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Article

Supramolecular Protein Stabilization with Zwitterionic Polypeptide–Cucurbit[7]uril Conjugates

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Cite This: *Biomacromolecules* 2023, 24, 481–488

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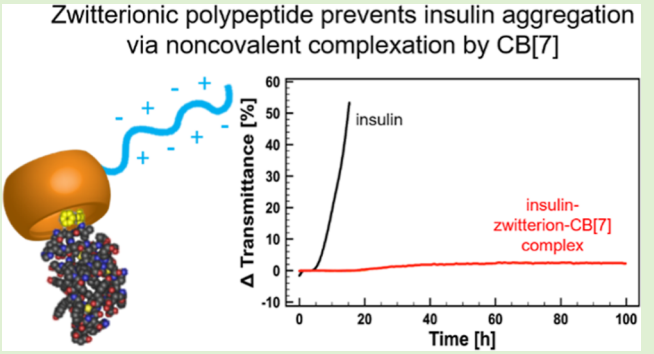
ABSTRACT: Protein aggregation is an obstacle for the development of new biopharmaceuticals, presenting challenges in shipping and storage of vital therapies. Though a variety of materials and methods have been explored, the need remains for a simple material that is biodegradable, nontoxic, and highly efficient at stabilizing protein therapeutics. In this work, we investigated zwitterionic polypeptides prepared using a rapid and scalable polymerization technique and conjugated to a supramolecular macrocycle host, cucurbit[7]uril, for the ability to inhibit aggregation of model protein therapeutics insulin and calcitonin. The polypeptides are based on the natural amino acid methionine, and zwitterion sulfonium modifications were compared to analogous cationic and neutral structures. Each polymer was end-modified with a single cucurbit[7]uril macrocycle to afford supramolecular recognition and binding to terminal aromatic amino acids on proteins. Only conjugates prepared from zwitterionic structures of sufficient chain lengths were efficient inhibitors of insulin aggregation and could also inhibit aggregation of calcitonin. This polypeptide exhibited no cytotoxicity in human cells even at concentrations that were five-fold of the intended therapeutic regime. We explored treatment of the zwitterionic polypeptides with a panel of natural proteases and found steady biodegradation as expected, supporting eventual clearance when used as a protein formulation additive.



INTRODUCTION

Protein biopharmaceuticals are a rapidly growing therapeutic class due to their advantages in specificity and in native signaling processes.[1] However, a serious challenge encountered in nearly all stages of protein drug development is aggregation and compound stability.[2] Proteins can aggregate by physical and/or chemical associations through van der Waals, hydrophobic, or electrostatic forces, or by formation of new covalent bonds. Such events typically convert the therapeutic molecules into non-active and potentially immunogenic substances.[3,4] Protein aggregation thus presents a challenge that reduces efficacy of on-market drugs and has impeded clinical application of potentially life-saving new therapies.

Insulin is one such therapeutic that can undergo both physical and chemical aggregation. Insulin is a 51-residue protein that has been the cornerstone of diabetes treatment for over a century.[5] Through physical associations and covalent bond formation, monomeric insulin can form soluble hexamers and dimers, as well as insoluble fibrils, aggregates, and covalent polymeric species.[2,6,7] Insoluble species present a major hurdle because they are no longer therapeutically active. Such aggregation events are exacerbated by mechanical agitation in solution, which is a routine occurrence during shipping and transport.[8] Therefore, the development of novel insulin formulations with enhanced stability and without the need for continuous cold-chain delivery is highly desired.

The use of neutral, hydrophilic polymers has been investigated as a strategy to reduce or inhibit the aggregation of therapeutic proteins and peptides. For example, the covalent modification of protein therapeutics with polyethylene glycol (PEG), an approach known as PEGylation, is a widely explored method to increase the stability and circulation half-life of biopharmaceuticals,[9] including for anti-aggregation applications with insulin.[10] However, an emerging body of literature has demonstrated the presence of anti-PEG antibodies in response to PEGylated therapeutics and has suggested that this modification is associated with increased immunogenicity and drug clearance.[11−13] Additionally, PEG is not biodegradable and can accumulate intracellularly.[14,15]

Received: November 5, 2022
Revised: November 10, 2022
Published: December 13, 2022

An approach that has been proposed to circumvent some of these challenges is supramolecular PEGylation, which is the modification of proteins with PEG chains through non-covalent interactions. One such approach demonstrated PEG conjugated to a cucurbit[7]uril (CB[7]) macrocycle, which preferentially recognizes and binds the N-terminal phenylalanine residue on insulin (K_a = 1.5 × 10^6 M^{-1}),[16] as a dynamic non-covalent route to stabilize insulin formulations.[17] Covalent modifications of insulin at this site are not typically associated with a loss of activity,[18] and routes for supramolecular formulation of insulin with CB[7]-PEG likewise demonstrated activity of aged insulin that was indistinguishable from freshly dissolved insulin.[17] Prior work by Urbach and coworkers demonstrated binding of CB[7] to N-terminal aromatic residues arising from the inclusion of the R-group as a guest within the cavity of the CB[7] macrocycle in concert with electrostatic interactions between the protonated N-terminal amino group and the electronegative carbonyl-lined portal.[16] The affinity and rapid exchange dynamics of the insulin−CB[7] interaction ensures that the complex remains bound at formulation concentrations in a vial, but rapidly dissociates once diluted in the body. Accordingly, this approach might reduce the risks associated with covalent PEGylation by separating the presentation of PEG from the therapeutic. This approach has thus been used to stabilize fast-acting insulin monomers and insulin−pramlintide co-formulations.[18,19] The affinity for supramolecular recognition can also be tuned by inclusion of higher affinity non-natural guests to afford extended pharmacokinetics.[20]

The growing issues surrounding the use of PEG still demonstrate a clear need for an expanded toolbox of biocompatible and relatively inert polymers for use in protein formulation, nanomedicine, biomedical device coatings, and other applications that are thus far dominated by PEG. Hydrophilic, neutral, and saccharide-bearing structures have been explored as protein stabilizers and as PEG alternatives.[21] For example, glycosaminoglycans were covalently conjugated to insulin via non-specific *N*-hydroxysuccinimide (NHS)-ester/maleimide chemistry.[22] The conjugation site was found to affect activity and the conjugates had variable efficacy in rodent models. Saccharide-functionalized methacrylate has also been conjugated to an insulin lysine residue, but these structures are not biodegradable.[23,24] More recently, hydrolyzable scaffolds were explored using polylactide backbones appended with saccharides and zwitterionic ammonium betaines.[25] Insulin aggregation upon shaking was mitigated when the solution was supplemented with a 10-fold excess of the polymer.

Similar to saccharides and PEG, zwitterionic structures have advantageous properties in that they are hydrophilic but overall neutral. Methacrylate-based sulfobetaine nanogels were shown to inhibit formation of toxic lysozyme nanofibrils.[26] Despite these collective efforts, the need remains for a material that is simple and economical to prepare, active in low concentration, biodegradable, and based on naturally occurring chemical motifs.

Kramer et al. previously reported a series of hydrophilic, neutral, or zwitterionic polypeptide structures based on the naturally occurring amino acid methionine (Met).[27−30] PolyMet (PMet) is attractive for biomedical applications because it can be prepared via a simple, scalable, and low-cost polymerization reaction.[28] Furthermore, Met itself is a relatively economical amino acid because it is used in livestock feed.[31] Compared to PEG and other previously explored materials, PMet is particularly appealing because it can degrade into a naturally occurring amino acid.

Met's thioether moiety can be oxidized to the sulfoxide or sulfone forms, or alkylated with a variety of electrophiles to generate stable sulfonium salts.[28,30] Sulfonium salts of Met are naturally occurring in foods and in cellular methylation processes.[32−36] Alkylation with a carboxymethyl group (Met^{CM}) yields a zwitterionic structure. Such alkylation reactions are simple, quantitative, can be performed in organic and aqueous solvents, and are chemoselective in peptides and proteins at low pH because Met nucleophilicity is not dependent upon protonation.[27−29] Oxidation of Met to Met sulfoxide (Met^{O}) in proteins is well-known and the reaction is believed to scavenge reactive oxygen species.[37−39] Met likely serves to prevent irreversible oxidation at other critical residues because Met^{O} can be reduced back to Met with natural enzymes.[40] Furthermore, polymers of Met^{O} have dimethyl sulfoxide (DMSO)-like solubilizing properties and are reported to be nontoxic at 2.0 g/kg when administered intravenously to mice.[41] $PMet^{O}$ is readily prepared from PMet by simple treatment with hydrogen peroxide. Due to the ease of preparation, we sought to explore the anti-aggregation properties of these neutral, hydrophilic, PMet structures based on naturally occurring building blocks.

To capture the beneficial properties of supramolecular protein formulation, a single CB[7] macrocycle was appended as a terminal group to different PMets, enabling evaluation of this approach for protein stabilization as a formulation additive. This strategy resulted in very high insulin stability, with no aggregation even upon continuous agitation for >100 h. We have demonstrated the ability to capture the functional benefits of PEG using a biodegradable, non-cytotoxic polymer based on natural amino acid motifs as a formulation additive to inhibit protein aggregation.

EXPERIMENTAL SECTION

Instrumentation and General Methods. Reactions were conducted under an inert atmosphere of N_2, using oven-dried glassware unless otherwise stated. Hexanes and dichloromethane were purified by first purging with dry nitrogen, followed by passage through columns of activated 3 Å molecular sieves, while being purged. Tetrahydrofuran (THF) was purified by passage through columns of activated alumina. Glassware was oven-dried at 120 °C. Infrared spectra were recorded on a Bruker Alpha ATR-FTIR spectrophotometer. Deionized water (18 MΩ cm) was obtained by passing in-house deionized water through a Thermo Scientific MicroPure UV/UF purification unit. Tandem gel permeation chromatography/light scattering (LS) was performed on an Agilent 1260 Infinity liquid chromatograph pump equipped with a Wyatt DAWN HELEOS-II LS detector and Wyatt Optilab T-rEX refractive index detectors. Circular dichroism (CD) measurements of the polypeptide solutions were recorded in quartz cells with a path length of 0.1 cm, on a JASCO J-1500 CD spectrophotometer. A Tecan Infinite M200 plate reader was used for absorbance assays.

Preparation of CB[7] Functional Poly(L-methionine). L-Methionine-*N*-carboxyanhydride, Met NCA, was prepared and polymerized according to a published procedure, with minor modifications as described in the Supporting Information.[42] Molecular weights were determined by ^{1}H NMR end-group analysis as previously reported.[42] After polymerization, the polypeptides were precipitated into 1 mM aqueous HCl, collected by centrifugation, and the precipitate was washed with two portions of deionized water. In THF, the polypeptides (10 mg/mL) were reacted with 5 equiv of pentynoic acid NHS ester and 1 equiv of sodium bicarbonate for 16 h at ambient temperature. The solutions were split for alkylation or

https://doi.org/10.1021/acs.biomac.2c01319
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oxidation without further purification. For oxidation, THF was removed and the polypeptide was subjected to 30% H_2O_2 with 1% AcOH (20 mg/mL) at 4 °C for 45 min. 1 M sodium thiosulfate was then added dropwise until the evolution of bubbles ceased for alkylation and an equal volume of water to THF was added to the THF−polypeptide solution from the previous step. Either bromoacetic acid or methyl iodide (3 equiv) was added and the reaction stirred for 48 h. All polymers were transferred to 2000 MWCO dialysis tubing and dialyzed first against 0.10 M NaCl, then Milli-Q water for 48 h, and finally lyophilized. For CB[7] functionalization, CB[7]-N_3 was synthesized and attached to $PMet^{CM}_{80}$-Alk via copper-catalyzed click chemistry according to previously published methods.[43] See the Supporting Information for representative procedures. The final product was transferred into dialysis tubing (MWCO = 3500) and dialyzed against Milli-Q water for 2 days and lyophilized to dryness.

Aggregation Assays. Peptide therapeutics were prepared[44] and aggregation was assessed[7,17] using previously published methods. Insulin or calcitonin solutions were prepared in phosphate-buffered saline (PBS) at pH = 7.4 at a final concentration of 1 mg/mL or 0.5 mg/mL respectively, and with and without the addition of 1.5 molar equiv of CB[7]-PMet for recombinant human (rHu) insulin and 5 molar equiv for human calcitonin (hCT). Samples were plated in a clear 96-well plate (Thermo Scientific Nunc) at a volume of 150 μL per well (n = 5 wells/group) and sealed with an optically clear and thermally stable seal. The plate was shaken continuously at 37 °C and absorbance readings at 540 nm were collected every 6 min duration of the experiment as reported. Absorbance values were subsequently converted to transmittance.

Calcitonin Modification. hCT (24 mg, 7.02 μmol) and $NaBH_3CN$ (5 equiv, 35.1 μmol, 2.2 mg) were dissolved in 2.7 mL citric acid buffer (pH = 6.1). 0.5 M *tert*-butyl 4-formylbenzylcarbamate in DMSO (2 equiv, 14.04 μmol, 28 μL) or 0.5 M *tert*-butyl 4-formylphenylethylcarbamate in DMSO (2 equiv, 14.04 μmol, 28 μL) was added into the system and stirred for 4 h at room temperature. The reaction solution was diluted with 1:1 acetonitrile/water (10.0 mL) and lyophilized. The lyophilized powder was deprotected in 1 mL of a solution of 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane for 10 min. The solution was further diluted with 3 mL of water, purified via preparative high- performance liquid chromatography and lyophilized to provide pure hCT A1 and hCT A2 samples.

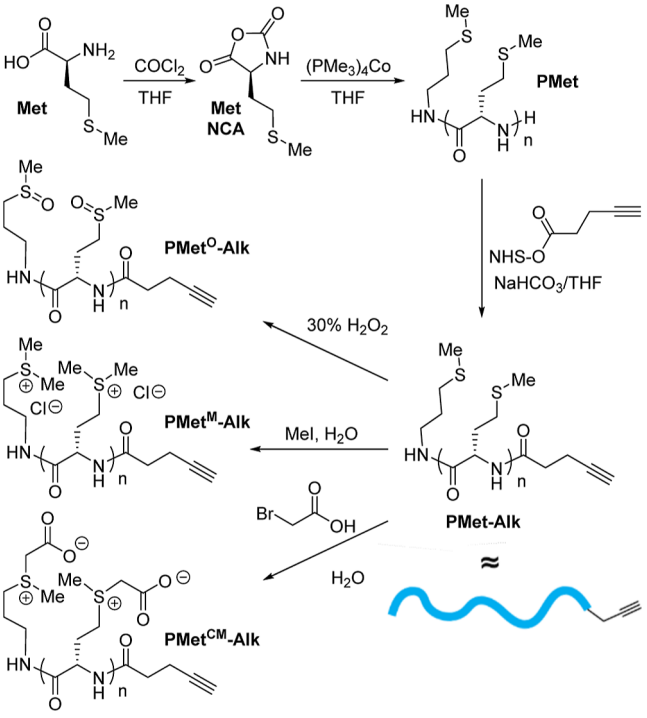
Cellular Viability. MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin. Upon reaching sufficient confluency, cells were trypsinized and suspended in medium. Cells were loaded 5 × 103 per well in a clear flat bottom 96-well plate. 24 h after plating, cells were treated with varied amounts of polypeptide for 24 h, then analyzed using a CCK-8 assay from Dojindo Molecular Technologies, Inc. The CCK-8 reagent was allowed to incubate with cells for 4 h prior to absorbance reading at 450 nm.

Protease Degradation. All digestions were performed with 40 μg of AF350-PMetCM80 at a final volume of 30 μL. 0.05% Gibco trypsin was used at varied enzyme/substrate (E/S) ratios in a reaction buffer of 50 mM NH_4HCO_3 at a pH of 8. Methionine aminopeptidase 2 (METAP2 from R&D Systems) was used at varied E/S ratios in a reaction buffer of 50 mM Hepes, 100 mM NaCl, 0.1 mM $CoCl_2$ at a pH of 7.4. Protease K was obtained from Thermo Fisher (#AM2542). Digestions with proteinase K (Pro K) were performed in 1× PBS at a pH of 7.4 with varied E/S ratios. Papain digestions were performed in McIlvaine buffer at a pH of 6 and preincubated with glutathione for 5 min before adding polypeptide. All digestions were allowed to proceed for between 24 h and 7 days at 37 °C sheltered from light.

RESULTS AND DISCUSSION

To investigate the use of PMet as the polymeric component of a supramolecular protein stabilization reagent, we prepared a panel of PMet chain lengths ranging from 12 to 320 residues. These were generated via polymerization of Met NCA (Scheme 1). Using previously reported methods, Met NCA was prepared from Met on multigram scale in one step.

Scheme 1. Preparation of Oxidized and Alkylated PMet Structures[a]



[a]Met was converted to Met NCA, which was polymerized to PMet using a Co^0 catalyst. After end-group functionalization, PMet-Alk was converted to the sulfoxide or the cationic or zwitterionic sulfoniums as shown.

Subsequent metal-catalyzed polymerizations to yield PMet were typically complete in <1 h and in quantitative yield (see Supporting Information).[42] PMet was easily separated from the catalyst by precipitation into acidic water and was collected as a white powder.

The PMet amino-terminal was functionalized with an alkyne moiety (PMet-Alk) via an NHS ester compound prepared from commercially available pentynoic acid. In the same pot, PMet-Alk was then either oxidized or alkylated. To generate neutral $PMet^O$-Alk, PMet-Alk was treated with H_2O_2 for 45 min at 4 °C (Scheme 1). Alternatively, PMet-Alk was treated with 3 equiv of methyl iodide or bromoacetic acid for 16 h at ambient temperature to generate the cationic methyl or zwitterionic carboxymethyl sulfonium salts $PMet^M$-Alk and $PMet^{CM}$-Alk, respectively (Scheme 1). All polypeptide structures were readily soluble in water. $PMet^M$-Alk was selected as a cationic comparison to neutral $PMet^O$-Alk and zwitterionic $PMet^{CM}$-Alk. All PMet-Alk species were purified by dialysis against NaCl which served to exchange counterions to chloride, followed by Milli-Q water. After lyophilization, ^{1}H NMR data confirmed that all conversions were quantitative (see the Supporting Information).

The azide-bearing monofunctional macrocycle, CB[7]-N_3, was synthesized according to previously published methods.[45] Subsequently, PMet-Alks of varied structures were covalently conjugated to CB[7]-N_3 via copper-catalyzed click reactions utilizing copper(II) sulfate pentahydrate, sodium ascorbate, and *N*,*N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine (Scheme

https://doi.org/10.1021/acs.biomac.2c01319
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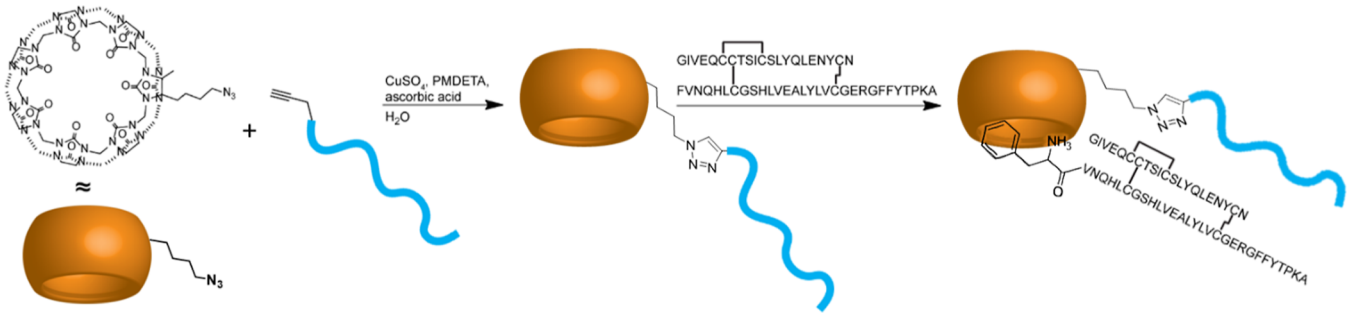
Scheme 2. Synthetic Scheme to Form CB[7]-PMet Conjugates via the Cu-Catalyzed Click Reaction of Azido-CB[7] and Alkyne-PMet[a]



[a]CB[7]-PMet can participate in host−guest complexation with the terminal Phe of human insulin.

2), following previously reported methods.[43] Reaction products were purified by dialysis and then lyophilized. Analysis by ^{1}H NMR indicated quantitative end-group modification and generation of the CB[7]-PMet panel (see the Supporting Information).

Aggregation of rHu insulin was assessed according to a reported procedure.[7,17] Briefly, 1 mg/mL insulin solutions were prepared in PBS buffer at pH = 7.4 with or without the addition of 1.5 molar equiv of CB[7]-PMets.[17] Samples were plated and sealed and then agitated continuously at 37 °C. Absorbance readings at 540 nm were collected every 6 min for 100 h. This wavelength was selected because it is removed from the typical absorbance of both protein and polymer, enabling protein aggregation to be monitored by light scattering and a concomitant reduction of sample transmittance.

We first examined medium chain lengths (80mers) of CB[7]-PMets in combination with rHu insulin. Solutions containing insulin with CB[7]-PMet$^{O}_{80}$ and CB[7]-PMet$^{M}_{80}$ became instantly turbid upon combination (Figure 1A). However, CB[7]-PMet$^{CM}_{80}$ maintained the same initial transmittance as that of pure rHu insulin, confirming formulation solubility. Based on this initial data, we chose to examine the full panel of chain lengths only for CB[7]-PMet^{CM} for their ability to inhibit insulin aggregation under stressed conditions.

Under continuous agitation at 37 °C, we observed that rHu insulin alone underwent rapid aggregation resulting in a substantial change in transmittance within the first few hours of the experiment. By contrast, rHu insulin in the complex with CB[7]-PMet^{CM} of chain lengths 80, 175, or 320 was stable with no evidence of aggregation over the 100 h period of agitation (Figure 1B). Shorter chain lengths of PMet^{CM} were less successful at preventing aggregation. The 25mer inhibited aggregation until ca. 50 h, whereas the 12mer offered even less stabilization. We verified that the effect was due to supramolecular modification of insulin with PMet^{CM} via the host−guest recognition because the same polymer without the conjugated CB[7] macrocycle (PMet^{CM}-Alk) offered no inhibition of insulin aggregation. CB[7] alone has already been shown to have no effect on rHu insulin stability.[17]

We speculate that differing solubilities of formulations from the neutral, cationic, and zwitterionic CB[7]-PMets can be rationalized by several considerations. The limited solubility of the cationic CB[7]-PMet$^{M}_{80}$ formulation is perhaps understood in the context of the clinically used insulin product, neutral protamine Hagedorn insulin. Neutral protamine

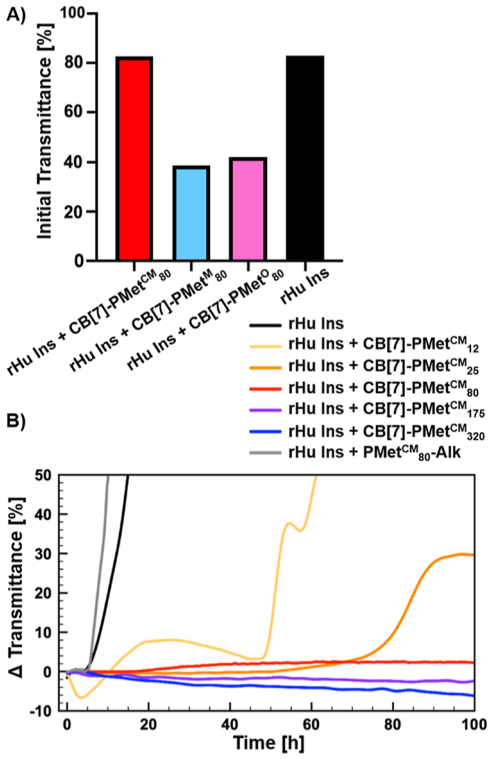


Figure 1. (A) Initial aggregation of rHu insulin alone or with addition of various CB[7]-PMet structures. (B) Aggregation over 100 h of rHu insulin or rHu insulin with CB[7]-PMet^{CM} of varied chain lengths complexed by host−guest chemistry or uncomplexed due to lack of CB[7] group.

Hagedorn insulin relies on electrostatic insulin aggregation from formulation with the cationic protein protamine. Similarly, cationic PMet^{M} could form electrostatic complexes with anionic residues on insulin.

We were, however, surprised at the stark difference in rHu insulin formulations of neutral CB[7]-PMet$^{O}_{80}$ and zwitterionic CB[7]-PMet$^{CM}_{80}$. Factors resulting in the difference between PMet^{O} and PMet^{CM} could potentially result from the DMSO-like properties of PMet^{O}.[41] Structural studies of DMSO-dissolved insulin indicated that the protein takes on multiple conformations including polyproline II-type helices, disordered structures, and α-helices, which could result in insoluble material.[46]

The differing formulation solubilities of the PMets are not likely ascribed to differences in their chain conformations. We

https://doi.org/10.1021/acs.biomac.2c01319
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analyzed the secondary structures by CD spectroscopy and found that PMet^{O}, PMet^{M}, and PMet^{CM} 80mers yield very similar patterns indicative of disordered morphologies (Figure 2).[47,48] Because the effects cannot be ascribed to chain

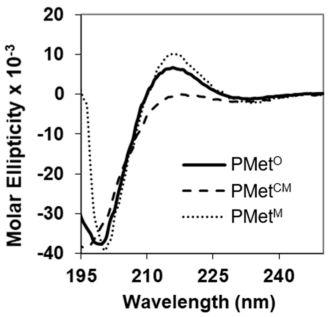

Figure 2. Aqueous CD analyses of 80-residue PMets at 20 °C.

conformation, we speculate that differing hydrogen bonding (H-bonding) and water ordering properties could play a role in insulin solvation. The sulfoxide structure takes on significant dipolar character,[49−51] and studies of DMSO−H_2O interactions indicate that one DMSO molecule forms two H-bonds, that the bonds are longer lived than water−water H-bonds, and that this induces linear ordering of water.[52−54] By contrast, spectroscopic and modeling data indicate that zwitterionic ammonium betaine polymers homologous to PMet^{CM} do not alter the structure of the H-bonded network of water molecules.[55−57] Water molecules at the polymer−material interface will be less oriented and similar to that of bulk water,[58] which could influence insulin H-bonding.

Next, we wanted to explore the stabilizing effects of CB[7]-PMet$^{CM}_{80}$ on other aggregation-prone proteins. We selected hCT (Figure 3A) as another model protein therapeutic. Because hCT lacks a terminal Phe for host−guest complexation, the N-terminal amine was selectively modified on-resin with a benzylic amine group using reductive amination chemistry (Figure 3B).[44] To optimize binding, two spacer lengths between the terminal amine and the benzyl ring were examined comprising either one or two methylene units (hCT A1 and A2, respectively). We examined aggregation behavior over 40 h using the methods previously described and with or without CB[7]-PMet$^{CM}_{80}$. Alone, hCT, hCT A1, and hCT A2 all underwent rapid aggregation as noted by a rapid increase in transmittance (Figure 3C). However, addition of CB[7]-PMet$^{CM}_{80}$ stabilized hCT A2 for at least 40 h with agitation, while hCT A1 was stabilized for ca. 34 h. Unmodified hCT lacking the terminal aromatic group was not stabilized by CB[7]-PMet$^{CM}_{80}$, indicating that supramolecular recognition of the protein by the CB[7] macrocycle is necessary to endow the protein with the anti-aggregation effects of the polymer.

Because insulin is often dosed by diabetic people multiple times each day over the course of a lifetime, it is ideal for formulation additives to be nontoxic and readily degraded and/or cleared. Therefore, we investigated the protease susceptibility and cytotoxicity properties of the lead anti-aggregation polymer, PMet$^{CM}_{80}$. We analyzed the cytocompatibility properties using a commercial CCK-8 assay and human epithelial cell line MDA-MB-231. We examined a broad concentration range from 0.1 to 5 g/L, and after a 24 h incubation period, PMet$^{CM}_{80}$ exhibited no statistically significant effect on cell viability at all concentrations studied

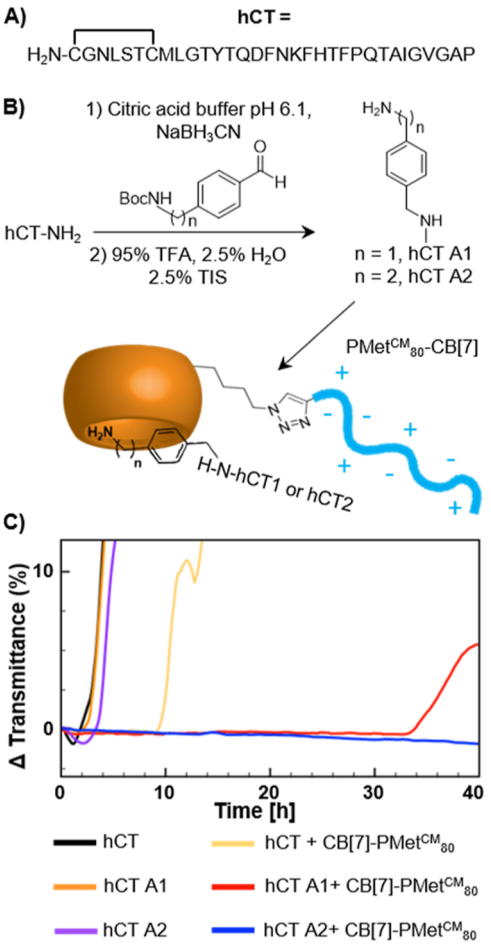

Figure 3. (A) Preparation of benzyl-functionalized hCT with one (hCT A1) or two (hCT A2) methylene spacers between the terminal amine. (B) Aggregation data for (B) hCT, hCT A1, and hCT A2 with and without CB[7]-PMet$^{CM}_{80}$ where only hCT A2 with the zwitterionic polypeptide showed resistance to aggregation.

(Figure 4A). CB[7] has an IC_{50} value of 0.53 ± 0.02 mM in Chinese hamster ovary cells and is tolerated in mice at up to 250 mg kg^{-1} intravenously or 600 mg kg^{-1} orally.[59] We speculate that the polypeptides will have low immunogenicity in vivo because a variety of zwitterionic polymers have avoided unwanted immune reactions and conjugates have dampened the response to known immunogenic proteins.[60−63] Overall, we expect this formulation to have excellent biocompatibility properties.

Next, we evaluated biodegradation of our PMet$^{CM}_{80}$ polypeptide polymer by four different proteases: trypsin, MetAP2, Pro K, and papain selected data are shown in Figure 4B−D and additional data is in the Supporting Information. Trypsin cleaves the peptide bond between the carboxyl group of arginine or lysine and the amino group of the adjacent amino acid, so we would expect no degradation.[64] MetAP2 catalyzes the hydrolytic removal of N-terminal Met residues,[65] so we were curious if the alkylated residues could be recognized despite the modification to the Met group. We chose Pro K and papain as two broad spectrum, non-specific proteases that we hypothesized might be promiscuous enough to digest Met^{CM} residues. Pro K is an endogenous serine protease in humans[66] and papain is a cysteine protease with similar activity to human cathepsins.[67,68]

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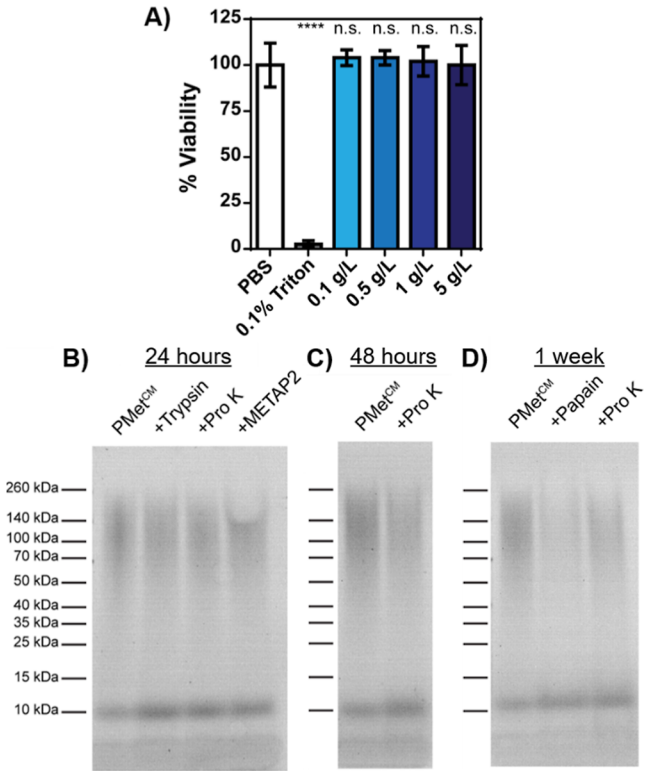

Figure 4. (A) Cell viability of MDA-MB-231 cells after treatment with $PMet^{CM}_{80}$. Data are represented as mean ± standard error. **** indicates $p < 0.0001$, and n.s. indicates not significant when compared to PBS control using Student's *t*-test. (B–D) Protease digestion of polypeptide polymer AF350-labeled $PMet^{CM}_{80}$ visualized on SDS–PAGE gels. (B) Digestion time of 24 h with an E/S ratio of 1:10 for trypsin and Pro K and 1:20 for METAP2. (C) Digestion time of 48 h with an E/S ration of 1:1 for Pro K. (D) Digestion time of 1 week with an E/S ratio of 1:1 for papain and Pro K.

Because zwitterionic $PMet^{CM}_{80}$ was resistant to many common staining methodologies, we end-functionalized with AF350-NHS to allow for in-gel visualization. After 24 h treatment with trypsin, Pro K, or MetAP2, the polypeptide fluorescent signal was only minimally decreased, suggesting negligible degradation (Figure 4B). Considering that we did not expect efficient cleavage from trypsin or MetAP2, this was not surprising. Therefore, we chose to explore further with only Pro K and papain. Increasing both the incubation time and the E/S ratio led to a decrease in the fluorophore signal in the 48 h Pro K digestion (Figure 4C). Finally, examination of the degradation of $PMet^{CM}_{80}$ after 1 week revealed partial degradation of $PMet^{CM}_{80}$ by Pro K and near complete proteolytic degradation by papain (Figure 4D). These data suggest that $PMet^{CM}_{80}$ will biodegrade and in vivo accumulation can be avoided.

Overall, we have developed conjugates of zwitterionic polypeptides with a supramolecular macrocycle that show potential for use as formulation additives to inhibit the aggregation of protein therapeutics. This zwitterionic polymer structure, $PMet^{CM}$, is derived from inexpensive natural amino acids and is readily synthesized via rapid and scalable NCA polymerization. Conversion of the natural amino acid Met to the zwitterionic sulfonium structure is simple and quantitative. We also examined neutral sulfoxide polypeptides and cationic sulfonium salts, but these were not efficient at inhibiting protein aggregation in the cases examined. At polymer lengths of 80mer or greater, zwitterionic $PMet^{CM}$ was efficient at preventing insulin aggregation, while shorter chain lengths had more limited impact. This zwitterionic polypeptide exhibited no cytotoxicity in a human cell line and was slowly degraded by non-specific natural proteases. We consider the slow degradation rate of $PMet^{CM}$ to be highly advantageous because the limited degradation and poor tissue clearance of formulation additives are accompanying challenges alongside aggregation in the development and distribution of protein therapeutics. Further studies on the in vivo application of these new therapeutic conjugates are underway.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.2c01319.

Detailed experimental procedures, spectral data, protease degradation, and related data (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

J.R.K. acknowledges financial support from the USA National Science Foundation via DMR-1848054 and CHE-1807651. M.J.W acknowledges funding support from JDRF (5-CDA-2020-947-A-N) and the American Diabetes Assocation (1-19-ACE-31). D.H.-C.C. acknowledges funding from USA National Institute of Health NIDDK DK120430.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge the lab of Prof. Michael Yu for use of their circular dichroism instrument.

■ ABBREVIATIONS

PEG, polyethylene glycol; Met, methionine; NCA, *N*-carboxyanhydride; NHS, *N*-hydroxysuccinimide; H-bonding, hydrogen bonding; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; Pro K, proteinase K; MetAP2, methionine aminopeptidase; PBS, phosphate-buffered saline

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