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Synthesis of Poly(NIPAM) for Efficient Trypsin Purification using Affinity Precipitation Technique

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Abstract

This day, the method of bio specific affinity for separation of polymeric substance from its mixture has gained more attention for further development. The affinity precipitation technique has been continuously refined due to its simplicity, economically appealing and ability to generate high purity product. Moreover, the polymers obtained can be reused and easily scaled up. This study was aimed to utilize ligand pairs for soluble liquid polymers based on macro ligands. The research was conducted in two stages making the resulting carboxylated Poly(NIPAM)-co MPA is ready for enzyme purification testing. First, the synthesis of N-Isopropylacrylamide (NIPAM) polymer, with NIPAM and 2, 2-Azobis-(isobutyronitrile) (AIBN) as fixed variables, while mercaptopropionic acid (MPA) addition was varied. Second, conjugation of the synthesized NIPAM polymer with the PABA ligand, making PABA characterization the changing variable in this phase. The dry weight of carboxylated Poly(NIPAM) obtained was 91.3%, carboxylated Poly(NIPAM)-co MPA 0.4 was 90.4%, and carboxylated Poly(NIPAM)-co MPA 0.6 was 88.9%. The SEM test results showed that the morphological structure of Poly(NIPAM) showed relatively wavier and more porous surfaces than the NIPAM monomer. FTIR test indicated a significant change in the spectra at 3300-2500 cm^{-1}, which some peaks became weaker due to the presence of carboxyl groups characterized in Poly(NIPAM). The spectrophotometer test revealed the LCST condition at a temperature of 40°C. The conjugation of PABA onto Poly(NIPAM)-co-MPA 0.6 with 50 mg PABA showed better conjugation efficiency, with a conjugation yield of 52.6%. This smart polymer is expected to provide an alternative solution for waste water treatment (WWT) and other chemical processing, which employ immobilized enzymes.

Keywords: affinity, Poly(NIPAM), precipitation, synthesis, trypsin

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INTRODUCTION

Trypsins are multifunctional enzymes that catalyze protein hydrolysis into polypeptides, oligopeptides, and amino acids. These enzymes are nearly 60% intact and have been broadly employed in various applications, specifically in the production of detergents, fertilizers, food, pharmaceuticals, textiles, and other bioprocessing products (Lanzalaco & Armelin, 2017). Basically, trypsins can be isolated from plants, animals, and microorganisms. Among these sources, microorganisms show great potential for trypsin production due to their broad biochemical diversity and susceptibility to genetic manipulation. It is predicted that microbial trypsins comprise approximately 40% of the total enzyme sold in the global market (Janser *et al*., 2014).

Biochemical characterization of enzymes provides essential information related to their biotechnological potential. Studies on the trypsin properties, such as substrate specification, optimum pH, temperature, and stability, can be utilized to predict their suitability for specific industrial applications (Janser *et al*., 2015, Halperin *et al*., 2015). Nowadays, the identification of trypsin-producing microorganisms is usually conducted using pure isolates and cultures. Then, further analysis is required to obtain their physiological and biochemical properties (Madani *et al*., 2024). However, the uncultured microorganisms cannot be identified. With advancements in molecular biology, the identification of all organisms can be performed using 16s-rRNA gene analysis because this gene is present in every single organism (Ding *et al*., 2014).

Proteins are one of the most important substances abundantly found in nature because animals, plants, and humans utilize them to support their life. The highly significant roles of proteins have attracted scientists' attention to continually develop more effective and efficient protein separation techniques. In addition to isolation using conventional techniques, enzymes or proteins isolation can also be carried out by utilizing polymer synthesis. Protein-polymer conjugates have long been utilized to manipulate the inherent properties of proteins (Cummings *et al*., 2013). This study aims to utilize ligand pairs for soluble liquid polymers separation based on macroligands that are easily developed for commercial-scale applications.

MATERIALS AND METHODS

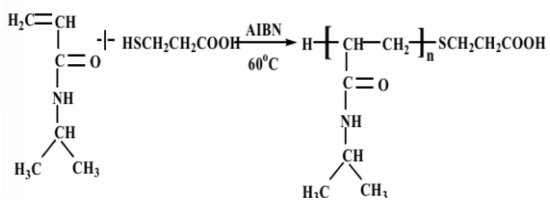
MATERIALS

High purity (≥99.98%) N-Isopropylacrylamide (NIPAM), mercaptopropionic Acid (MPA), 2, 2-Azobis-(isobutyronitrile) (AIBN), Para-aminobenzoic acid (PABA), 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), ethanol, diethyl ether, trypsin, and bovine serum albumin (BSA) with were the products of Sigma-Aldrich (Singapore). Meanwhile, deionized water and other reagents were procured from local chemical store in Banda Aceh, Indonesia.

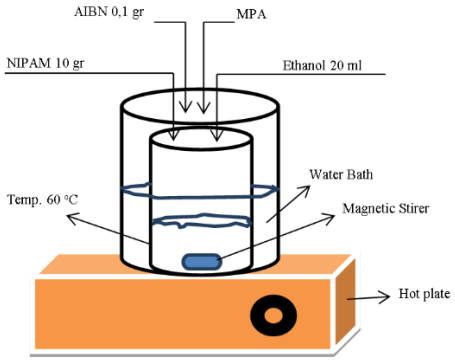
METHODS

NIPAM Polymer Synthesis

Polymer synthesis (Figure 1 (a)) was carried out by dissolving 10 g of N-Isopropylacrylamide (NIPAM) in 20 mL of ethanol in a 250 mL capacity beaker glass. Then, a predetermined mass (according to the ratio) of mercaptopropionic acid (MPA) along with 0.1 g of 2, 2-Azobis-(isobutyronitrile) (AIBN) were added together into the solution. Accordingly, the resulting mixture was incubated at 60°C for 20 hours (Figure 1 (b)). To obtain the precipitate, diethyl ether was carefully added to the solution. At the optimum temperature, the precipitate can be measured at a wavelength of 470 nm using a UV spectrophotometer after heating the polymer solution in water for 10 minutes. The recovery of the dry weight of the precipitate is calculated based on the dry weight of the polymer solution in water at 37°C for 15 minutes.



(a)



(b)

Figure 1. (a) Reaction of NIPAM Polymer Synthesis, (b) Experimental Set-Up

Analytical Procedures

The physical and chemical characteristics and performance of the polymer assays were subjected to the pristine NIPAM and the resulting poly(NIPAM). The accomplishment of the polymerization process to convert the monomer to polymers can be identified based on their functional groups and surface microstructure using FTIR and SEM. Meanwhile, the polymer's precipitation temperature was determined using UV spectrophotometer testing.

Morphology and Surface Microstructure Analysis

The surface morphology of NIPAM polymer was observed at the Geological Survey Central Laboratory in Bandung - Indonesia using a scanning

electron microscope (SEM) employing JEOL JSM 6360LA apparatus (JEOL Ltd, Japan). All samples were tested using an electron beam with a kinetic energy of 10 kV with an image magnification of 10,000×.

Functional Groups Analysis

The functional groups of the polymer were identified using a Fourier Transform Infrared (FTIR) instrument (Shimadzu FTIR 8400, Shimadzu Corporation – Kyoto, Japan). The NIPAM polymer sample was analyzed at wave numbers ranging from 400 cm^{-1} to 4000 cm^{-1}, which transmittance was selected as the response of the resulting spectra.

Lower Critical Solution Temperature (LCST) Analysis

The NIPAM polymer is a polymer that is sensitive to temperature in its solvent, commonly referred to as LCST (Halperin *et al*., 2015, Lee *et al*., 2014). Physically, the LCST is the lowest critical temperature of the solution, where the solution is well-homogenized that functions as the optimal condition for performing the precipitation technique. Therefore, it is necessary to find the LCST of the Poly(NIPAM) solution to achieve optimal precipitate formation during the precipitation process (McFaul *et al*., 2014, Pasparakis & Tsitsilianis, 2020). To determine this precipitation temperature, absorbance testing of the NIPAM polymer was conducted using a UV spectrophotometer.

Para-aminobenzoic acid (PABA) Ligand Conjugation Procedure Against PolyNIPAM

Two grams of NIPAM polymer were dissolved in 15 mL of deionized water with the addition of 450 mg of p-aminobenzamidine (PABA). Subsequently, the pH of the solution was adjusted to 6.5 through a cautious addition of dilute NaOH solution. Then, 550 mg of 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was gradually added in three portions over 12 hours and stirred at room temperature. The PABA-conjugate (Figure 2) was precipitated by raising the temperature above 38°C. The precipitate was washed twice using cold water and once with 10 mM Tris-HCl buffer (pH 8.1). The polymer was dried in an oven at 80°C for 5 hours. The polymer was dissolved in water to obtain a 10% weight/volume solution for storage as a sample for further experiments.

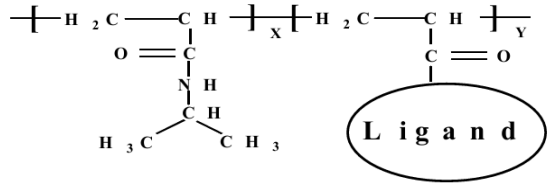


Figure 2. Ligand Conjugation against PolyNIPAM

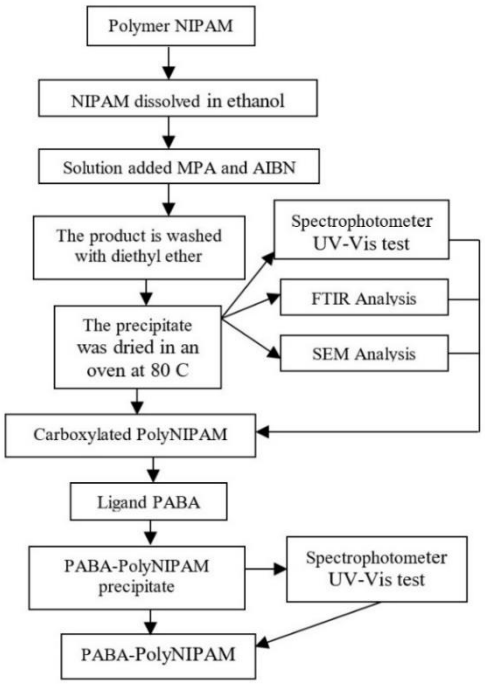


Figure 3. Scheme for synthesis of PolyNIPAM nanoparticles

After obtaining the PABA-polyNIPAM precipitate, the next step was to quantify the amount of PABA bound to poly(NIPAM) using a UV-vis spectrophotometer. It is expected that this quantitative analysis can also indirectly represent a qualitative analysis of the PABA-poly(NIPAM) precipitate sample (Marchenko *et al*., 2016). Finally, the PABA-poly(NIPAM) was analyzed using pure enzymes. All of these experimental steps are illustrated in Figure 3.

Recovery of Trypsin

One milliliter of PABA-polymer solution in water (10%, w/v) was mixed with 1 mL solution containing 48 μg of trypsin and 960 μg of bovine serum albumin (BSA) in 50 mM Tris, 10 mM $CaCl_2$, with a pH of 8.1. Then, the mixture was incubated at 25ºC for 15 min and the precipitate was recovered by centrifugation. The precipitate was dissolved in 2 mL solution containing 50 mM Tris, 10 mM $CaCl_2$, with a pH 8.1 and 50% v/v ethylene glycol. The PABA-polymer was thermoprecipitated and separated by centrifugation. The residual activity of trypsin in the supernatant was assayed according to Erlanger *et al*. (1961) using BAPNA as a substrate for trypsin.

RESULTS AND DISCUSSION

NIPAM Polymer Synthesis

The polymer synthesis of NIPAM, characterized by the addition of MPA, is referred to as carboxylated poly(NIPAM) (Lalita *et al*., 2017). The synthesis of NIPAM polymer used the non-polar solvent ethanol because the initiator used is only soluble in non-polar solvents. The poly(NIPAM) yields achieved in this research were 91.3%, 90.4%,

and 88.9%, for poly(NIPAM) synthesis without addition of MPA, and with the addition of MPA at 0.4 mL and 0.6 mL, respectively. The results show that the addition of MPS decreased the poly(NIPAM) yield. Furthermore, a higher amount of MPS added to NIPAM during poly(NIPAM) synthesis resulted in a lower poly(NIPAM) yield.

Morphology and Surface Microstructure

One way to develop or characterize the mechanical properties of poly(NIPAM) is by substituting organic and inorganic components into the network (Ming *et al*., 2015, Litowczenko *et al*., 2021). The SEM analysis was carried out to examine the effect of MPA concentration variation on the microstructure of Poly(NIPAM).

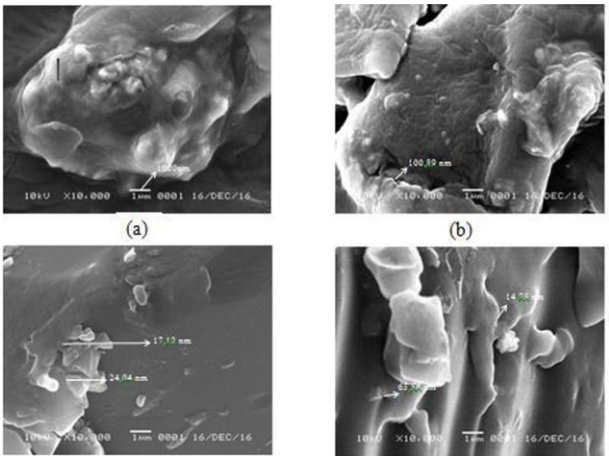


Figure 4. SEM test results

As seen in the micrograph obtained from SEM analysis presented in Figure 4 (a), it is clear that the pristine NIPAM exhibited the existence of particle clumps. Obviously, the particle surface is smooth with some ulcer-like protrusions. However, with the addition of MPA, the morphological structure becomes smoother. This is due to the hydrophilic nature of NIPAM and the oleophilic nature of MPA (Ming *et al*., 2015). As depicted in Figure 4 (b), Poly(NIPAM) with added 0 MPA, demonstrated a relatively flat, rough, wavy, and slightly porous particle surface. Some pores around 100 nm in diameter can be observed at its surface. However, the addition of 0.4 MPA to NIPAM resulted in considerable changes of Poly(NIPAM) particle surface morphology as the particle surface became flatter and smoother with slight waviness (Figure 4 (c)). In can also be observed that the pore size reduced to about 17.12 nm to 24.04 nm. Meanwhile, Figure 4 (d) shows that the addition of 0.6 MPA to NIPAM during Poly(NIPAM) synthesis, shows a similar structure with particles obtained from addition of 0.4 MPA. The particle surface is flat, smooth, and slightly wavier, with varied pore sizes, which are generally smaller (14.75 nm).

As expected, the more amount of MPA molecules are bound to Poly(NIPAM) structure, the smaller the pore size is created leading to the formation of a smoother surface. Therefore, the resulting polymer (denotes as smart polymer) can be able to bind more PABA ligands. Previous research suggested that MPA is an efficient medium to bind PABA ligands, which can be utilized to bind specific enzymes (Stratton *et al*., 2015). Finally, this smart polymer can be applied in waste water treatment and other chemical processing, which employs immobilized enzymes.

Fourier Transform Infrared (FTIR)

The results of the FTIR analysis are displayed in Figure 5.

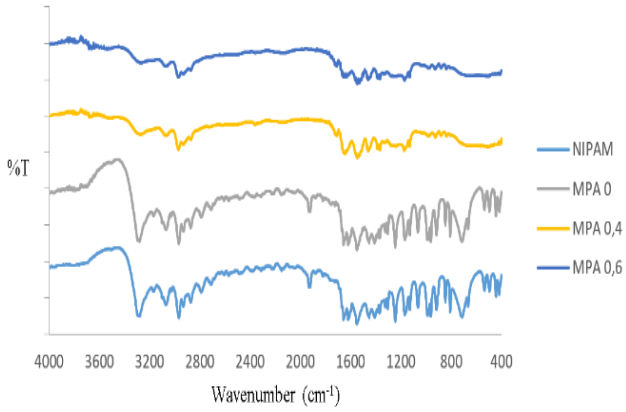


Figure 5. Fourier Transform Infrared (FTIR) Spectra

Figure 5 illustrates the FTIR spectra of carboxylated poly(NIPAM) and NIPAM monomer. The IR spectrum of NIPAM polymer is characterized by specific frequencies: (3300-3100 cm^{-1}) for the N-H bond of a secondary amide, (1675-1665 cm^{-1}) for C=C of alkenes, (2971-2861 cm^{-1}) for C-H bonds in isopropyl groups, and (1650 cm^{-1}) for C=O bonds. Meanwhile, the specific characterization of carboxylated Poly(NIPAM) appears in the spectrum at 3300-2500 cm^{-1} for O-H of carboxylic acid, 1310-1250 cm^{-1} strong for C-O, and 2600-2550 cm^{-1} for S-H. The results agree well with FTIR spectra of carboxylated poly(NIPAM) and NIPAM monomer reported by Ziaee *et al*. (2016).

Figure 5 also exhibits a relatively stronger NIPAM monomer FTIR spectrum compared to the two carboxylated Poly(NIPAM) spectra. This can be seen clearly in the weaker spectrum at 3300-2500 cm^{-1}, which indicates the presence of O-H groups from carboxylic acid, a group found in MPA that has been characterized in Poly(NIPAM) (Maeda *et al*., 2000). Similar findings were also documented by Futscher *et al.* (2017) and Gobeze *et al*. (2020).

LCST Analysis with UV Spectrophotometer

One of the synthesized NIPAM polymers underwent UV spectrophotometer analysis to determine its precipitation temperature. During the UV spectrophotometer measurement, the sample was conditioned at the LCST temperature following the previous procedure carried out by Patrick *et al*. (2017).

Optical Density (OD) measurement was conducted at a wavelength of 470 nm. Before measuring the OD, the polymer solution was heated (for several minutes) in a water bath at varying temperatures, as presented in Figure 6. The LCST value for the Poly(NIPAM)-based copolymer measured during heating through changes in particle size is typically observed at around 35°C (Tomasz *et al*., 2017, Mathieu *et al*., 2024).

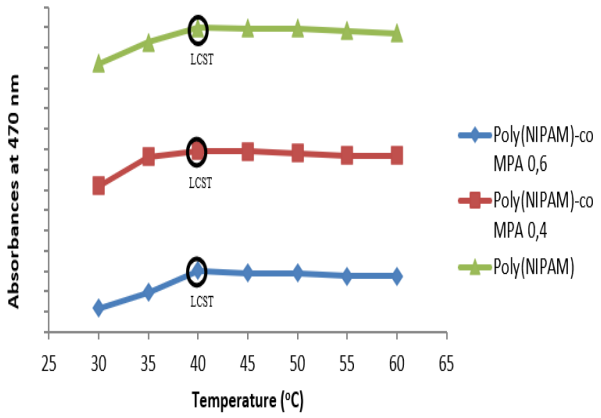


Figure 6. Curve NIPAM Polymer Collaboration

At a wavelength of 470 nm, different absorbance values were obtained based on the system's temperature variations. It should be noted that the LCST value of the copolymer obtained in this research is slightly higher than the average LCST value documented in the literature for NIPAM-based copolymers (Pelton, 2000). Figure 6 shows that the absorbance at 40°C has the highest value compared to other temperatures, while the lowest absorbance is at 30°C. Therefore, Figure 6 indicates that the precipitation temperature of the NIPAM-based polymers synthesized in this research is 40°C. For that reason, to perform the precipitation technique on this polymer, the polymer solution must be conditioned at 40°C.

Conjugation of PABA Ligand on Poly (NIPAM)

Figure 7 displays the physical illustration during conjugation of carboxylated poly(NIPAM) with PABA ligand. The purpose of this ligand conjugation is to facilitate the binding or capture of specific enzymes that will later bind directly and specifically to PABA.

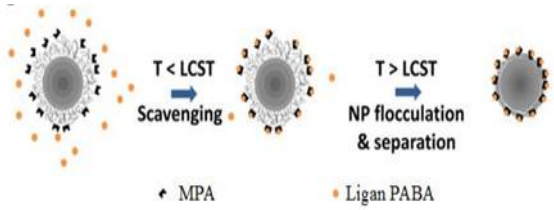


Figure 7. Conjugation of PABA Ligand

At the LCST condition, carboxylated poly(NIPAM) binds and pairs with its ligand. Above the LCST, a coagulation process occurs. Based on the smart polymer properties mentioned, the engineering of PABA-poly(NIPAM) follows and utilizes these three conditions. The preparation of the PABA-poly(NIPAM) solution was conducted below the LCST condition to ensure a perfectly homogenized solution. Then, the temperature of the solution was raised to the LCST condition (40°C). At this condition, it is expected that a significant amount of PABA ligand will bind to carboxylated poly(NIPAM). Next, the coagulation process is carried out at a temperature above the LCST, which was at 60°C (Schmidt *et al*., 2008).

The calibration curve of p-Aminobenzamidine is shown in Figure 8. The conjugation of PABA was analyzed using a UV spectrophotometer at a wavelength of 280 nm. The conjugation with NIPAM was performed using 50 mg and 450 mg of PABA at a pH of 6.5. The precipitate obtained was tested using a UV spectrophotometer by dissolving 10 mg of the precipitate in 8 mL of distilled water.

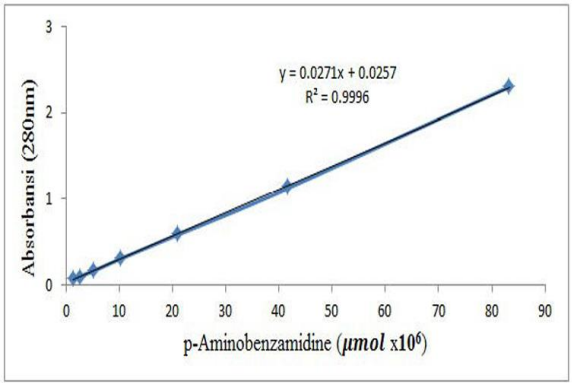


Figure 8. The PABA Calibration Curve

Based on the calibration curve displayed in Figure 8, testing was conducted on carboxylated poly(NIPAM) to determine the amount of conjugated PABA ligand. The percentage of conjugated PABA are summarized in Table 1.

Table 1. The percentage of conjugated PABA

No.	Sample	PABA (mg)	Conjugation Yield (%)
1	Poly(NIPAM)co-MPA 0.4	50	42.3
2	Poly(NIPAM)co-MPA 0.6		52.6
3	Poly(NIPAM)co-MPA 0.4	150	46.9
4	Poly(NIPAM)co-MPA 0.6		54.2
5	Poly(NIPAM)co-MPA 0.4	250	46.2
6	Poly(NIPAM)co-MPA 0.6		55.6

Table 1 shows that irrespective to the amount of PABA addition to the Poly(NIPAM)co-MPA 0.4

and Poly(NIPAM)co-MPA 0.6, nearly the same yield of PABA can be conjugated, with only a slight increase. This is because the amounts of 150 mg and 250 mg of PABA are too large to bind to 2 g of carboxylated poly(NIPAM). In terms of efficiency, the use of 50 mg of PABA is more advantageous than both 150 and 250 g of PABA. The resulting PABA-poly(NIPAM) can be used for enzyme exploration. After ligand conjugation, PABA-poly(NIPAM) can be tested against specific enzymes. Specific and commercial testing of poly(NIPAM) in waste treatment can be conducted in future studies. Figure 9 illustrates the specific enzyme exploration on PABA-poly(NIPAM).

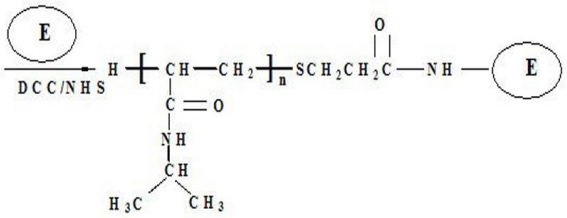


Figure 9. The enzyme is absorbed/binds to the ligand on poly(NIPAM)

CONCLUSION

Based on the research conducted, the dry weight of carboxylated Poly(NIPAM) obtained was 91.3%, carboxylated Poly(NIPAM)-co MPA 0.4 was 90.4%, and carboxylated Poly(NIPAM)-co MPA 0.6 was 88.9%. Poly(NIPAM) characterized with MPA showed a smoother and more even surface morphology compared to the NIPAM monomer and the pristine polymer. Meanwhile, the characterization of Poly(NIPAM) resulted in a weaker spectrum at 3300-2500 cm^{-1}. This is due to the carboxyl O-H group of MPA being substituted onto Poly(NIPAM). Other characterizations were also observed at 3300-2500 cm^{-1} for the O-H carboxylic acid, 1310-1250 cm^{-1} strong for C-O, and 2600-2550 cm-1 for S-H. Meanwhile, the LCST of the Poly(NIPAM) solution was found at a temperature of 40°C. The conjugation of PABA onto Poly(NIPAM)-co-MPA 0.6 with 50 mg of PABA showed better conjugation efficiency, with a conjugation yield of 52.6%. Thermoprecipitating of carboxylated NIPAM polymer was synthesized and conjugated with paminobenzamidine. Finally, further studies should be focused to the testing and scale-up of the conjugated PABA-poly(NIPAM) against specific enzymes, specifically for application in waste treatment and other chemical processing, which employ immobilized enzymes.

REFERENCES

Cummings, C., Murata, H., Koepsel, R., and Russell, A. J., (2013), Tailoring Enzyme Activity and Stability using Polymer-based Protein Engineering, Biomaterials, 34(30), pp. 7437–7443.

Ding, Z., Kang, L., and Cao, X., (2014), Application of Docking Methods for Metal Chelate Affinity Precipitation of Endo-Glucanase using Ph-Response Polymer. Colloids and Surfaces B: Biointerfaces, 113, pp. 412–420.

Erlanger, B.F., Kokowsky, N., and Cohan, W., (1961), The Preparation and Properties of Two New Chromogenic Substrates of Trypsin, Archieves of Biochemistry and Biophysics, 95, pp. 271–278.

Futscher, M.H., Philipp, M., Müller-Buschbaum, P., and Schulte, A., (2017), The Role of Backbone Hydration of Poly(N-isopropyl acrylamide) Across the Volume Phase Transition Compared to its Monomer, Scientific Reports, 7, 17012

Gobeze, H. B., Ma, J., Leonik, F. M., and Kuroda, D. G., (2020), Bottom-Up Approach to Assess the Molecular Structure of Aqueous Poly(N-Isopropylacrylamide) at Room Temperature via Infrared Spectroscopy, The Journal of Physical Chemistry B, 124 (51), pp. 11699–11710.

Halperin, A., Kroger, M., and Winnik, F.M., (2015), "Poly (N-isopropylacrylamide) phase diagrams: fifty years of research,", Angewandte Chemie International Edition, 54(51), pp. 15342–15367.

Janser, R, Soares, D. C., Tânia, G. N., and Hélia, H. S., (2014), Biocatalysis and Agricultural Biotechnology Production and Biochemical Properties of Trypsins Secreted by Aspergillus Niger under Solid State Fermentation in Response to Different Agroindustrial Substrates, Biocatalysis and Agricultural Biotechnology, 3(4), pp. 236–245.

Janser, R, Soares, D. C., Tânia, G. N., and Hélia, H. S., (2015), Biocatalysis and Agricultural Biotechnology a Versatile System Based on Substrate Formulation Using Agroindustrial Wastes for Trypsin Production by Aspergillus Niger under Solid State Fermentation, Biocatalysis and Agricultural Biotechnology, 4(4), pp. 678–684.

Lalita, Singh., A.P., and Sharma, R.K., (2017), Synthesis and Characterization of Graft Copolymer of Chitosan with NIPAM and Binary Monomers for Removal of Cr(VI), Cu(II), and Fe(II) Metal Ions from Aqueous Solutions. Biological Macromolecules, 99, pp. 409–426.

Lanzalaco, S., and Armelin, E., (2017), Poly(N-isopropylacrylamide) and Copolymers: a Review on Recent Progresses in Biomedical Applications, Gels, 3 (4), pp. 36.

Lee, Y., Hong, M. C., Ahn, H., Yu, J., and Rhee, H., (2014), Pd Nanoparticles Immobilized on Poly (NIPAM-Co-4-VP) Hydrogel: Highly Active and Reusable Catalyst for Carbon–Carbon Coupling

Reactions in Water, Journal of Organometallic Chemistry, 769, pp. 80–93.

Litowczenko, J., Gapiński, J., Markiewicz, R., Woźniak, A., Wychowaniec, J. K., Peplińska, B., Jurga, S., and Patkowski, A., (2021), Synthesis, Characterization and In Vitro Cytotoxicity Studies of Poly-N- Isopropyl Acrylamide Gel Nanoparticles and Films, Materials Science & Engineering C, 118, pp. 111507.

Madani, S., Nikfarjam, N., Mamipour, Z., Kompany-Zareh, M., (2024), Preparation of a Surface Molecularly Imprinted Thermo-Sensitive Polymer for Selective Trypsin Capture, Microchemical Journal, 206, pp. 111530.

Maeda, Y., Higuchi, T., and Ikeda, I., (2000), Change in Hydration State During the Coil−Globule Transition of Aqueous Solutions of Poly (N-isopropylacrylamide) as Evidenced by FTIR Spectroscopy, Langmuir, 16(19), 7503–7509.

Marchenko, N.Y., Sikorskaya, E.V., Marchenkov, V.V., Kashparov, I.A., and Semosotnov, G.V., (2016), Affinity Chromatography of Chaperones Based on Denatured Proteins: Analysis of Cell Lysates of Different Origin. Protein Expression and Purification, 119, pp. 117–123.

Mathieu, C., Issa, S., and Richaud, E., (2024), Effect of isothermal aging on Poly(N-isopropylacrylamide): Correlation between chemical structure and phase transition temperature property, Materials Today Chemistry, 38, pp. 102127.

McFaul, C.A., Drenski, M.F., and Reed, W.F. (2014), Online, Continuous Monitoring of the Sensitivity of the LCST of NIPAM-Am Copolymers to Discrete and Broad Composition Distributions, *Polymer*, 55(19), pp. 4899–4907.

Ming, L. Z., Qu, L. L., Kang, Z. M., Yi, F. M., and Yong, J., (2015), Preparation and Properties of Controllable Amphiphilic P(NIPAM-co-LMA) Gel for Drug Delivery, Journal of Drug Delivery Science and Technology, 29, pp. 245–250.

Pasparakis, G. and Tsitsilianis, C., (2020), LCST Polymers: Thermoresponsive Nanostructured Assemblies Towards Bioapplications, Polymers, 211, pp. 123146.

Patrick, A.L., Adam, P.C., Larry, F.T., and Nicolas, C.P. (2017), Effects of ESI Conditions on Kinetic Trapping of The Solution-Phase Protonation Isomer of p-aminobenzoic Acid in the Gas Phase. International Journal of Mass Spectrometry, 418, pp. 148–155.

Pelton, R., (2000), Temperature-sensitive aqueous microgels, Advances in Colloid and Interface Science, 85, pp. 1-33.

Schmidt, S., Hellweg, T., and Klitzing, R.V., (2008), Packing Density Control in P(NIPAM-co-AAc) Microgel Monolayers: Effect of Surface Charge, pH, and Preparation Technique, Langmuir, 24(21), pp. 12595–12602.

Stratton, C.F., Namanja-Magliano, H. A., Cameron, S. A., and Schramm, V. L., (2015), Binding Isotope Effects for para-Aminobenzoic Acid with Dihydropteroate Synthase from *Staphylococcus aureus* and *Plasmodium falciparum*, ACS Chemical Biology, 10 (10), pp. 2182–2186.

Tomasz, Ś., Maciej J., Ewa A., Mikołaj S., and Jacek, G., (2017), Uptake and Controlled Release of a Dye from Thermo-sensitive Polymer P(NIPAM-co-Vim), Reactive and Functional Polymers, 115, pp. 102–108.

Ziaee, M., Zahedi, P., Abdouss, M., Zarandi, M. A., Manouchehri, S., and Mozdoori, N., (2016), Electrospun poly (N-isopropylacrylamide- co -acrylic acid)/cellulose laurate blend nanofibers containing adapalene: Morphology, drug release, and cell culture studies, International Journal of Polymeric Materials and Polymeric Biomaterials, 65 (9), pp. 477– 486.