Communication

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Supramolecular Glycosylation Accelerates Proteolytic Degradation of **Peptide Nanofibrils**

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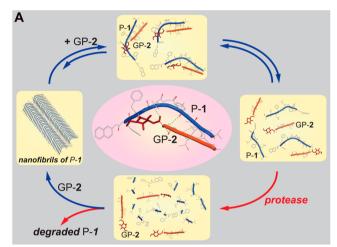
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Supporting Information

ABSTRACT: Despite the recent consensus that the oligomers of amyloid peptides or aberrant proteins are cytotoxic species, there is still a need for an effective way to eliminate the oligomers. Based on the fact that normal proteins are more glycosylated than pathogenic proteins, we show that a conjugate of nucleobase, peptide, and saccharide binds to peptides from molecular nanofibrils and accelerates the proteolytic degradation of the molecular nanofibrils. As the first example of the use of supramolecular glycosylation to dissociate molecular nanofibrils and to accelerate the degradation of peptide aggregates, this work illustrates a new method that ultimately may lead to an effective approach for degrading cytotoxic oligomers of peptides or aberrant proteins.

Teurodegenerative diseases represent a great challenge in modern medicine, and currently there is no effective treatment for these diseases, especially Alzheimer's diseases (AD). Recent conceptual advances in AD suggest that β amyloid $(A\beta)$ oligomers² are the most neurotoxic species³ or the initiators⁴ of the A β cascade, which has stimulated the development of approaches to prevent the early assemblies of amyloid peptides or aberrant proteins⁵ and to generate neuroprotective plaques.⁶ Another promising approach is to accelerate the proteolysis of the pathogenic proteins or peptides. Recently, Landreth and co-workers reported that apolipoprotein E (ApoE) promotes the degradation of soluble $A\beta$ both inside and outside of cells. However, the oligomers of these pathogenic proteins or peptides are usually inaccessible by endogenous proteases in vivo due to their aggregated state. Thus, it is necessary to develop a new strategy to degrade the cytotoxic oligomers. Based on the early reports by Prusiner that pathogenic prion proteins (PrPSc), compared to normal prion proteins (PrP^C), contain decreased levels of glycans, we reckon that the glycosylation of a pathogenic peptide may reduce its ability to aggregate, thus allowing the peptide to be degraded by proteases.

To test the above hypothesis and to avoid the synthetic difficulty associated with glycosylation, we decide to examine a process that we term "supramolecular glycosylation-assisted proteolysis" (sGAP). As shown in Figure 1A, a conjugate containing saccharide (GP-2) is added to the nanofibrils of a small peptide (P-1, which serves as a model system of amyloids of pathogenic peptides or proteins). The binding of GP-2 with P-1, via non-covalent interactions, disturbs the nanofibrils of P-1 and produces monomeric P-1, which acts as the substrate for



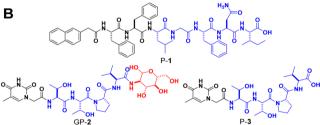


Figure 1. (A) Illustration of supramolecular glycosylation-assisted proteolysis, accelerating the degradation of molecular nanofibrils via supramolecular interactions. (B) Molecular structures of the selfassembling peptide P-1 and its binding partner conjugated with (GP-2) or without (P-3) a saccharide.

proteolytic degradation. Unlike P-1, the protease-resistant conjugate GP-2 re-enters the cycle to promote the dissociation of the nanofibrils of P-1. Thus, the overall effect of GP-2 is to non-covalently attach a saccharide to P-1 and to facilitate the liberation of free P-1 from the nanofibrils of P-1, thus accelerating the proteolytic degradation of the nanofibrils of P-1.

Based on the concept illustrated in Figure 1A, we design a pair of molecules to validate our hypothesis. We choose two short binding peptide sequences, Leu-Gly-Phe-Asn-Ile (LGFNI) and Thr-Thr-Pro-Val (TTPV), to establish intermolecular non-covalent interactions based on the known heterodimerization of these two sequences.9 To ensure LGFNI self-assembles, we covalently conjugate 2-(naphtha-

Received: June 7, 2015 Published: August 3, 2015 len-2-yl)acetic-Phe-Phe (Nap-FF), a well-established motif for self-assembly, 10 to LGFNI to form a heptapeptide derivative, P-1. To reduce the tendency of self-assembly of the TTPV motif, we covalently link thymine and glucosamine at the N-terminal and C-terminal of TTPV, respectively, to form GP-2. Our results confirm that a glycoconjugate made of nucleobase, peptide, and saccharide GP-2 binds to P-1 even when P-1 is assembled, disrupting the nanofibrils in the hydrogel matrix of P-1. This binding between GP-2 and P-1 not only promotes gel-sol transition but also accelerates the proteolytic degradation of the nanofibrils of P-1. As the first example of using a glycoconjugate to promote the degradation of molecular nanofibrils, this work contributes useful insight for ultimately developing a supramolecular glycosylation approach for degrading cytotoxic oligomers of peptides or aberrant proteins.

Using solid-phase peptide synthesis (SPPS),11 we first synthesize conjugates P-1 and P-3 from 2-chlorotrityl chloride resin and N-Fmoc-amino acids with protected side chains. To obtain the conjugate GP-2, we use the coupling reagent HBTU/DIEA to connect D-glucosamine to the side-chainprotected P-3, followed by deprotection of the tert-butylprotected Thr side chain. After the use of reversed-phase highperformance liquid chromatography (HPLC) for purification, we obtain the target molecules P-1, GP-2, and P-3. Molecule GP-2 differs from P-3 by only a single saccharide (i.e., glucosamine) at the C-terminal. We expect that the saccharide motif increases the solubility and biostability of GP-212 compared to P-3. On the other hand, P-3 also serves as a non-glycosylated control of GP-2.

After their synthesis, we examine the ability of these three molecules to self-assemble in water. Since one of the consequences of the self-assembly of small molecules in water is to form supramolecular hydrogels, 13 we use hydrogelation as a simple assay to report the molecular self-assembly in water.¹ We find that P-1 self-assembles to form a hydrogel at a low concentration in water (622 μ M, pH = 6.4) overnight (Figure 2A). Interestingly, the gelation concentration of P-1, by mass, is

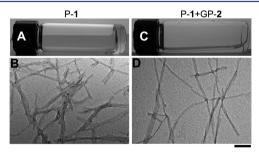


Figure 2. Optical images and corresponding TEM images of (A,B) hydrogel of P-1 and (C,D) solution of P-1 with 4 equiv of GP-2. Hydrogel of P-1 is at a concentration of 622 μ M (0.063 wt %) and pH = 6.4 in 300 μ L of H₂O. After addition of 50 μ L (4 equiv) of GP-2 (pH = 6.4) for 7 days, the corresponding optical images were taken. Scale bar is 100 nm.

637 μ g/mL, which is comparable with the aggregation concentrations¹⁵ of A β peptide (500 μ g/mL)¹⁶ and polyQ (105 μ g/mL).¹⁷ Transmission electron microscopy (TEM) reveals that the hydrogel consists of nanofibrils of P-1 that are 9 ± 2 nm in width (Figure 2B), which act as the hydrogel matrices. Unlike P-1, both GP-2 and P-3 show excellent solubility in water at the same conditions. Moreover, the TEM

images of the solutions of GP-2 and P-3, even at 16.6 mM (Figure S4) show hardly any ordered nanostructures. These results confirm that GP-2 and P-3 are unable to self-associate to form homotypic nanoscale assemblies.

We use isothermal titration calorimetry (ITC) to quantify the interactions of P-1 with GP-2 (or P-3). As listed in Table 1,

Table 1. ITC Analysis of GP-2 or P-3 Binding to P-1 in PBS Buffer^a

titrant	2.98 mM GP-2	2.98 mM P-3
titrand	149 μM P- 1	149 μM P- 1
ΔH (kJ/mol)	-17.21 ± 0.17	-13.09 ± 0.13
n	1.04 ± 0.01	1.38 ± 0.01
$K_{\rm d}$ (M)	$(2.615 \pm 0.026) \times 10^{-4}$	$(2.351 \pm 0.024) \times 10^{-4}$
$-T\Delta S$ (kI/mol)	-3.24 ± 0.03	-7.62 ± 0.08

 $^{a}\Delta H$ = change in enthalpy, n = number of binding sites, $K_{\rm d}$ = dissociation constant, $-T\Delta S$ = change in entropy.

we obtain the thermodynamic parameters of the interactions using an independent binding mode. Binding of GP-2 (or P-3) with P-1 produces an enthalpy change of -17.21 kJ/mol (or -13.09 kJ/mol), indicating that it is an exothermic reaction. The dissociation constant (K_d) is 261.5 μ M between P-1 and GP-2, suggesting sufficient interactions between P-1 and GP-2. In addition, K_d between P-1 and P-3 is 235.1 μ M. These results indicate that the interaction between P-1 and P-3 is slightly tighter than that of P-1 and GP-2. Furthermore, the binding ratio between GP-2 (or P-3) and P-1 is about 1:1 (Table 1), which is consistent with a previous report.

To test whether GP-2 would reduce the self-assembly ability of P-1, we add a solution of GP-2 (15 mM, pH = 6.4, 50 μ L) to the hydrogel of P-1 (622 μ M, pH = 6.4, 300 μ L), giving a final molar ratio of GP-2 to P-1 of 4:1. The resulting hydrogel starts to collapse on the first day and becomes completely fluid on the third day (Figure S5). TEM indicates that the amount of aggregates in the solution (Figure 2C) on the seventh day decreases dramatically, and the diameters of the nanofibrils (with an average width about 5 ± 2 nm, Figure 2D) are almost half of those in the hydrogel of P-1 (Figure 2B). The thinner nanofibrils and less-entangled networks confirm that the binding between GP-2 and P-1 disrupts the nanofibrils of P-1 and causes the gel-sol transition.

To further quantify the effect of GP-2 binding with P-1, we investigate the rheological properties of the hydrogel of P-1 after the addition of different ratios of GP-2 (or P-3). We first prepare the hydrogel of P-1 (622 μ M, pH = 6.4, 300 μ L) and then add GP-2 or P-3 (pH = 6.4, 50 μ L, in 3.7, 7.5, or 15 mM) to make final molar ratios of GP-2 (or P-3) to P-1 of 1:1, 2:1, or 4:1. We also add H_2O (pH = 6.4, 50 μ L) to the hydrogel of P-1 as a control. After 7 days of incubation, we test their rheological properties (Figure S7) and summarize the maximum storage moduli (max G') of the mixtures in Figure 3. The addition of GP-2 results in the most significant drop of the max G' of hydrogel of P-1. The G' decreases in a dosagedependent manner. The hydrogel of P-1 becomes weaker with increasing molar ratios of GP-2 to P-1. For the case of 4 equiv of GP-2, G' = 0.06 Pa, which is about 300 times smaller than that of hydrogel of P-1 (18.4 Pa, Figure 3). This feature is consistent with monomeric binding between P-1 and GP-2. This monomeric binding is critical because its reverse reaction should provide monomeric P-1 as a substrate for proteolysis. However, upon treatment with H_2O , the G' of hydrogel

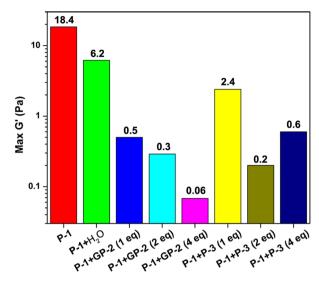


Figure 3. Maximum storage moduli of the hydrogels of P-1 and P-1 + H_2O , the solutions of P-1 + GP-2 (GP-2 in 1, 2, or 4 equiv of P-1), and the solutions of P-1 + P-3 (P-3 in 1, 2, or 4 equiv of P-1).

(Figures S5 and S7) decreases only slightly (e.g., a factor of 3) compared to that of the hydrogel of P-1, likely due to dilution. These results confirm that GP-2 breaks up the aggregation of P-1 via monomeric binding. After the addition of 1 equiv of P-3, the G' (2.4 Pa) is of the same order of magnitude as the G' measured after the addition of H_2O . Apparently, P-3 exhibits much less ability to break up the assemblies of P-1. In fact, the addition of 4 equiv of P-3 results in the G' (0.6 Pa) of the mixture being higher than in the case of the addition of 2 equiv of P-3 (0.2 Pa). This kind of "bell-curve" ¹⁸ implies that P-3 itself may aggregate in the presence of P-1. These results suggest that incorporation of the saccharide in the binding partner of P-1 is critical.

After confirming the binding of P-1 and GP-2, we examine whether GP-2 would accelerate proteolytic degradation of nanofibrils of P-1. We first assess the proteolytic stability of P-1, GP-2, and P-3 by incubating the solution of P-1, GP-2, or P-3 (0.2 mg/mL in HEPES buffer) with proteinase K (a powerful protease, 3.2 U/mL) at 37 °C for 24 h. As shown in Figure 4A, P-1 undergoes complete proteolysis upon treatment with protease K at 24 h. Liquid chromatography—mass spectrometry

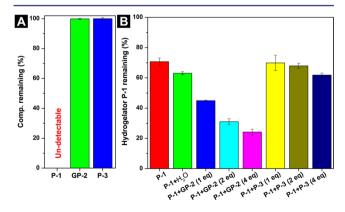


Figure 4. (A) Compound P-1, GP-2, or P-3 remaining after treatment with proteinase K in HEPES buffer (3.2 U/mL) at $37\,^{\circ}$ C for 24 h at an initial concentration of 0.2 mg/mL. (B) Hydrogelator P-1 remaining in the mixtures of P-1 and GP-2 (or P-3) after treatment with proteinase K (3.2 U/mL) at $37\,^{\circ}$ C for 24 h.

(LC-MS) displays only one peak of NapF after 24-h incubation of P-1 with protease K, agreeing with previous reports demonstrating that proteinase K is an endopeptidase. 19 In contrast to P-1, GP-2 and P-3 exhibit excellent proteolytic stability when being incubated with proteinase K (99.9 \pm 0.4% of GP-2 or $100.1 \pm 0.6\%$ of P-3 remains after 24 h). Apparently, the peptide epitope^{9b} dictates the proteolytic stability (or instability) of the conjugate P-1, GP-2, or P-3. Second, we examine the proteolytic stability of the nanofibrils of P-1 in the hydrogel of P-1. As shown in Figure 4B, there is 70.7 + 2.4% of P-1 remaining in the hydrogel state (622 μ M. pH = 6.4) after the treatment with protease K at 24 h. This result is consistent with the resistance of aggregates of peptides to proteolysis, as is the case with amyloid fibrils, ²⁰ because the self-assembly of P-1 in the hydrogel state greatly reduces the exposure of proteolytic sites of P-1 to proteinase K. The addition of H₂O slightly dilutes the hydrogel of P-1, resulting in $63.1 \pm 1.0\%$ of P-1 remaining undigested. In addition, the hydrogel of P-1 incubated with P-3 at different molar ratios exhibited similar values of hydrogelator P-1 remaining, 69.9 ± 5.1%, $68.0 \pm 1.6\%$, or $61.9 \pm 1.3\%$ for 1, 2, or 4 equiv of P-3, respectively. These values are comparable with those obtained upon addition of H2O, indicating that P-3 has little impact on the proteolytic stability of the nanofibrils of P-1. However, upon treatment of the hydrogel of P-1 with GP-2, the hydrogel of P-1 was much more susceptible to proteolysis. Increasing the molar ratio of GP-2 and P-1 from 1:1 to 4:1 results in dramatically increased degradation of P-1—the remaining amount of P-1 decreases from $45.0 \pm 0.4\%$ to $24.2 \pm 2.0\%$. This result agrees with TEM and rheological measurements indicating that GP-2 promotes the dissociation of the nanofibrils of P-1. The different abilities of GP-2 and P-3 for promoting the dissociation and the proteolysis of the nanofibrils of P-1 confirm that the conjugation of the saccharide is essential in the process of accelerating the proteolytic degradation of the nanofibrils of P-1.

In conclusion, based on the supramolecular interactions between peptide epitopes in water, we demonstrate a new concept of supramolecular glycosylation that uses saccharides to bias the equilibrium of self-assembly in the direction of dissociation, which generates monomeric peptides for proteolysis. As natural building blocks for biopolymers, nucleobases and saccharides not only reduce the self-assembly ability of the conjugate itself but also increase the biostability of the resulting conjugate. 9b,21 While the presence of saccharide is essential, the necessity of the nucleobase remains to be determined. In principle, more hydrophilic amino acid residues (e.g., Lys or Glu) may be introduced into the conjugate, but it is critical to maintain the proteolytic stability of the conjugate. Moreover, a key prerequisite for the design in Figure 1 is that the K_d values for the binding of GP-2 and P-3 to P-1 are less than the apparent saturation concentration/gel point of P-1. This novel and facile approach contributes a useful insight that may assist molecular design for degrading cytotoxic oligomers of peptides or aberrant proteins that are plausible causal agents of neurodegenerative diseases.

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05888.

Detailed NMR and LCMS characterizations, optical images of gelation tests, TEM images, and rheological measurements (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was partially supported by grant from NIH (R01CA142746) and NSF (DMR-1420382). We thank the Brandeis EM facility at Brandeis University. D.Y. is grateful for a scholarship from the Chinese Scholarship Council (2010638002). D.Y. thanks Mr. Richard Haburcak for grammatical correction.

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