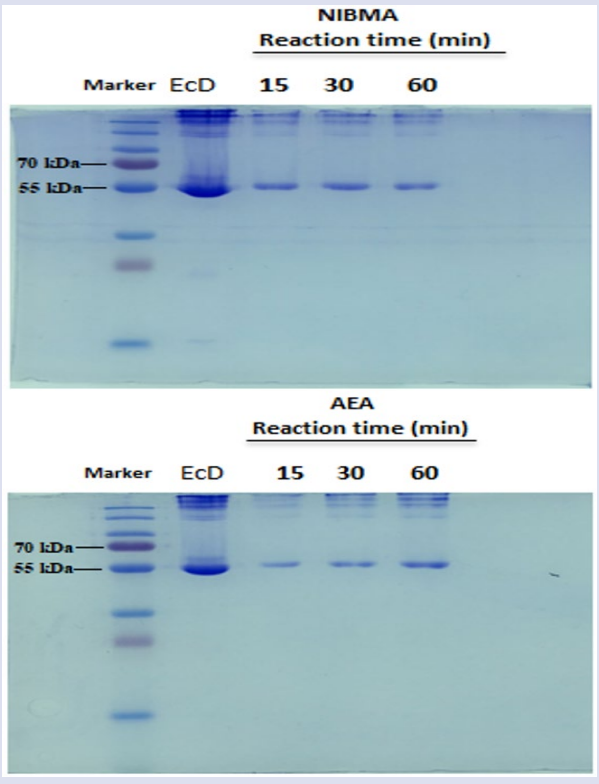


Figure 3. SDS-PAGE images of the obtained bio-conjugates at different reaction time

To evaluate the enzymatic activity of the EcD-pNIBMA and EcD-AEA bioconjugates, the enzyme activity was determined by using N-Acetyl-D-leucine as substrate with a method of 2, 4, 6-trinitrobenzene sulfonic acid (TNBS). TNBS is a highly sensitive and rapid chemical that is used to quantitate the free amino groups. A highly chromogenic product generated by the reaction of TNBS with primary amines was used for monitoring at 335 nm. Enzymatic activities of EcD, EcD-pNIBMA, and EcD-pAEA were measured at 335 nm using UV-visible spectroscopy and also observed changing the colour of substrate from colourless to orange. The activity results of bioconjugates were normalized comparing with enzymatic activity of native D-aminoacylase (EcD) and statistical analysis was carried out between groups using Graphpad 8.0 Prism Software as drawn in Fig 4.

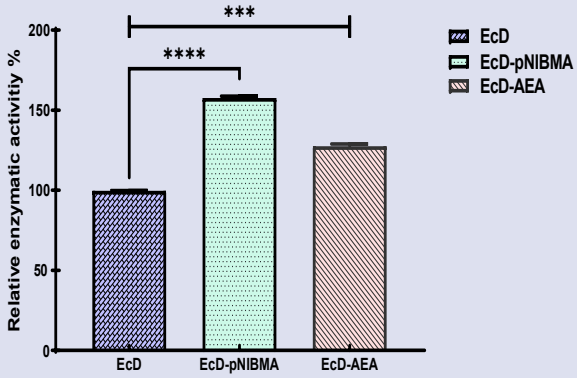


Figure 4. Relative enzymatic activity of native D-aminoacylase and D-aminoacylase-polymer conjugates at a possible ambient condition. Statistically high significance at $p < 0.001$(***) and $p < 0.0001$ (****).

The bioconjugates exhibited high enzymatic activities than native enzyme. Moreover, EcD-pNIBMA occurred using hydrophobic monomer was more efficient than the other and the results were also found statically significant. Our results were similar to the studies in the literature [46]. Polymeric amine groups bounded to proteins increased enzymatic activity by removing water molecules from the hydrophobic regions of the protein, forming a local environment that increases enzyme-substrate-hydrophobic-hydrophobic interactions.

The thermal stabilities of the EcD-pNIBMA, EcD-AEA, EcD were investigated by thermal stability assay. For this reason, each material was subjected to an optimum temperature and an optimum pH by incubating each sample at different times (30, 60, 90,120 min). D-aminoacylase and the bio-conjugates were analyzed using TNBS method, using N-acetyl-D-leucine as substrate. The native enzyme and bioconjugates were incubated for a certain period at specified conditions and then the substrate was added to each group (EcD-pNIBMA, EcD-AEA, EcD) and measured at 335 nm. The results were calculated using Graphpad 8.0 Prims Software as shown in Fig 5. The results revealed that grafting from method

could increase the thermal stability of enzymes. While the thermal stability of the native enzyme decreased linearly depending on the incubation time, it was determined that the grafted enzymes maintained their thermal stability. In addition, comparison of bio-conjugates EcD-pNIBMA was found more stable than EcD-pAEA and EcD-pNIBMA was remained stable as the time-dependent manner while thermal stability of EcD-pAEA was very slightly changed.

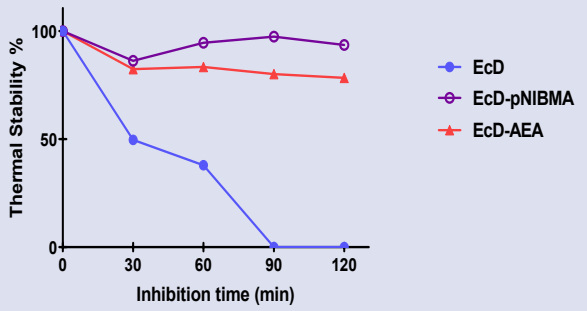


Figure 5. Thermal stabilities of EcD, EcD-pNIBMA and EcD-AEA versus pre-inhibition time.

Conclusions

Well-defined, monodisperse protein-polymer bioconjugates have the potential to be important macromolecules for biotechnology and biomedicine. In particular, polymers could be synthesized by grafting from the method without damaging the structure and function of enzymes, increasing the activities of enzymes and allowing them to work in extreme conditions. In the present study, D-aminoacylase was first produced by the grafting from method and by PET-RAFT polymerization of protein-polymer bioconjugates using two different monomers. The PET-RAFT technique was successfully used to produce modified protein with extended bioactivity at optimum temperature and optimum pH. The PET-RAFT technique offered a versatile and high-throughput approach with effective control over polymerization while allowing the reaction to take place under room conditions. At the same time, the RAFT technique offered the opportunity to compare monomers with different properties by testing. Side chain lengths could be controlled over time for both grafted polymers, pNIBMA and pAEA. Compared with pAEA, pNIBMA was found to be more effective in biological activity. pNIBMA is more hydrophobic than pAEA and leads to better substrate conversion due to its protective effect on the enzyme environment. In addition, the bioconjugates were purified in one step by dialysis without the need for post-polymerization purification, and the absence of the need for serious chemicals makes this method both inexpensive and environmentally friendly. This study reveals that the bioconjugates obtained by the grafting from method using monomers with different properties of D-aminoacylase have increased activity compared to D aminoacylase. Polymers synthesized by PET-RAFT polymerization over D-aminoacylase by grafting from method acted as a thermal shield for D-aminoacylase and it was determined that the thermal stability of bioconjugates increased compared to D-aminoacylase.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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