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Complete List of Authors:	Kovaliov, Marina; Allegheny Health Network, Neuroscience Disruptive Research Lab Cheng, Cooper; Allegheny Health Network, Neuroscience Distuptive Research Lab Cheng, Boyle; Allegheny Health Network, Neuroscience Institute Averick, Saadyah; Allegheny Health Network, Neuroscience Distuptive Research Lab

Polymer Chemistry

PAPER

Grafting-From Lipase: Utilization of a Common Amino Acid Residue as a New Grafting Site

Marina Kovaliov,[a,b] Cooper Cheng,[a,b] Boyle Cheng,[b] and Saadyah Averick[a,b*]

Protein-polymer hybrids are used in a variety of fields including catalysis, detection, and therapeutics. The grafting-from method for the synthesis of these biohybrids has gained popularity due to the ease of synthesis and purification. In this method, an initiator or chain transfer agent (CTA) is ligated onto an amino acid residue, typically lysine or cysteine, and polymers are subsequently grown *in situ*. In this manuscript, we report the preparation of protein polymer hybrids by grafting-from a previously overlooked acidic amino acid residue (glutamic and aspartic acid) and compare our results to protein polymer hybrids, grafted from the traditional lysine residue. Herein, we conjugated an atom transfer radical polymerization (ATRP) initiator to acidic amino acid residues and lysine residues and grew polymers from Thermomyces lanuginosa lipase (TL). *N*-[3-(*N*,*N*-Dimethylamino)propyl] acrylamide was grafted from the TL initiator, and the enzymatic activity of protein polymer hybrids was compared. We found that the acidic residues are easily modified with multiple ATRP initiators and polymers are readily grown. Additionally, the hybrids grafted from acidic residues demonstrated a 50% increase in enzyme activity compared to those grafted from lysine residues. Moreover, the activity was higher than that of native lipase TL in both cases. The polymers that were grafted-from the acid residues tended to provide the hybrids with a higher activity at elevated temperatures. These results point to a new amino acid ligation strategy for preparing protein polymer hybrids *via* a grafting-from method.

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Introduction

Protein-polymer hybrids are an important class of biomaterials with applications in sensors, diagnostics, drug delivery vehicles and catalysts.[1-26] These biomaterials may be prepared using the grafting-to method wherein preformed polymers are attached to a protein, or by the grafting-from method,[27-31] by directly growing a polymer from polymerization initiators conjugated to a protein. Each method has distinct advantages and disadvantages. However, with regards to synthesis, purification, and yields, the grafting-from method generally exhibits greater yields and higher polymer per protein loading. This is due to the relatively lower steric effects from growing a small molecule (monomer) from a macromolecule (protein).[27-29] A key advantage of having a higher polymer per proteins loading is the ability to extend the use of enzymes from native to non-native environments, as a more dense polymer shell helps to stabilize and solubilize enzymes under non-native conditions.[29, 30, 32]

The grafting-from approach, both reversible-deactivation radical polymerization (RDRP) and ring opening metathesis polymerization (ROMP),[33] have been used to graft polymers from proteins under biologically relevant conditions (BRC). The BRC strategy is designed to preserve the structure, functionality, and utility of the protein. Functional enzyme polymer hybrids have been prepared under BRC, using Atom transfer radical polymerization (ATRP) and low [Cu] ATRP methods such as initiators for continuous activator regeneration (ICAR) and activators regenerated by electron transfer (ARGET) ATRP.[34, 35] Reversible addition-fragmentation chain transfer (RAFT)[36, 37] and metal free photoinduced electron transfer (PET) RAFT polymerization,[32, 38] have also been used to grow polymers from proteins. Both RDRP methods provide access to functional biohybrids.[29]

[a] *Neuroscience Disruptive Research Lab, Allegheny Health Network Research Institute, Allegheny General Hospital, Pitts-burgh, Pennsylvania 15212, USA.*
[b] *Neuroscience Institute, Allegheny Health Network, Allegheny General Hospital, Pittsburgh, Pennsylvania 15212, USA.*
** E-mail: Saadyah.Averick@ahn.org; Tel:+1-412-359-4943.*
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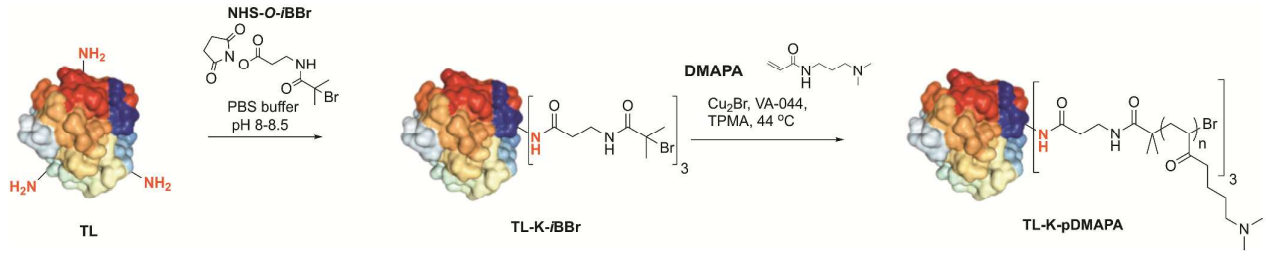


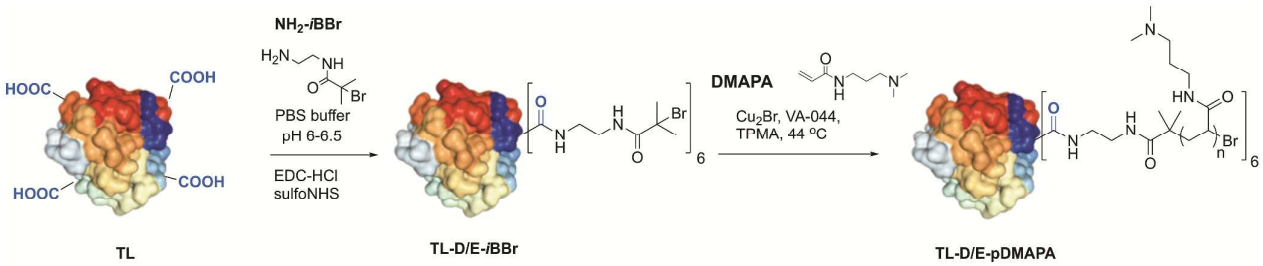
Scheme 1. Synthetic pathway to obtain the lipase TL-K-*i*BBr and subsequent grafting-from polymerization using ICAR ATRP to generate polymer-protein conjugates TL-K-pDMAPA.

The grafting-from strategy requires the covalent ligation of ATRP initiator or chain transfer agent (CTA), to a protein surface. The desired amino acid reactive group should be freely accessible on the protein surface for initiator/CTA ligation, and modification must not affect the three-dimensional structure of the protein, to maintain its native state, binding pockets or recognition areas.[2] The lysine side chain is most commonly modified amino acid residue,[39] due to its relative selective coupling with activated esters on a protein surface.[7, 38, 40] Cysteine residues, although less abundant on protein surfaces, can be readily reacted with an alkene, i.e., acrylate or maleimide bearing an initiator or CTA moiety.[27, 41-43] The N- or C-terminal groups can be selectively modified using appropriate pH to yield one polymer per protein.[40, 44, 45] In addition the N-terminal amine, when solvent accessible, more likely to be modified due to lower pKa (7.8) during the modification of the amine group present on lysine side chains (pKa 10.5–12) that are typically distributed on the protein surface.[46-50] The genetic introduction of non-canonical amino acid moieties, such as initiator for ATRP, is much less common.[51] The acid bearing residues, such as glutamic and aspartic acid, are common residues that are seemingly overlooked for bioconjugation. A literature review reveals limited references for PEGylation *via* the grafting-to strategy, with no work on grafting-from this relatively abundant amino acid.[39, 40] Linking polymers to acidic amino acid residues, may provide a method to increase the grafting density of polymers onto proteins. In particular, in cases where lysine modification on the surface of protein could lead to enzyme inactivation, or those proteins unstable to the alkaline conditions required for the conjugation.

Lipase is an enzyme with medical and industrial importance, due to its vital role in the metabolism of lipids, and in the hydrolysis variety of esters and amides.[52-55] Recent research has demonstrated the viability of a lipase derived from *Thermomyces lanuginose* (TL) to serve as a model enzyme for grafting-from. The lysine residues on TL were modified with trithiocarbonate CTA groups and various monomers were grafted-*from* using PET-RAFT.[32] The resultant biohybrids demonstrated differing activity depending on polymer grafting density and polymer composition. Furthermore, the availability of a colorimetric enzyme activity assay facilitates the rapid evaluation of biohybrid activity.

Here, TL lipase[56] was selected as a model system to explore the effect of polymer grafting-from ATRP initiator (-*i*BBr) on acidic and basic amino acid residues. For this purpose, we modified TL lipase with ATRP initiators either at the amine side chain of lysine (K) or acid residues of aspartic (D) and glutamic (E) amino acids, and *N*-[3-(*N,N*-Dimethylamino)propyl] acrylamide (DMAPA) was grafted-from it using ICAR ATRP. ICAR ATRP in aqueous media has advantages over other low copper ATRP methods, such as ARGET ATRP, because it does not require the continuous infusion of reducing agents, simplifying the experimental procedures.[34, 35]

Protein activity was assessed using a colorimetric-based hydrolysis assay under normal and elevated temperature. To minimize possible confounding variables, a two-carbon spacer



Scheme 2. Synthetic pathway to obtain the lipase TL-D/E-*i*BBr and subsequent grafting-from polymerization using ICAR ATRP to generate polymer-protein conjugates TL-D/E-pDMAPA.

between the protein and ATRP initiator was used as an initiator, which was ligated to both lysine and acid residues (Scheme 1 and 2). We not only found that the acidic residues were able to be modified with ATRP initiators, but the grafted biohybrids demonstrated retained hydrolytic activity. These results indicate that these residues should be examined for use in other enzymes and as a part of the design of biohybrid materials.

Experimental section

Materials and Instrumentations

All materials were used without further purification. *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (EDC·HCl, 98%) were purchased from Carbosynth. Lipozyme® TL was purchased from Strem. *N*-[3-(*N,N*-Dimethylamino)propyl] acrylamide (DMAPA) was purchased from Polysciences Inc and was passed through basic alumina to remove inhibitors prior to polymerization. Solvents and deuterated solvents were obtained from Worldwide Medical Products. SDS-poly(acrylamide) gel electrophoresis was performed using Bio-Rad Mini PROTEAN TGX 4-20% gradient gels. GelCode Blue protein stain was obtained from Thermo Scientific. Unmodified protein concentrations were determined using a Direct Detect® FTIR spectrometer (Millipore). Water was deionized with a Millipore system as a Milli-Q grade. Monomers were passed over a column of basic alumina prior to use to remove the inhibitor. 1×PBS (Phosphate buffered saline) = 2 mg/mL KCl , 2 mg/mL KH_2PO_4, 80 mg/mL NaCl, 11.5 mg/mL Na_2HPO_4. ^{1}H NMR spectra were recorded on a Bruker Advance 500 MHz NMR spectrometer using the residual solvent signal as a reference. UV-vis spectra were collected on a DeNovix DS-11 spectrophotometer.

Synthesis of TL-K-*i*BBr

Lipase TL was dissolved in PBS buffer pH 8-8.5, at concentration 10 mg/mL (2.5 mL, 25 mg lipase, 0.8 µmol). Then, NHS-*O*-*i*BBr (55 mg, 0.16 mmol, 200 equiv.) was dissolved in 0.5 mL of DMSO and slowly added of the enzyme solution. The reaction was stirred at room temperature for 4 h. The crude reaction mixture was purified by dialysis (25k MWCO dialysis membrane Spectrum Labs) against 1×PBS (50 mM, pH 7.4), at 4 ^{o}C for 24 h to remove unreacted reagents.

Synthesis of TL-D/E-*i*BBr

Lipase TL was dissolved in PBS buffer pH 6-6.5, at concentration 5 mg/mL (5 mL, 25 mg lipase, 0.8 µmol). Then, NH_2-*i*BBr (35 mg, 0.16 mmol, 200 equiv.) was dissolved in 1 mL of buffer and slowly added to the enzyme solution. (7 mg, 0.032 mmol, 5 mM final) sulfo-NHS (*N*-hydroxysulfosuccinimide) and EDC-HCl (60 mg, 0.32 mmol 0.05 mM final) in 0.5 mL, of water was added to the enzyme solution. The reaction was stirred at room temperature for 4 h. The crude reaction mixture was purified by dialysis (25k MWCO dialysis membrane Spectrum Labs) against 1×PBS (50 mM, pH 7.4), at 4 ^{o}C for 24 h to remove unreacted reagents.

ICAR ATRP from TL-K-*i*BBr

TL-K-*i*BBr (4 mg/mL) 1.67 mL (6.7 mg (protein), 0.20 µmol (0.60 µmol initiator, 0.35 mM)), monomer DMAPA (140 mM, 0.24 mmol, 40 µL), CuBr2 (0.23 mg, 0.00096 mmol) TPMA (0.61 mg, 0.00044 mmol) (0.066 mL of 6.4 mg/ml stock solution of CuBr2 and 18.4 mg/mL TPMA in DMF) and VA-044 (0.067 mg, 0.00019 mmol) (0.066 mL of 1 mg/ml stock solution in DDW) were added to 5 mL microwave vial, total volume of 1.85 mL. The reaction mixture was purged with N_2 for 20 minutes then placed in a water bath at 44 ^{o}C for 15, 30, 45, 60, 75 and 90 min. ^{1}H NMR measurement of monomer conversion (DMF standard). The crude reaction mixture was purified by dialysis (30k MWCO centricon) against 1×PBS (50 mM, pH 7.4), to remove unreacted reagents.

ICAR ATRP from TL-D/E-*i*BBr

TL-D/E-*i*BBr (3 mg/mL) 1.67 mL (5 mg (protein), 0.16 µmol (0.95 µmol initiator 0.40 mM)), monomer DMAPA (163 mM, 0.38 mmol, 60 µL), $CuBr_2$ (0.32 mg, 0.00144 mmol, TPMA (0.92 mg, 0.00067 mmol) (0.1 mL of 6.4 mg/ml stock solution of $CuBr_2$ and 18.4 mg/mL TPMA in DMF) and VA-044 (0.1 mg, 0.00029 mmol) (0.1 mL of 1 mg/ml stock solution in DDW) were added to 5 mL microwave vial. 400 µL of phosphate buffer was added to total volume of 2.33 mL. The reaction mixture was purged with N_2 for 20 minutes then placed in a water bath at 44 ^{o}C for 15, 30, 45, 60, 75 and 90 min. ^{1}H NMR measurement of monomer conversion (DMF standard). The crude reaction mixture was purified by dialysis (30k MWCO centricon) against 1×PBS (50 mM, pH 7.4), to remove unreacted reagents.

Gel Permeation Chromatography (GPC)

Aqueous GPC analyses were conducted in 0.05 M aqueous Na_2SO_4/acetonitrile (80:20) at a flow rate of 1 mL/min (Viscotek GPCMax VE 2001 module; columns: TSKgel PWXL guard column (Tosoh) + TSKgel G4000PWXL analytical column (Tosoh; 7.8 mm × 30 cm, 10 µm particle size)).

Matrix-Assisted Laser Desorption/Ionization Mass Spectroscopy (MALDI-ToF-MS)

MALDI-ToF data acquisition was performed on a Bruker AutoFlexIII MALDI-ToF mass spectrometer. All samples were mixed with the sinapinic acid (SA) matrix. Samples were analyzed in the positive ion linear mode to detect $[M+H]^+$ ions. In general, 3.0 µL of sample (1 mg/mL) was mixed with 3.0 µL of saturated sinapinic acid solution (0.1% TFA, 40%

acetonitrile) and 1 µL was spotted directly on the target plate and allowed to dry at room temperature.

Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were cast and run using Mini-PROTEAN® systems (Bio-Rad), TGX 4-20% gradient gels. Samples were prepared in SDS-PAGE sample buffer containing 1% DTT and heated 10 min to denature proteins prior to running at 100 V for 1 h to separate the proteins, which were visualized with GelCode™ Blue staining.

In Gel Digestion

5µg of the sample was processed by SDS-PAGE using a 4-12% Bis-Tris NuPage Mini-gel with the MOPS buffer system. The target bands were excised and processed by In-gel digestion with trypsin using a ProGest robot (DigiLab) using manufacturer recommended protocol. Briefly, bands were trimmed as close as possible to minimize background polyacrylamide material. Gel pieces were washed with 25mM ammonium bicarbonate followed by acetonitrile. The proteins were reduced with 10mM dithiothreitol at 60°C followed by alkylation with 50mM iodoacetamide at RT. The proteins were digested with trypsin (Promega) at 37°C for 4h. The digestion was stopped by quenching with formic acid and analyzed directly without further processing.

Nano-Liquid chromatography-tandem mass spectrometry (Nano-LC-MS/MS)

Half of each gel digest was analyzed by nano LC-MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. Peptides were loaded on a trapping column and eluted over a 75µm analytical column at 350nL/min; both columns were packed with Luna C18 resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with the Orbitrap operating at 70,000 FWHM and 17,500 FWHM for MS and MS/MS respectively. The fifteen most abundant ions were selected for MS/MS.

Sequence analysis

Data were searched using a local copy of Mascot with the following parameters: Enzyme: Trypsin/P; Database: UniProt Trichocomaceae (concatenated forward and reverse plus common potential contaminants); Mass values: Monoisotopic; Peptide Mass Tolerance: 10 ppm; Fragment Mass Tolerance: 0.02 Da; Max Missed Cleavages: 3; The elemental formula for the target ligands, after excluding the contribution from the acceptor amino acid are *Br C(6) H(11) N(2) and **Br C(7) H(10) N O(2) Mascot DAT files were parsed into Scaffold (Proteome Software) for validation, filtering and to create a non-redundant list per sample. Data were filtered at 1% protein and peptide FDR and requiring at least two unique peptides per protein. Scaffold results were exported as mzIdentML and imported into Scaffold PTM in order to assign site localization probabilities using A-Score,[57] a minimum localization filter of 50% was applied.

Assay of lipolytic activity and thermal stability of lipase TL and lipase-polymer hybrids

Lipolytic activity was determined spectrophotometrically by using *p*-nitrophenyl palmitate (*p*-NPP) as substrate. *p*-NPP stock solution (3 mM) was prepared. First, *p*-NPP was dissolved in 3 mL isopropanol and 50 mL of 0.01 M potassium phosphate buffer (pH=7.4) (3% Triton X-100) was added. A volume of 50 µL of buffered *p*-NPP solution was added to 10 µL enzyme solution and incubated for 20 min at 37 °C. The *p*-nitrophenol (*p*-NP) release was monitored at 405 nm in 96-well Nunc clear polystyrene plate on a BioTek Cytation 3 plate reader. A blank sample was always used containing distilled water instead of enzyme solution. The molar absorption coefficient of *p*-NP ($\varepsilon=1.2475\cdot10^4$ M^{-1} cm^{-1}) was estimated from the absorbance of *p*-NP standard solutions measured at 405 nm. Lipase activity was defined as the concentration of the product *p*-NP (µmol) per min, per mg of enzyme.

The optimal temperature for both native TL and the polymer hybrids was studied at the range from 30 to 70 ^{o}C for 20 min. A volume of 50 µL of buffered *p*-NPP solution which was incubated for 5 min at different temperatures was added to 10 µL enzyme solution. The lipase activities under different temperatures were measured as described above.

Thermal stability was investigated by monitoring the concentration of *p*-NP formed at different temperatures. The thermal inactivation was carried out by using 1 mg/ml solution of lipase and lipase-protein conjugate in 1×PBS pH 7.4 and incubating the solution at 25, 37, and 50 °C. Samples of the solution were withdrawn at 1, 3, 5, 10, 24 and 48 hours, and their activities were determined.

Determination of TL-pDMAPA hybrids solubility in organic solvents

The solubility of lipase TL, TL-K-pDMAPA and TL-D/E-pDMAPA was determined by quantifying the total amount of protein using the Direct Detect® FTIR spectrometer. 200 µL of Lipase TL (1.5 mg/mL), TL-K-pDMAPA (3.2 hybrid mg/mL, 1.5 enzyme mg/mL) and TL-D/E-pDMAPA (3.6 hybrid mg/mL, 1.5 enzyme mg/mL) in buffer solution were lyophilized and resuspended in water and organic solvents (DMF, methanol, ethanol, acetonitrile, dichloromethane and tetrahydrofuran). The mixture was stirred overnight at 4 ^{o}C and filtered. The comparative solubility of lipase TL was determined by measuring the concentration of the sample solution using Direct Detect® FTIR spectrometer and comparing it to solubility in aqueous media.

Results and Discussion

Initiator ligation to lipase

Table 1. Grafting from Lipase TL using ICAR ATRP polymerization.[a]

TL-polymer hybrid	Reaction time (min)	Conv.[b] (%)	*biohybrid*		*polymer*	
			$M_{n,\text{theory}}$[c] (kDa)	$M_{n,\text{expt}}$[d] (kDa)	$M_{n,\text{theory}}$[c] (kDa)	$M_{n,\text{expt}}$[d] (kDa)
TL-K-pDMAPA	15	48	59	50	9.3	6.5
	30	62	67	56	12	8.3
	45	74	76	58	15	8.8
	60	77	78	61	15.3	9.9
	75	80	80	67	16.5	12
	90	81	81	68	16.7	12.1
TL-D/E-pDMAPA	15	40	56	53	4	3.7
	30	46	59	60	4.5	4.7
	45	53	64	63	5.3	5.3
	60	60	68	65	6	5.5
	75	66	72	69	6.7	6.1
	90	69	75	76	7.1	7.4

[a]Conditions: [DMAPA]:[TL-*i*BBr]:[$CuBr_2$]:[TPMA]:[VA-044] = 400:1:3:6.6:0.3; [TL-K-iBBr]=0.35 mM, [TL-D/E-iBBr]=0.40 mM,. [b]Monomer conversions were calculated using ^{1}H NMR spectroscopy. [c]$M_{n,\text{theory}}$ (theoretical molecular weight of the *biohybrid*) = [M]/[TL-iBBr]×conv.%×MW_M+$MW_{TL\text{-}iBBr}$. [d]$M_{n,\text{expt}}$ were calculated using UV-Vis spectroscopy and Direct Detect® FTIR spectrometer (see SI).

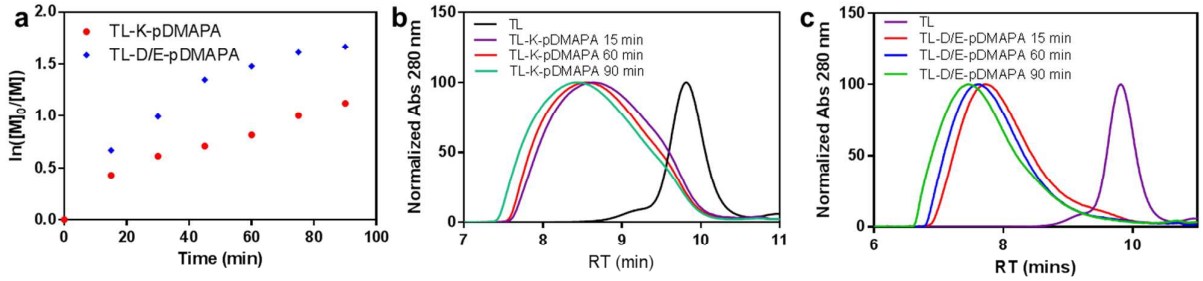


Figure 1. Polymerization of DMAPA by grafting from iBBr-modified lipase TL. (a) Kinetics plots for the polymerization of DMAPA from TL-K-*i*BBr and TL-D/E-*i*BBr GPC chromatograms of lipase-polymer hybrids, TL-K-pDMAPA and TL-D/E-pDMAPA (GPC was conducted in 0.05 M aqueous Na_2SO_4/acetonitrile (80:20) as the eluent, and the normalized refractive index (RI) signals were plotted versus elution time) (b) TL-K-pDMAPA (c) TL-D/E-pDMAPA.

To prepare lysine residue modified TL, TL-K-*i*BBr, an *N*-hydroxy succinimide amide based ATRP initiator (NHS-*O*-*i*BBr), that was synthesized according to the previously published procedure (see Supporting Information for detailed synthesis), was mixed with TL at pH 8.5 for four hours at room temperature and purified using dialysis (Scheme 1). MALDI-ToF mass spectroscopy indicates that three initiators are attached per lipase (Figure S1-S3), A range of reaction conditions were explored (Table S1).[7, 32, 58] We tried to decrease the concentration of the protein and increase the concentration of the small molecule, NHS-*O*-*i*BBr, to prevent the aggregation and increase the modification rate. Yet, we consistently achieved ~3 conjugated initiating groups per protein. This may be due to a limited number of solution exposed lysine groups on the lipase TL surface. Trypsin digestion of the lipase TL-K-*i*BBr, followed by liquid chromatography mass spectroscopy (LC-MS/MS), showed that the initiators were attached to lysine residues 49 and 149 with the N-terminus being the most likely final modified position.[12, 49, 59, 60]

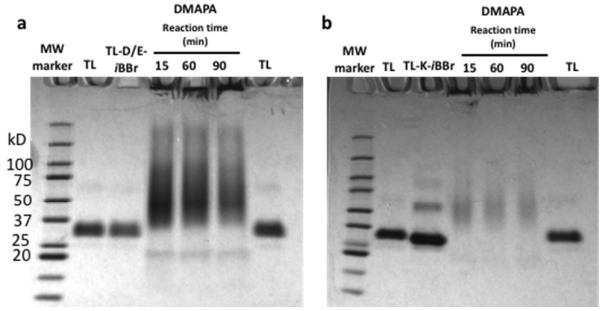


Figure 2. SDS-PAGE (4-20% denaturing PAGE gel) results of TL-K-pDMAPA and TL-D/E-pDMAPA.

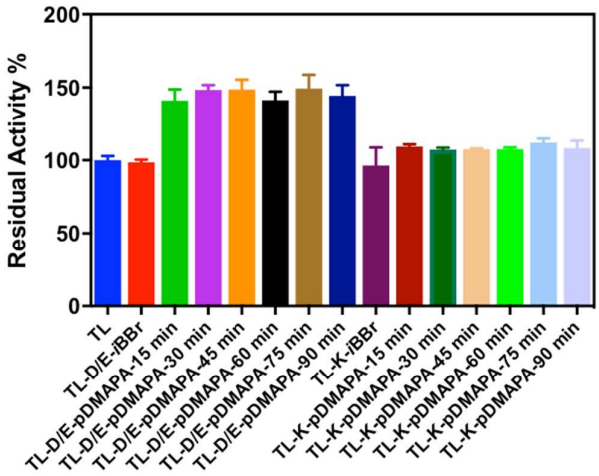


Figure 3. Lipolytic activity of lipase-polymer hybrids (TL-D/E-pDMAPA, TL-K-pDMAPA compared to native lipase TL,). (pH = 7.4, 37 °C). Activity considered 100% was 132.47 ± 9.15 µmol *p*-NP min^{-1} mg^{-1}

Lipase TL that modified on acid residues of glutamic and aspartic amino acids (TL-D/E-*i*BBr) was prepared by mixing an amine functional amide based ATRP initiator, NH_2-*i*BBr (see Supporting Information for detailed synthesis) with TL at pH 6.5, using one pot EDC/sulfo-NHS (Scheme 2).[61-63] The lipase initiator was isolated using 25k MWCO dialysis membrane. MALDI-ToF indicates that 6 initiators are attached per lipase (Figure S1-S3), while using a less concentrated solution of protein and 200 equivalent of initiator molecule (Table S2). Trypsin digestion of the lipase TL-D/E-*i*BBr followed by LC-MS/MS showed that the initiators were attached to aspartic acid residues 49 and 187 and glutamic acid residues 109, 151, 156 and 206. Although the acidic modified residues yielded 3 more initiators per protein than the lysine modified enzyme, the goal of this manuscript was to determine the viability of the acidic residues as grafting-from sites. Therefore, we did not examine the impact of the total number of grafting sites or the impact of polymer degree of polymerization of enzyme activity.

ICAR ATRP was used to graft DMAPA from lipase TL-K-*i*BBr and TL-D/E-pDMAPA due to the ease of catalyst removal and good control over polymer molecular weight and molecular weight distribution.[29, 34, 64] DMAPA was selected as the monomer due to water solubility and compatibility with lipase (due to the acrylamide bonds stability to lipase's hydrolytic activity) (Table 1). We used aqueous GPC to follow the polymerization reaction. In both cases, linear evolution of elution time with conversion was observed accompanied by linear monomer conversion with time (Figure 1). The grafted samples were purified using dialysis to yield a pure protein polymer hybrid. The M_n of the final grafted samples was determined using our previously reported infrared (IR) spectroscopy based method[32] (Table 1). This was done by determination of the total amide bond concentration using a Direct Detect FTIR spectrometer calibrated to determine the concertation of molecules bearing amide bones (i.e. proteins and acrylamides). Protein concentration was determined using UV-vis from a calibration curve (See SI for a detailed explanation). SDS PAGE showed that the polymer was attached to the protein and there is no unreacted TL left (Figure 2). According to Table 1, the lipase polymer hybrids TL-D/E-pDMAPA and TL-K-pDMAPA have similar M_n at different time points. Migration on SDS-PAGE that does not correlate with the results presented in Table 1, "gel shifting", as a result of differences in SDS binding,[65, 66] might be due to the dissimilarity in grafting density between TL-D/E-pDMAPA and TL-K-pDMAPA, and amino acid modification (Figure 2). The lower band at 20-25 kDa is a minor impurity (less than 5%) that is also present in the native enzyme and does not affect the activity of lipase. The upper band at 50 kDa is a lipase dimer (Figure S1 and S4).

In addition, the discrepancy between Table 1 and Figure 1b

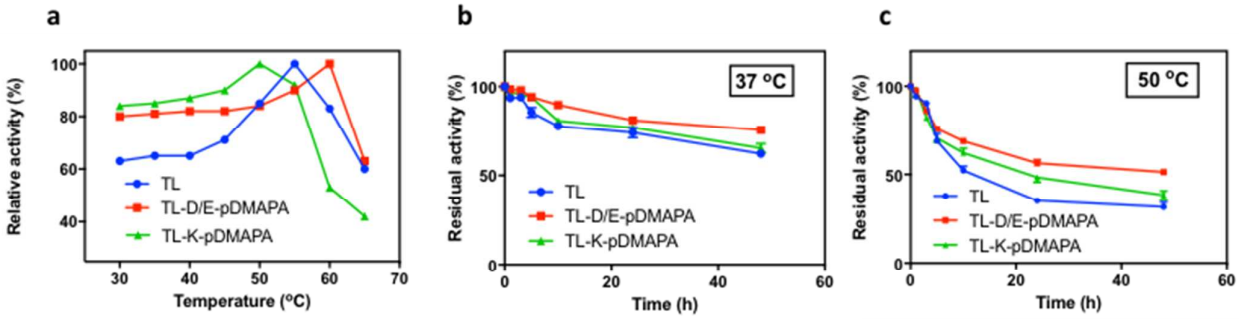


Figure 4. (a) Optimal temperature for native TL and polymer hybrids, at the range of 30 to 70 °C. The activity of native TL and lipase polymer hybrids (TL-D/E-pDMAPA, TL-K-pDMAPA) at different temperatures was compared to the activity at the temperature with maximum activity (optimal temperature) . Time course of thermal inactivation of lipase TL at 37 °C (b) and 50 °C (c), in comparison to lipase polymer hybrids (TL-D/E-pDMAPA, TL-K-pDMAPA). Thermal stability was investigated by monitoring the concentration of p-NP formed after the incubation at a given time compared to the activity at zero time of incubation at given temperature (expressed as a percentage). Each point is the average of triplicates ±SD.

and 1c, can also be explained by the impact of grafting density on polymer hydrodynamic radius and the effect on size exclusion chromatography (SEC) elution times on GPC spectra.

We observed that molecular weights of the polymers predicted by monomer conversions had variance with the experimental molecular weights. A possible explanation for the disparity in the molecular weight values may be that the ICAR ATRP process generates free chains from the decomposition of the azo imitator reacting with free monomer instead of reducing the copper-ligand complex.[64, 67, 68] In both cases the total molecular weight of the biohybrids was nearly equivalent as the TL-D/E-pDMAPA grafted polymers, which had an M_n of 5.5 kDa while the TL-K-pDMAPA grafted polymers had an M_n of 9.5 kDa.

The activity of TL-D/E-pDMAPA and TL-K-pDMAPA was measured by the rate of pNPP hydrolysis to yield nitrophenol in buffered media, which was measured by the increase of solution absorbance at 405 nm. The concentration of TL in all samples was 1.5 mg/ml, determined by the calibration curve using the absorbance at 280 nm and Direct Detect® FTIR spectrometer (see Supporting Information). Our results demonstrate that in all cases, polymer grafting does not decrease enzyme activity. Although, TL-D/E-iBBr and TL-K-iBBr have similar activities to native TL the grafted TL-D/E-pDMAPA had 50% greater activity compared to TL-K-pDMAPA. This result was confirmed by repeating each experiment in triplicate (Figure 3 and Table S3).

In our prior studies where PET-RAFT was used to grow DMAPA from TL-CTA, we observed a significant increase in biohybrid activity compared to native enzyme. We speculate that the reason we do not observe a similar increase in biohybrid activity, in the case of the ICAR ATRP grafted polymers, is because the degree of polymerization was approximately 2 to 4 times greater in the case of the PET-RAFT grafted TL. In our current studies, each time point taken during the polymerization for TL-D/E-pDMAPA and TL-K-pDMAPA hybrids was isolated and analyzed for enzyme activity. No difference in enzyme activity was observed when comparing the time points, presumably because the difference in the degree of polymerization was not sufficiently large enough to enhance the activity.

As was mentioned before, we did observe a clear difference in lipase activity between TL-D/E-pDMAPA and TL-K-pDMAPA hybrids. This difference may be explained by the greater grafting density of the D/E modified TL versus K modified TL.

The modification of lipase with DMAPA polymer facilitated enhanced activity in hydrolysis reactions lipase TL. We posit that this is due to enhanced solubility/affinity of the hydrophobic enzyme substrate in the polymer shell, thus increasing the effective concentration of substrate in the immediate vicinity of the enzyme. [69, 70] In addition, polymeric tertiary amine moieties attached to proteins increase enzymatic activity by pulling water molecules away from hydrophobic regions of the protein, creating a local environment that increased enzyme–substrate hydrophobic–hydrophobic interactions.[71]

The effect of temperature on the activities of native TL and modified TL were investigated at different temperatures (ranging from 30-70 ^{o}C). The optimal conditions required elevated temperatures (from 50 to 55 ^{o}C) (Figure 4a). The increased optimal temperature and the residual activity for TL-D/E-pDMAPA conjugate indicated that the DMAPA polymer increases the stability of lipase at high temperatures. Conversely, in the case of TL-K-pDMAPA the optimal temperature was lower, but the residual activity was higher at lower temperatures. The impact on optimal activity may be residue specific and this will be further explored in future work.

Thermal stability of the conjugates was investigated at temperatures 25, 37, and 50 °C (Figure 4b,c and Figure S5). We measured the ability of the conjugates to retain the activity as a function of heating time. Some denaturation is expected for both the native and polymer modified TL.[72, 73] No change in enzymatic activity at room temperature was observed after 48 hours (Figure S5). The enzymatic activity at 37 ^{o}C of TL and its conjugates had slightly increased from 60% to 75% after 48 hours in case of TL-D/E-pDMAPA conjugate. Interestingly, at 50 ^{o}C both conjugates showed improved functional thermal stability of the protein. The activity of the native TL dropped to 30% after 48 h, while TL-D/E-pDMAPA still retained 50% activity after the same period of time and TL-K-pDMAPA 40%. The enhanced thermal stability of TL-D/E-pDMAPA might be due to stabilization of the enzyme from the additional polymer grafted chains.[74, 75]

Due to the amphiphilic nature of pDMAPA and relatively high grafting density especially for TL-D/E-pDMAPA, we thought that the lipase polymer hybrids may be soluble in organic media. We examine the solubility of the largest hybrids, 3 pDMAPA polymers with M_n of 12 kDa for TL-K-pDMAPA, and 6 polymers with M_n of 7.4 kDa in 6 different organic solvents (Figure S6). The concentration of the enzyme was measured using Direct Detect® FTIR spectrometer and normalized to the amount of enzyme in water. The solubility of the native lipase TL and the hybrids in DMF were similar to the solubility in water. Although we observed no solubility of lipase TL in methanol and ethanol, TL-pDMAPA hybrids had good solubility in both. TL-pDMAPA hybrids were also more soluble then native lipase TL in acetonitrile, dichloromethane, and tetrahydrofuran.

These results highlight the novelty of our use of carboxylic acid residue of D/E amino acids as a new initiator ligation site. Grafting-from both K and D/E amino acid residues provided biohybrids with a greater activity than native enzyme as well as a dramatic increase in organic solvent solubility. These results point to the advantages of grafting-polymers from enzymes and the use of a novel tool for characterization of biohybrid solubility that requires low sample amounts. Overall,

our research demonstrates a new synthetic strategy to prepare protein polymer hybrids by the grafting-from method.

Conclusions

In conclusion, we have determined that previously overlooked acidic amino acid residues are excellent moieties for modification with ATRP initiators. Our research shows that a ubiquitous amino acid residue class can be modified with ATRP initiators without impacting enzyme activity.- as confirmed by protein digestion assays. Moreover, we found that the grafted TL-D/E-pDMAPA had a significantly higher activity than TL-K-pDMAPA. Furthermore, our results indicate that this new amino acid modification strategy may be employable in other enzymes, thereby granting access to new biohybrid modification schemes. The preparation of amine bearing ATRP initiators is synthetically simpler due to the inherent instability of NHS-activated esters upon long-term storage or in aqueous solutions. In future work, we will focus on expanding the number of proteins modified with the aspartic acid residue scheme and expand to dual labeled Lys Asp acid initiators for densely grafted biohybrids.

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GRAPHICAL ABSTRACT