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Tailoring Site Specificity of Bioconjugation using Step-Wise ATRP on Proteins (SWAP)

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KEYWORDS

Protein-ATRP, initiator, inhibitor, bioconjugates, step-wise, site-specific

ABSTRACT

Protein-polymer conjugates are powerful combinations of the biotic and abiotic worlds that impact many industries. Predicting the site and impact of polymer growth from the surface of proteins are only useful if we can use that information to choose which site to modify synthetically. We have explored the combination of a predictive algorithm with a unique step-wise atom transfer radical polymerization to selectively move the predominant modification sites around a model enzyme. Lysozyme was modified with defined stoichiometric ratios of polymerization initiators and initiation inhibitors to selectively and strategically grow poly-(carboxybetaine methacrylate) polymers from different protein sites. Electrospray ionization mass spectrometry was used to examine the uniformity of the lysozyme-initiator and lysozyme-inhibitor complexes prior to polymer growth. Bioactivity of the lysozyme-polymer conjugates was examined as a function of polymer location on the enzyme surface. Step-wise atom transfer radical polymerization from proteins provides a versatile and modular approach that can be extended to the rational and selective design of other protein-polymer conjugates.

INTRODUCTION

Over the last four decades, protein-polymer conjugates have found applications in research laboratories, industrial facilities and medical clinics[1,2]. The modification of proteins with polymers yields exciting biomacromolecular systems that exhibit properties from both individual components. The function of the bioconjugates depends on the nature, size and location of the polymer on the protein surface. Early work with protein-polymer conjugates relied on the use of poly(ethylene glycol) (PEG) to impart greater stability, solubility and decrease the immune response of proteins[3,4]. Recent advances in the field of protein-polymer chemistry have led to the predictable modification and enhancement of enzyme structure and function under non-native conditions. Researchers[5,6,15,7–14], including ourselves, have exploited the use of stimuli-responsive polymers for the preparation of bioconjugates with increased pH and temperature stability, increased substrate affinity and stability in non-aqueous conditions.

Despite these numerous benefits and exponential increase in applications, challenges remain in the rational design of bioconjugates. Indeed, the site(s) of modification and number of polymer chains bound to the protein surface have a direct impact on protein function[16–19]. A significant reduction or complete loss in biological function can occur upon modification, particularly when the polymer is located close to the active center[20]. Thus, a delicate balance of polymer distribution on the protein surface is pivotal for optimal protein function. A complete understanding requires knowledge of the conjugation site(s) on the protein surface; the conjugation strategy; the nature of the polymer; protein and polymer conformations during and after modification; and the degree of intra- and intermolecular interaction between polymer and protein. Traditional structure determination methodologies are not always suitable because of the

intrinsic complexity and size of these bioconjugates[21–24]. Additionally, protein modification often results in conjugates with high levels of heterogeneity[22].

One approach to generate more uniformly defined protein-polymer conjugates with high grafting densities, when desired, is surface-initiated atom-transfer radical polymerization (ATRP)[10,25]. Using amine-reactive chemistry, small molecule initiators are coupled onto a protein surface, from where ATRP is used to grow polymers, in situ. ATRP reaction conditions can be fine-tuned to obtain desired polymer chain lengths at a controlled density. Elegant, though time consuming, genetic techniques that introduce non-natural amino-acid ATRP initiators have been used to target polymers to specific sites[26]. In addition, a variety of chemistries have been introduced to grow polymers from single sites on the protein[11,12]. Unfortunately, however, there has remained a need for a straightforward way to influence the site of modification or to protect a desired location from ATRP.

We recently used the tertiary structure of proteins to predict the outcome of protein-ATRP[27]. Our technique enabled us to predict where and how fast ATRP initiators react with proteins. Knowing the speed and location of ATRP initiator reactions encouraged us to design a synthetic strategy through which we could use this information to target particular reaction sites. We decided to take a model protein, lysozyme, use the algorithmic predictions of modification rate at each available reaction site and then synthetically switch off fast reacting sites and target selective growth at slow reacting sites. We synthesized a protein-reactive "ATRP-inhibitor" that was structurally similar to our ATRP initiator. The initiation inhibitor, that lacked the halogen atom required for initiation of ATRP reactions, was designed to react with amine groups (N-terminal and lysine side chains) on proteins by N-hydroxysuccinimide chemistry. We

hypothesized that the strategic step-wise use of the initiator and inhibitor would lead to tailored polymer distributions on the surface of lysozyme.

Herein, we describe Step-Wise ATRP on Proteins (SWAP) with lysozyme. Lysozyme is an antimicrobial enzyme and an important component of the innate immune system that has been widely used for protein conjugation, has a well characterized amine-ATRP initiator reactivity pattern and a method for evaluating bioactivity[28–30]. These features provide lysozyme with ideal characteristics to explore the potential of SWAP and the implications of polymer distribution on protein function. Amine-ATRP initiator and amine-initiation inhibitor reactivities were first predicted by tertiary structure analysis. The site(s) of modification for both active esters were confirmed experimentally by enzymatic digestion studies followed by peptide mass mapping. With a defined reactivity pattern for lysozyme, we attempted three general approaches to strategic polymer growth (Figure 1). For the modification of the fast-reacting groups in lysozyme, a stoichiometric approach of ATRP initiator was used. To modify slow-reacting amine groups, lysozyme was first reacted with a defined stoichiometric amine-to-inhibitor ratio followed by the addition of excess ATRP initiator. Lastly, to inhibit polymer growth at an amine group with intermediate reactivity, a multi-step addition ATRP initiator and initiation inhibitor was used. Electrospray ionization (ESI-MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS) were used to probe the uniformity of the lysozyme-initiator and -inhibitor complexes. After poly-(carboxybetaine methacrylate) (pCBMA) growth by ATRP, the bioactivity of the family of SWAP lysozyme-polymer conjugates was measured using a *M. lysodeikticus* cell wall degradation assay. This is, to the best of our knowledge, the first step-wise ATRP approach towards controlled polymer distribution on

proteins. The versatility and ease of SWAP allows for this methodology to be applied to other protein-polymer conjugates.

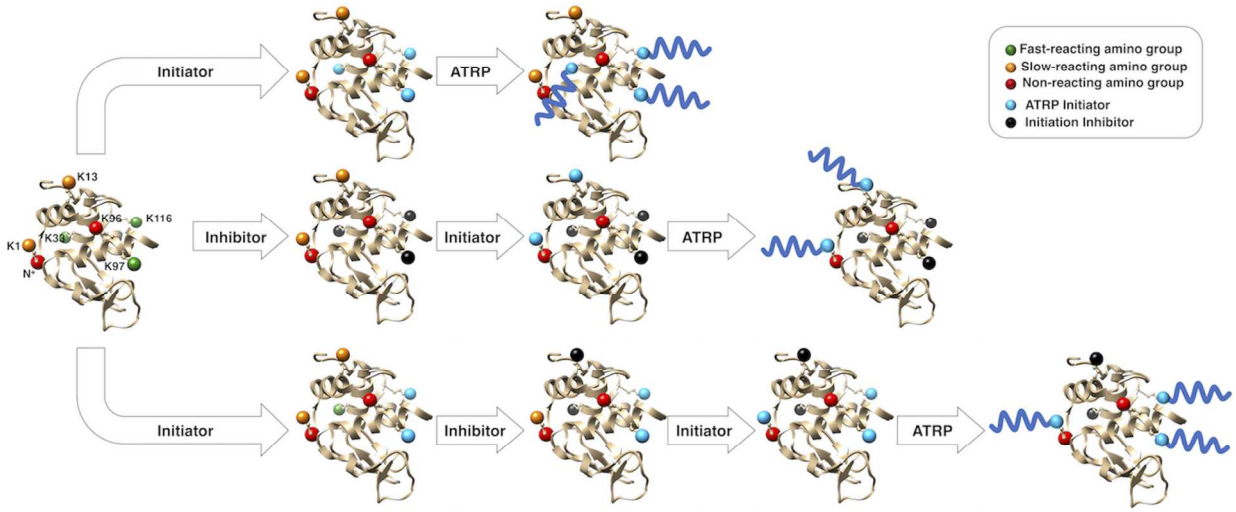

Figure 1. Step-Wise ATRP for Proteins (SWAP) for tailored polymer distribution on the surface of lysozyme. Fast-, slow- and non-reacting amine groups (shown in green, orange and red, respectively) are first determined using a tertiary structure based prediction algorithm. Spheres in blue indicate amino groups modified with ATRP initiator while those in black denote amino groups with initiation inhibitors.

EXPERIMENTAL SECTION

Materials.

Hen egg-white lysozyme (Lyz) from Gallus gallus and lyophilized *Micrococcus lysodeikticus* were purchased from Sigma-Aldrich (St Louis, MO) and used without further purification. Carboxybetaine methacrylate was purchased from TCI America. Bromine-functionalized N-hydroxysuccinimide ATRP initiator **1** synthesis was carried out as described previously[10]. Dialysis tubing (molecular weight cut off 15 kDa Spectra/Por, Spectrum Laboratories Inc., CA) for macro-initiator and macro-inhibitor isolation, ZipTipsC_{18} microtips (Millipore, catalogue no. ZTC1 8M0 08), and In-Solution Tryptic Digestion and Guanidination Kits (catalogue no. 89895) were purchased from Thermo Fisher Scientific (Pittsburgh, PA).

Methods.

Synthesis of N-isobutyryl-β-alanine N'-oxysuccinimide ester 2.

Isobutyryl chloride (10.5 mL, 100 mmol) was slowly added into a solution of β-alanine (8.9 g, 100 mmol) and triethylamine (30.7 mL, 220 mmol) in mixture of deionized water (100 mL) at 0 °C. The mixture was stirred at room temperature for 1 h. The water phase was washed with diethyl ether (50 mL x 3) and adjusted to pH 2 with 6 N HCl aq. at 0 °C. The product was extracted with ethyl acetate (50 mL × 5). The organic phase was dried with $MgSO_4$ and evaporated to remove solvent. N-isobutyryl-β-alanine was isolated by recrystallization from a mixture of ethyl acetate and diethyl ether (1:1 volume ratio); yield 9.2 g (58 %), mp 100 – 102 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.14 (d, 3 H, J = 6.9 Hz, NHC=OCH$(CH_3)_2$), 2.36 (m, 1 H, J = 6.9 Hz, NHC=OCH$(CH_3)_2$), 2.59 (t, 2 H, J = 6.0 Hz, HOOCCH_2CH_2NHC=O), 3.52 (q, 2 H, J = 6.0 Hz, HOOCCH_2CH_2NHC=O), and 6.18 (broad s, 1 H, amide proton) ppm. IR (KBr) 3385, 2970, 2927, 1713, 1632, 1614, 1553, 1435, 1372, 1355, 1307, 1278, 1223, 1199 and 1127 cm^{-1}.

N,N'-diisopropylcarbodiimide (3.4 mL, 22 mmol) was slowly added to the solution of N-isobutyryl-β-alanine (3.2 g, 20 mmol) and N-hydroxysuccinimide (2.5 g, 22 mmol) in dichloromethane (100 mL) at 0 °C. The mixture was stirred at room temperature overnight. After filtration, the solution was evaporated to remove solvent. N-isobutyryl-β-alanine N'-oxysuccinimide ester **2** was purified by recrystallization from 2-propanol with a yield of 4.1 g (80 %), mp 128 – 131 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.13 (d, 3 H, J = 6.9 Hz, NHC=OCH$(CH_3)_2$), 2.37 (m, 1 H, J = 6.9 Hz, NHC=OCH$(CH_3)_2$), 2.86 and 2.82 (s and t, 4 H and 2 H, J = 6.0 Hz, ethylene of succinimide and NHSOOCCH_2CH_2NHC=O), 3.65 (q, 2 H, J = 6.0 Hz, NHSOOCCH_2CH_2NHC=O), and 6.26 (broad s, 1 H, amide proton). IR (KBr) 3304, 2969, 2876, 1816, 1709, 1741, 1649, 1551, 1428, 1381, 1366, 1310, 1247, 1208 and 1087 cm^{-1}.

HRMS (m/z): $[M+Na]^+$ calcd. for $C_{11}H_{16}N_2O_5$, 279.09; found 278.93; $[M+2ACN+H]^+$ calcd. for $C_{11}H_{16}N_2O_5$, 339.17; found 338.20.

Stoichiometric Reaction between ATRP initiator 1 and Lyz.

A family of lysozyme macro-initiators with varying degrees of modification was prepared using bromine-functionalized ATRP initiator **1**. ATRP initiator **1** (1.6−24.6 mg, 0.005−0.07 mmol) and Lyz (50 mg, 0.004 mmol protein, 0.02 mmol NH_2 group in lysine residues and N-terminus) were dissolved in 10 mL of 0.1 M sodium phosphate buffer, pH 8.0. The solution was stirred at 4 °C for 2 h. After this time, the reaction was dialyzed against deionized water, using dialysis tubing with a molecular weight cutoff of 15 kDa, for 24 h at 4 °C, and then lyophilized.

Reaction between initiator inhibitor 2 and Lyz.

A family of lysozyme macro-inhibitors with varying degrees of modification was prepared using initiator inhibitor **2**. Initiator inhibitor **2** (1.25−12.5 mg, 0.005−0.05 mmol) and Lyz (50 mg, 0.004 mmol protein, 0.02 mmol -NH_2 group in lysine residues and N-terminus) were dissolved in 10 mL of 0.1 M sodium phosphate buffer, pH 8.0. The solution was stirred at 4 °C for 2 h. After this time, the reaction was dialyzed against deionized water, using dialysis tubing with a molecular weight cutoff of 15 kDa, for 24 h at 4 °C, and then lyophilized.

Synthesis of $LyzBr_2I_1Br_3$.

Lysozyme (50 mg, 0.0035 mmol protein, 0.024 mmol –NH_2 group in lysine residues and N-terminus) and ATRP initiator **1** (2.5 mg, 0.007 mmol) were dissolved in 10 mL of 0.1 M sodium phosphate buffer, pH 8.0. The solution was stirred at 4°C for 30 minutes. After this time, initiator inhibitor **2** (1.8 mg, 0.007 mmol) was added to the reaction mixture and the solution was stirred for an additional 30 minutes at 4°C. Lastly, excess ATRP initiator **1** (24.6 mg, 0.07 mmol) was added to the lysozyme solution and the reaction was stirred for 1 hour. After the total time of 2

hours, the reaction was terminated and dialyzed against deionized water, using dialysis tubing with a molecular weight cutoff of 8 kDa, for 24 h at 4 °C, followed by lyophilization.

ATRP from the surface of Lysozyme.

ATRP was carried out as previously described[31]. In brief, a solution of CBMA and protein-initiator in 100 mM sodium phosphate, pH 8.0 (for a final concentration of protein-initiator of 0.5 mM) was sealed and bubbled with nitrogen gas for 30 min. 1 mL of deoxygenated catalyst solution (described below) was then added to the polymerization reactor under bubbling nitrogen. The mixture was sealed and stirred at room temperature for 2 h. The conjugate was isolated by dialysis with a 25 kDa molecular weight cutoff dialysis tube in deionized water in a refrigerator for 24 h and then lyophilized. Note: Approximate final concentrations in solution: Initiator = 0.5 mM, CuII = 5 mM, NaAsc = 0.5 mM and HMTETA = 6 mM.

Preparation of Cu-HMTETA as deoxygenated catalyst solution.

50 mM $CuCl_2$ in deionized water (1.2 mL, 60 μmol) was bubbled with N2 for 25 min and then 100 mM sodium ascorbate in deionized water (50 μL, 5 μmol) was added. HMTETA (18 μL, 70 μmol) was added to the copper suspension bubbled with N_2 for 3 min. The deoxygenated Cu-HMTETA solution was added to the synthesis vessel immediately.

Lysozyme Activity Assay.

Lysozyme activity was measured by the lysis of M. lysodeikticus cell walls as previously described[32]. Lyophilized *Micrococcus lysodeikticus* was used to monitor enzymatic catalysis of cell wall lysis. Absorption at 450 nm of suspended M. lysodeikticus (990 μL, 0.2 mg/mL) in 50 mM phosphate buffer (pH 6.0) was measured by UV-VIS spectrometer. 10 μL of Lyz-pCBMA solution (2.8 μM in 50 mM phosphate buffer (pH 6.0)) was added and the change of absorbance at 450 nm at room temperature was monitored.

Determination of Modification Site.

Trypsin digests were used to generate peptide fragments from which lysozyme macro-inhibitor attachment sites could be determined using ElectroSpray Ionization (ESI) Mass Spectrometry. Samples were digested per the protocol described in the In-Solution Tryptic Digestion and Guanidination Kit. In brief, 20 μg of lysozyme or lysozyme macro-inhibitors (10 μL of a 2 mg/mL protein solution in ultrapure water) were added to 15 μL of 50 mM ammonium bicarbonate with 1.5 μL of 100 mM dithiothreitol (DTT) in a 0.5 mL Eppendorf tube. The reaction was incubated for 5 min at 95 °C. Thiol alkylation was conducted by the addition of 3 μL of 100 mM iodoacetamide aqueous solution to the protein solution and incubation in the dark for 20 min at room temperature. After this time, 1 μL of 100 ng of trypsin was added to the protein solution, and the reaction was incubated at 37 °C for 3 h. An additional 1 μL of 100 ng of trypsin was subsequently added. Digested samples were purified using ZipTipC_{18} microtips and eluted with 1 μL of 50% acetonitrile with 0.1% formic acid into a 0.5 mL Eppendorf tube and diluted 100-fold with 50% acetonitrile with 0.1% formic acid. The molecular weight of the expected peptide fragments before and after blocking agent attachment was predicted using PeptideCutter (ExPASy Bioinformatics Portal, Swiss Institute of Bioinformatics). A complete list of identified tryptic peptides can be found in supporting information.

^{1}H NMR spectroscopy.

^{1}H NMR spectra were recorded on a spectrometer (300 MHz Bruker AvanceTM 300) in the NMR facility located in Center for Molecular Analysis, Carnegie Mellon University, Pittsburgh, PA, with $CDCl_3$.

Fourier-Transform Infrared Analysis.

Routine FT-IR spectra were obtained with a Nicolet Magna-IR 560 spectrometer (Thermo), in the Department of Chemical Engineering at Carnegie Mellon University.

Melting points.

Melting points (mp) were measured with a Laboratory Devices Mel-Temp.

MALDI-ToF-MS Analysis.

Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) measurements were recorded using a PerSeptive Voyager STR MS with nitrogen laser (337 nm) and 20 kV accelerating voltage with a grid voltage of 90%. At least 300 laser shots covering the complete spot were accumulated for each spectrum. For the determination of the molecular weight of synthesized lysozyme complexes, sinapinic acid (20 mg/mL) in 50% acetonitrile with 0.1% trifluoroacetic acid was used as matrix. Protein solution (1.0 mg/mL) was mixed with an equal volume of matrix, and 2 μL of the resulting mixture was loaded onto a silver sterling plate target. Apomyoglobin, cytochrome C, and aldolase were used as calibration samples. The extent of modification was determined by subtracting the lysozyme complexes' m/z values from the protein lysozyme m/z and dividing by the molecular weight of the ATRP initiator or initiator inhibitor (220.9 or 142.18 g/mol, respectively).

ESI-MS Analysis.

Experiments were performed by using a Finnigan LCQ (Thermo-Fisher) quadrupole field ion trap mass spectrometer with electrospray ionization (ESI) source. Each scan was acquired over the range m/z 150-2000 by using a step of 0.5 u, a dwell time of 1.5 ms, a mass defect of 50 pu, and an 80-V orifice potential. Samples at a protein concentration of 50 μM and eluted using 50% acetonitrile and 0.1% formic acid at a flow rate of 20 μL/min.

Dynamic Light Scattering (DLS).

The DLS data were collected on a Nanoplus Zeta/Nano Particle Analyzer (Particulate Systems). The concentration of the sample solution was kept at 1.0 mg/mL. Hydrodynamic diameters (D_h) of native lysozyme and conjugates were measured three times (25 runs/measurement) in 100 mM sodium phosphate buffer (pH 8.0) at 25 °C.

Bicinchonic Assay (BCA).

Sample solution in deionized water (25 μL, 1.0 mg/mL) was mixed with mixture of bicinchonic acid (BCA) solution (1.0 mL) and copper (II) sulfate solution (50:1 vol:vol). The sample solution was incubated at 60 °C for 15 min. Absorbance of the sample at 562 nm was recorded by UV-VIS spectrometer. Lysozyme concentration (wt%) of the conjugates was determined by comparison of the absorbance to the standard curve. Standard curve was obtained from native Lysozyme with different concentration ratio in deionized water. Estimation of molecular weight for lysozyme-polymer conjugates was determined as previously described[33].

RESULTS AND DISCUSSION

Lysozyme contains seven amine groups; one α-amino group of N-terminal (K1) and the six ε-amino groups of the lysine residues (K1, K13, K33, K96, K97, and K116)[34,35]. Previous analysis of the structural and chemical environment surrounding these amine groups allowed us to determine the occurrence and sequence of amine modifications with ATRP initiator **1**[27]. We hypothesized that the distinct reactivities of the amine groups in lysozyme could be used to target modification and tailor polymer growth using the SWAP synthetic strategy (Figure 1).

Structure-Reactivity Characterization of the Amine-Initiation Inhibitor Reaction

To aid in the selectivity and precision of amine modifications, initiation inhibitor **2** was synthesized and characterized by ^{1}H-NMR and ESI-MS. Initiation inhibitor **2** was designed to be comparable to the ATRP initiator **1** in terms of structure and reactivity, but prevent polymer growth by ATRP. Thus, the N-hydroxysuccinimide ester group allowed the reaction with lysine residues and N-termini while the absence of a halogen atom prevented polymer growth at this site.

We exposed lysozyme to increasing amounts of initiation inhibitor **2** in 0.1 M sodium phosphate buffer, pH 8, at 4 °C for 2 h to generate a small family of macro-inhibitors (Lyz-I). As a comparison, a family of macro-initiators (Lyz-Br) was also prepared under the same stoichiometric and reaction conditions. We were interested in confirming whether the ATRP initiator **1** and initiation inhibitor **2** would react with the same rate and selectivity with the amine groups in lysozyme. Lysozyme macro-initiators and macro-inhibitors were purified by dialysis and analyzed by MALDI-TOF (Supporting information, Figures S1 and S2).

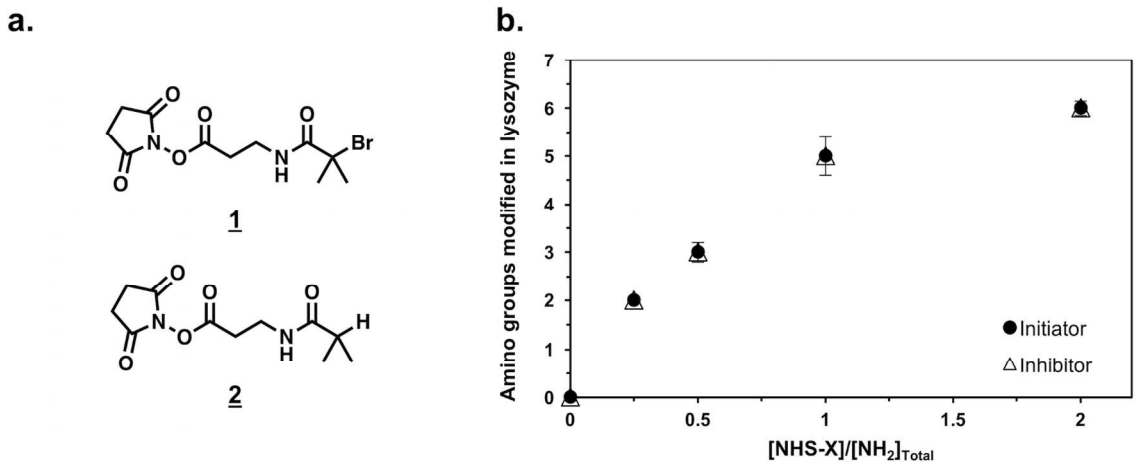

Figure 2. Dependence of ATRP initiator **1** versus initiation inhibitor **2** reacting with the surface of lysozyme as a function of the initiator or inhibitor to the amino groups' stoichiometry. Experimentally, native lysozyme was incubated with increasing amounts of ATRP initiator **1** or

inhibitor **2** in 0.1 M sodium phosphate buffer, pH 8.0, at room temperature for 2 hours. After this time, the lysozyme complexes were purified by dialysis and analyzed by MALDI-TOF to determine the number of amino groups modified. In plot, [NHS-X] represents the concentration of ATRP initiator **1** or inhibitor **2**.

The number of initiators in each lysozyme complex was calculated by subtracting native lysozyme m/z from the Lyz-Br or Lyz-I m/z values and dividing by the molar mass of ATRP initiator **1** or initiation inhibitor **2** (Mw = 220.9 g/mol or 142.0 g/mol, respectively). As anticipated, the number of initiator or inhibitor molecules on lysozyme increased when increasing molar excess of the respective active ester. Moreover, MALDI-ToF analysis indicated that, on average, both active esters reacted similarly with the amine groups. We were now interested in determining where initiation inhibitor **2** reacted with the surface of lysozyme.

The location of the amine groups that were modified by initiation inhibitors were identified by trypsin digestion followed by mass spectrometry analysis (see supporting information, Tables S2 and S3) as described in detail previously[27]. Lysozyme contains six lysine residues that may be cleaved by trypsin. Previous studies[27,36–38] have shown that the order of amine modification in lysozyme with ATRP initiator **1** was K116, K97 ~ K33, K13, and finally K1. Furthermore, we also observed that K96 was non-reactive. MS analysis on the tryptic digests for the various Lysozyme macro-inhibitors showed that this order of modification was maintained, indicating identical reactivities between both initiator and inhibitor. Interestingly, an additional modification was identified on K1 for lysozyme macro-inhibitor ($LyzI_6$), suggesting that two modifications on the same lysine residue were possible (ε-amino side chain and the N-terminus). This was not observed previously for lysozyme macro-initiators[27] and was attributed to steric impediment and low surface exposure of the N-terminus, which would prevent modification at this site. To further understand this finding, a short 20 ns molecular dynamics

simulation was performed on the lysozyme macro-inhibitor with one modification at the ε-amino group on K1 and compared to native lysozyme as well as lysozyme macro-initiator also with one modification at the ε-amino group on K1 (see supplementary information, Figure S3). End-to-end distances between the α- and ε-amino groups were measured over the simulation time and a greater distance between both amino groups was observed when K1 was modified with initiation inhibitor **2**. We inferred that the absence of the bromine atom in the inhibitor molecule decreased the steric impediment previously seen with ATRP initiator **1**.

To further confirm the specificity of amine modifications with both active esters, excess ATRP initiator **1** was added to $LyzI_2$ (modified with inhibitor at K116 and K97). Subsequent MS analysis of trypsin digestion of peptide fragments revealed the initiator now reacted first with K33, K13 and K1 (see supporting information, Figure S4). Thus, in a SWAP process we could block the growth of polymer from the most reactive lysine residues.

Structural Characterization of Lyz Macro-Initiators and -Inhibitors

In our approach to surface-initiated ATRP, we target modifications at lysine residues and N-terminus in proteins. Due to the high natural abundance and nucleophilicity of the α- and ε-amino groups, this strategy ensures dense modification to produce nanoarmored enzymes. However, when more selective modifications are needed, this high abundance of nucleophiles could yield a statistical population of many products with variable biological outcomes[39–41]. Such heterogeneity has been observed in early research with first-generation PEGylated products[39], but has not been the focus of extensive research with protein-ATRP. We next looked to assess the promise that site-specific polymer growth synthetic strategies could yield more homogeneous conjugates with retained and predictable biological effects[42–44].

To examine the uniformity of lysozyme macro-initiators and macro-inhibitors, we initially performed MALDI-ToF-MS analysis. MALDI-ToF mass signals for macromolecules correspond to the convolution of the intrinsic isotope distribution. Thus, for lysozyme complexes (and proteins in general), we observed an envelope of masses due to the distribution of ^{13}C isotope. This translated visually into a broad spectrum where we assumed that the actual mass was at the center of the envelope. This value corresponded to the weighted average of all isotope peaks. Unfortunately, this average mass was not sufficiently precise to develop a real insight into any heterogeneity of the macro-initiators and macro-inhibitors. To obtain more detailed structural information on the uniformity of the lysozyme complexes, we developed an ESI-MS technique with the bioconjugates.

ESI-MS is a powerful tool for investigating the covalent modification of proteins with small molecules. The use of this technique leads to spectra with multiply charged ions allowing the detection of large proteins using mass analyzers with low mass-to-charge (m/z) ranges. Moreover, the overall mass accuracy and precision of ESI-MS allows low abundance molecules with small mass differences to be efficiently detected. This can be particularly advantageous when determining the stoichiometry and uniformity of protein-initiator or protein-inhibitor complexes.

ESI-MS analyses of lysozyme covalently modified with 0.5 and 2.0 equivalents of initiator inhibitor **2** confirmed the formation of lysozyme macro-inhibitors (**Error! Reference source not found.**c and d). Upon reaction of lysozyme with 0.5 equivalents of initiator inhibitor **2**, by MALDI analysis we detected the presence of three initiator inhibitors on each protein molecule (Figure 2b). When analyzed by ESI-MS, we detected a family of products varying in inhibitor content from one to four per molecule of enzyme (Figure 3c). For lysozyme that was

reacted with molar excess of initiation inhibitor **2**, two distinct populations were identified by ESI-MS (Figure 3d).

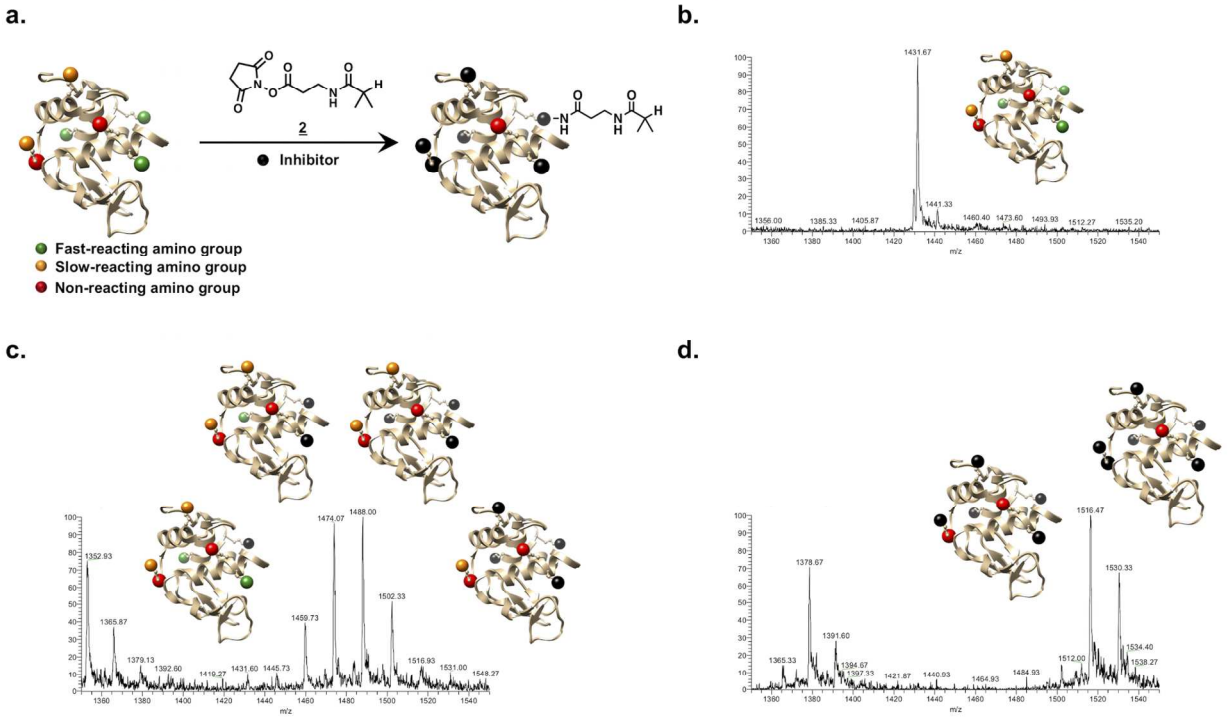

Figure 3 a. Initiation inhibitor reaction with lysozyme. Fast, slow and non-reacting amine groups on lysozyme are depicted in green, orange and red, respectively. Amine groups modified with initiation inhibitor **2** are represented in black. ESI-MS spectrum for **b.** native lysozyme, **c.** lysozyme macro-inhibitor with 1-4 inhibitors and **d.** lysozyme macro-initiator with 5-6 ATRP inhibitors. Spectra range shows protein at $[M+10]^{10+}$ charge state.

The sensitivity of ESI-MS compared to MALDI allowed us to fully understand the uniformity of the starting materials for protein ATRP. When reacted with excess ATRP initiator **1**, lysozyme-initiator complexes with 3 to 5 initiators per molecule of enzyme were observed (see supporting information, Figure S5). Because of the natural abundance of bromine isotopes, the mass spectra resolution was found to decrease dramatically with increasing initiator modification.

Tailored Polymer Growth by Surface-Initiated ATRP

To test our hypothesis that Step-Wise ATRP on Proteins could be used to tailored polymer distribution, we next grew pCBMA from the initiating sites of the previously prepared lysozyme macro-initiators. Since we were interested in controlling polymer growth to specific locations on the surface of the protein, we maintained the ATRP reaction conditions to obtain similar polymer chain lengths. The protein content for each conjugate was determined by the bicinchoninic acid assay (BCA) which allowed us to determine the apparent molecular weight and degree of pCBMA polymerization (DP) for each of the lysozyme-pCBMA conjugates (Table 1). As the number of polymer chains on the protein surface increased, the overall conjugate molecular weight increased.

Table 1. SWAP Synthesis of Active Lysozyme-pCBMA Conjugates. The nomenclature for the lysozyme- conjugates includes the number of ATRP initiators or initiation inhibitors and the determined DP. For example, Lyz-($pCBMA_{11}$-Br)$_2$ refers to lysozyme-pCBMA conjugate with two bromine initiators and a DP of 11. The degree of the polymeric carboxybetaine was controlled by maintaining constant the initial molar concentration of the CBMA monomer in solution, which was reflected in the similar DP values for all lysozyme conjugates. The protein content for each conjugate was determined by bicinchoninic acid assay (BCA), from which the apparent molecular weight and DP for each conjugate were calculated. The hydrodynamic diameter values (D_h) were determined using dynamic light scattering (DLS) in 100 mM sodium phosphate at pH 8.0. For native lysozyme, the diameter was consistent with that reported in the literature[45].

	Inhibited –NH_2	Initiated –NH_2	DP	Conjugate Mw (Da)	D_h (nm)	*M. lysodeikticus*	
						ΔA_{450}/min ($\times 10^{-5}$)	Ratio of lysis (Conjugate/Native)
Native Lysozyme					3.2 ± 0.7	56.7 ± 5.8	1.00 ± 0.0
Lyz-($pCBMA_{11}$-Br)$_2$	-	K116, K97	11	19,235.25	3.6 ± 0.7	23.3 ± 5.8	0.41 ± 0.08

Lyz-(pCBMA$_9$-Br)$_4$	-	K116, K97, K33, K13	9	22,174.62	4.2 ± 1.0	5.7 ± 1.2	0.10 ± 0.03
Lyz-(pCBMA$_{13}$-Br)$_6$	-	K116, K97, K33, K13, K1, N^+	13	32,235.25	4.1 ± 1.2	0.9 ± 0.2	0.02 ± 0.00
LyzI$_2$-(pCBMA$_{12}$-Br)$_2$		K33, K13	12	19,883.58	3.1 ± 0.7	20.0 ± 0.0	0.36 ± 0.04
LyzI$_2$-(pCBMA$_9$-Br)$_3$	K116, K97	K33, K13, K1	9	22,477.09	4.0 ± 0.8	1.6 ± 1.2	0.03 ± 0.02
LyzI$_2$-(pCBMA$_{15}$-Br)$_4$		K33, K13, K1, N^+	15	28,472.60	4.6 ± 0.9	1.9 ± 1.1	0.03 ± 0.02
LyzI$_1$-(pCBMA$_{13}$-Br)$_5$	K33	K116, K97, K13, K1, N^+	13	29,142.76	4.1 ± 0.9	2.3 ± 0.6	0.04 ± 0.02

The bioactivity of the family of polymer protein conjugates was assayed in a cell wall hydrolysis assay. At physiological pH Lysozyme has a net positive charge, while *Micrococcus lysodeikticus*, like most other bacterial cells, has a negatively charged outer surface[46]. We attributed the decrease in activity of the lysozyme conjugates (when compared to native lysozyme) to differences in surface charge of the conjugate, steric impediment of the catalytic site to large substrates by the polymers or a combination of the two. Considering the importance of the difference in charge between the enzyme and the substrate in this lytic assay, we were not surprised to observe the dependence of activity decrease on increasing initiator and pCBMA coverage of the enzyme. The activity of lysozyme was inversely proportional to the number of polymer chains attached (Table 1 and Figure S6 in supporting information). The active site of lysozyme consists of a cleft on the exterior of the enzyme in which only two residues (Glu 35 and Asp 52) are involved in the catalytic action[47]. The lysine residue that was closest to the

active site cleft, Lys 33, was not close enough, however, to eliminate activity when conjugated to a polymer chain.

Previous studies have reported that lysozyme interacts with a negative surface using its largest positively charged patch, which includes Lys 1, Lys 13, Lys 96, Lys 97, Arg 14 and Arg 128. We were not surprised, therefore, to note that Lyz-(pCBMA$_9$- Br)$_4$ (in which Lys 116, Lys 97, Lys 33 and Lys 13 were modified with pCBMA), had a more pronounced activity reduction when compared, for example, to LyzI$_2$ -(pCBMA$_{12}$-Br)$_2$ (in which only Lys 33 and Lys 13 were modified with pCBMA). Our results demonstrated that we could engineer lysozyme-pCBMA conjugates in which polymer chains were grown by SWAP from targeted locations, thereby rationally tailoring the activity of the enzyme.

CONCLUSIONS

We have used SWAP to synthesize lysozyme-pCBMA conjugates at sites targeted by a knowledge of lysine reactivity and enzyme structure-function relationships. ATRP initiators and initiation inhibitors were used in a modular approach to tailor the polymer distribution on the surface of lysozyme. Macro-initiators and macro-inhibitors were characterized by mass spectrometry techniques. ESI-MS proved to be a powerful and insightful tool to characterize the uniformity of the prepared macro-initiators and macro-inhibitors. Structural and mechanistic insights allowed us to engineer enzyme variants that minimized the loss of critical protein surface charges (at Lys 97, Lys 33 and Lys 13). When combined with algorithms that can predict where and how fast individual sites react with ATRP initiators, SWAP can be used to selectively tailor polymer distribution on the surface of enzymes. We are now exploring the use of SWAP

with a range of proteins that require site-selectivity when being subject to grown-from protein-ATRP.

SUPPORTING INFORMATION

Molecular dynamics simulation study of native lysozyme, lysozyme macro-initiator and lysozyme macro-inhibitor; MALDI-TOF spectra for the characterization of lysozyme-initiator and lysozyme-inhibitor complexes; data for the trypsin digestion studies; ESI-MS spectra characterization for lysozyme-initiator complexes.

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Author Contributions

S.C. predicted amine reactivity of lysozyme, prepared lysozyme-initiators and lysozyme-inhibitors, performed the trypsin digestion studies and analyzed MS data. H.M. synthesized initiator inhibitor. A.R and K.M supervised the project and provided guidance. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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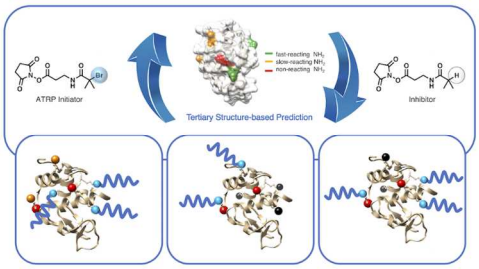
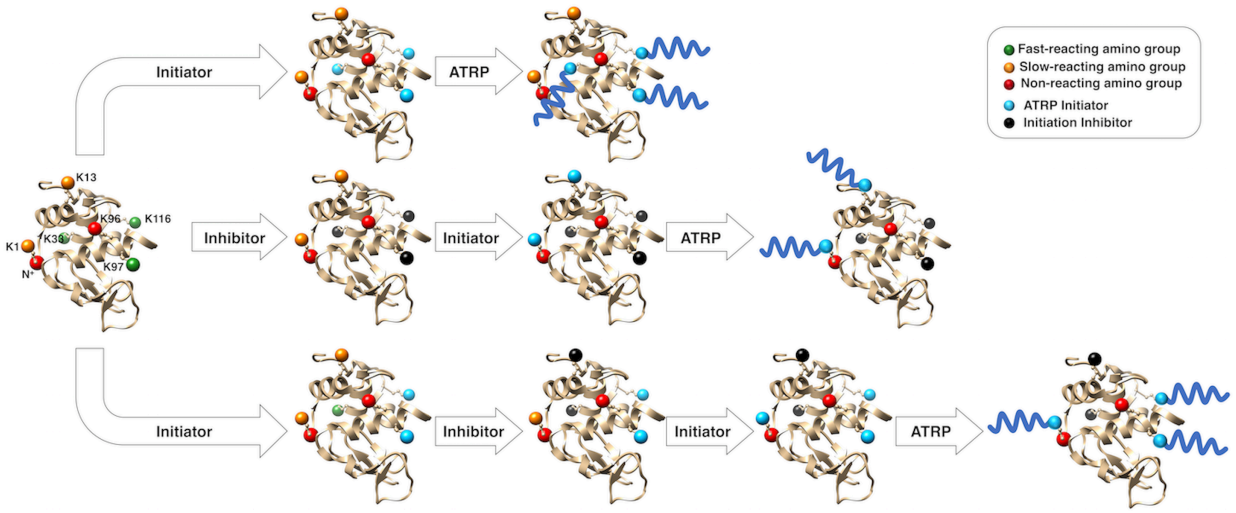
TABLE OF CONTENTS GRAPHIC

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ATRP
Inhibitor
Initiator
ATRP
Initiator
Inhibitor
Initiator
ATRP
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K96
K116
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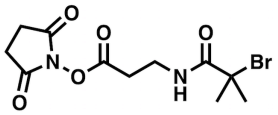
Slow-reacting amino group
Non-reacting amino group
ATRP Initiator
Initiation Inhibitor

a.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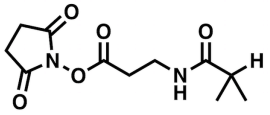
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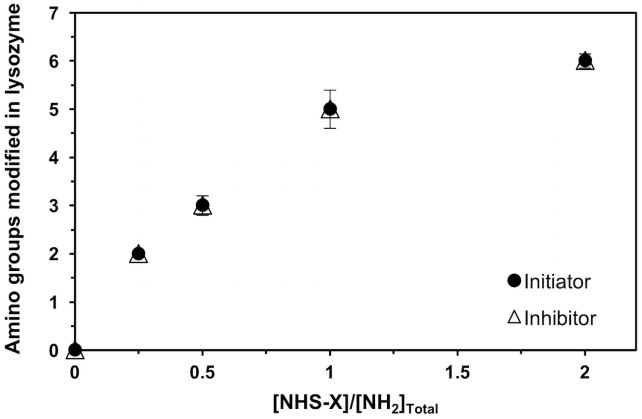
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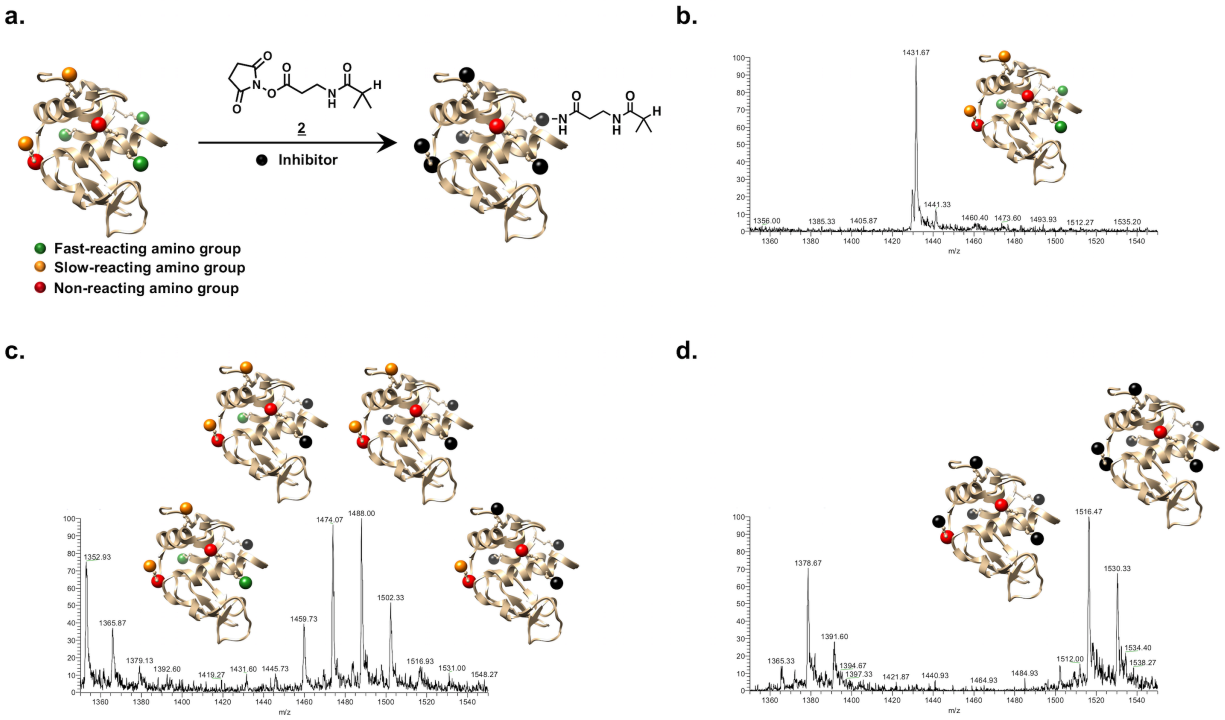
[NHS-X]/[NH2]Total
Initiator
Inhibitor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Non-reacting amino group
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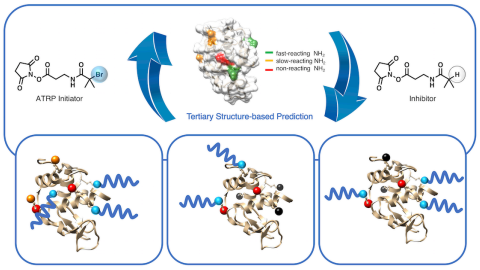
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