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Glycopeptide Self-Assembly Modulated by Glycan Stereochemistry through Glycan-Aromatic Interactions

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ABSTRACT: Carbohydrates are often utilized to provide hydrophilicity and hydroxyl-based hydrogen bonds in self-assembling glycopeptides, affording versatile scaffolds with wide applicability in biomedical research. However, how stereochemistry of carbohydrates impacts the self-assembling process remains unclear. Here we have established a dimeric tyrosine-rich glycopeptide system for probing the corresponding hydrogelating behavior under the influence of site- and stereospecific glycosylations. Comparison of eighteen glycoforms bearing monosaccharides at Tyr_4 and $Tyr_{4'}$ shows that the glycopeptides with either α- or β-anomers exhibit contrary gelating abilities, when the glycan moieties contain axial hydroxyl groups. A high-resolution X-ray crystallographic structure of the β-galactose-containing gelator, along with other results from spectroscopic, microscopic and rheological experiments, indicate an unusual carbohydrate-aromatic CH-π bonding that promotes glycopeptide self-assembly. These mechanistic findings, particularly evidences obtained at the angstrom scale, illuminate an unconventional role that carbohydrates can play in building supramolecules. Potential biomaterials exploiting the CH-π bond-based stabilization, as exemplified by an enzyme-resistant hydrogel, may thus be developed.

1. INTRODUCTION

Self-assembly is an important process in living organisms that builds up polymeric structures from repetitive "building blocks", often through non-covalent interactions, introducing increased diversity and complexity.[1] Tubulins, for example self-assemble dynamically to form microtubules as long chains or filaments which serve as an essential component of the cytoskeleton.[2] Inspired by microtubules and other natural systems, such as actin filaments, clathrin and ribosomes, the use of self-assembly of proteins or peptides to produce novel materials has emerged as a fast-growing research area.[3] Particularly, because readily accessible peptide monomers could assemble to form various nanostructures including spheres, fibers or tubes,[4, 5, 6] peptide-based systems with useful properties have attracted significant attention, and been used in cancer therapy,[7] tissue engineering,[8] drug delivery,[9] as well as development of novel catalysts,[10] pigments,[11] semiconductors,[12] and so on.[13, 14]

Unlike synthetic polymers with fixed bond formation, self-assembling structures built with non-covalent interactions offer the potential to dynamically control polymerization and de-polymerization. However, with assemblies lacking covalent bonds, it is challenging to predict the organization of monomers, and the design of peptide self-assembly still is mostly empirical.[15] For example, to mimic the modification by glycans of eukaryotic proteins and improve their biocompatibility, glycosylation of peptide skeletons has been achieved, and is generally considered as a hydrophilic poly-hydroxyl motif.[16, 17] Evidence has been collected showing that the size and structure[18, 19] of glycans can influence the self-assembling ability of peptides, as well as the chirality and micromorphology of the resulting supramolecules.[20, 21] The carbohydrate components in these systems also provide O–H based hydrogen bonding along with steric effects that can interfere with other hydrophobic forces such as π-π interactions.[22, 23] This rationalization however, mostly neglects the stereochemistry of carbohydrates and lacks a structural basis to support the hypothetical properties. Thus it could not fully explain the distinct impacts glycosylation can generate in comparison to other hydroxyl-containing structures or hydrophilic modifications, such as phosphorylation.

Despite the challenges in development of glycopeptide-based supramolecular assemblies, recent examples of utilizing such biomimetic scaffolds for cell adhesion and proliferation,[24] human mesenchymal cell therapy,[25] immunosuppression[26] and DNA delivery[27] illustrate their potential,

and emphasize the need for studies leading to an understanding of the irreplaceable roles of glycans. Here, we describe our research on the self-assembling behavior and hydrogel forming ability under the influence of different monosaccharides of a glycopeptide sequence. These experimental results suggest that the glycan moiety with suitable stereochemistry may provide crucial stabilization to the supramolecular assembly through a distinct carbohydrate-aromatic interaction (**Figure 1**).

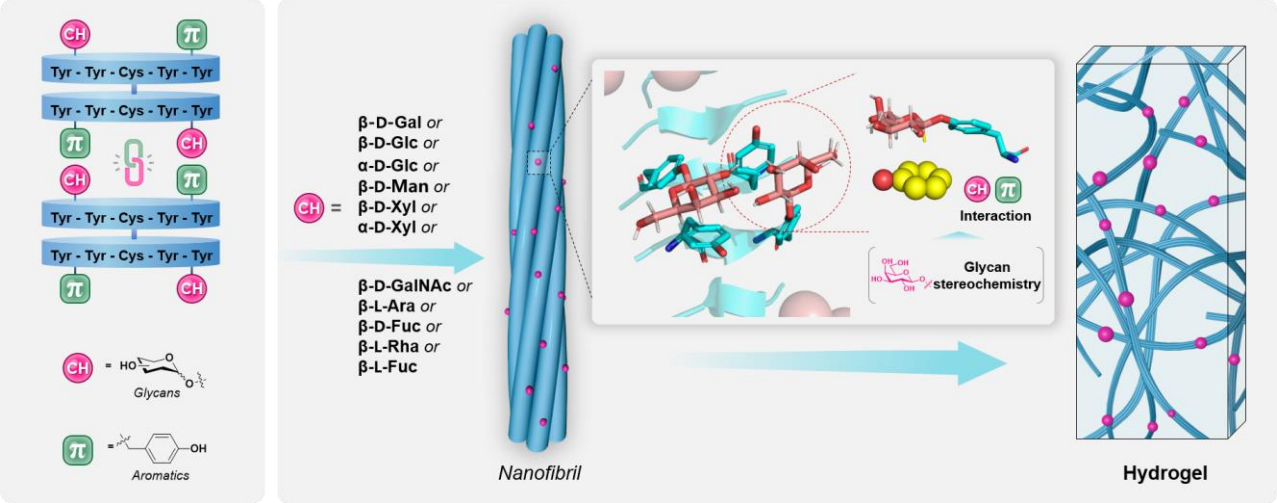


Figure 1. Glycopeptides may self-assemble to form hydrogels depending on the stereochemistry of contained monosaccharides. The self-assembly is presumably stabilized by carbohydrate-aromatic interactions.

2. RESULTS AND DISCUSSION

Experimental design and sequence screen. In order to explore the potential multifaceted functions of carbohydrates in a peptide self-assembly system, especially taking stereochemistry into account, a peptide sequence that shows self-assembling ability would be preferred as a start point. Such peptide could then be site-specifically modified using various monosaccharides with different stereochemistry of chiral hydroxyl groups and anomeric positions, and the properties of generated glycopeptides would be evaluated. As the tyrosine residue contains an aromatic moiety that is widely utilized in numerous self-assembling systems,[28] and a phenolic group that represents a common post-translation modification site for *O*-glycosylation,[29] a tyrosine-containing sequence could be used.

With these considerations in mind, we initiated our study on a sequence, Tyr-Tyr-Ala-Tyr-Tyr, which has previously been utilized in assembling supramolecular structures under UV irradiation.[30] We found that once a β-D-galactose (β-Gal) was appended to this pentamer peptide, the glycopeptide **1** (**Table 1**, entry 1) has good solubility in water and phosphate buffer (See **Figure S1** in the Supplementary Information). Drawing inspirations from a recent report from Lee *et al.*, where a disulfide bond-linked peptide dimer, [Tyr-Tyr-Cys-Tyr-Tyr]$_2$, can enjoy two-dimensional self-assembly,[31] a cysteine was introduced to derivatize peptide **1** in hope of altering its solubility while the size increases. After a survey of disulfide-linked dimers **2**-**5** derived from sequence Tyr-Tyr-Cys-Tyr-Tyr with every possible glycosylation site (entries 2-5), the glycopeptide [Tyr-Tyr-Cys-Tyr(β-Gal)-Tyr]$_2$ (**4**), was found to self-assemble under a heating/cooling regimen, forming a white opaque hydrogel in phosphate buffer (**Figure 2a**). In contrast, the glycopeptides **2**, **3** and **5** generated only a white suspension. This suggests that a glycosylation site is crucial for the self-assembly of peptide derivatives. To investigate the requirements of amino acid residues, an alanine scan of the sequence was carried out (**Table 1**, entries 6-10), which showed that peptide **10** could also form a hydrogel while peptides **6** and **8** generated white suspension and peptides **7** and **9** afforded a clear solution (**Figure S1**). These results suggest that tyrosine at sites 2, 4, and 5 play important roles in maintaining the self-assembling ability of the glycopeptides. Notably, peptide **8** with a thioether linkage does not support any organized self-assembly, indicating that the disulfide is required to construct the dimer with a preferred orientation. It is also noteworthy that the unexpected gel-forming properties of glycopeptides **4** and **10** offered additional perspectives for studying their supramolecular self-assembly, because gelation could be easily evaluated through unequivocal manifestation such as vial inversion, and quantitative indicators such as rheology or melting point. The broad applications of hydrogels in many fields, in particular biomaterials,[32] prompted us to further examine the hydrogel derived from glycopeptide **4**. Its critical gelation concentration, pH tolerance, and thermostability (**Figure S2**) were all considered, and the peptide sequence of **4** was used in studies of other glycopeptides bearing various monosaccharides.

Table 1. Screen of Glycopeptide Sequences

Entry	Sequences	Results[a]
1	Tyr-Tyr-Ala-Tyr(β-Gal)-Tyr **(1)**	clear solution
2	[Tyr(β-Gal)-Tyr-Cys-Tyr-Tyr]$_2$ **(2)**	white suspension
3	[Tyr-Tyr(β-Gal)-Cys-Tyr-Tyr]$_2$ **(3)**	white suspension
4	[Tyr-Tyr-Cys-Tyr(β-Gal)-Tyr]$_2$ **(4)**	hydrogel
5	[Tyr-Tyr-Cys-Tyr-Tyr(β-Gal)]$_2$ **(5)**	white suspension
6	[Tyr-Tyr-Cys-Tyr(β-Gal)-Ala]$_2$ **(6)**	white suspension
7	[Tyr-Tyr-Cys-Ser(β-Gal)-Tyr]$_2$ **(7)**	clear solution
8[b]	[Tyr-Tyr-Xaa-Tyr(β-Gal)-Tyr]$_2$ **(8)**	white suspension
9	[Tyr-Ala-Cys-Tyr(β-Gal)-Tyr]$_2$ **(9)**	clear solution
10	[Ala-Tyr-Cys-Tyr(β-Gal)-Tyr]$_2$ **(10)**	hydrogel

[a]Visualizable results after a heating-cooling operation (85 °C to r.t.) of glycopeptides in phosphate buffer (0.25% *w*/*w*). [b]Xaa = L-lanthionine.

Evaluation of glycopeptides with different glycoforms and characterization of generated hydrogels. Besides the galactosylated peptide **4**, seven glycopeptides (**11-17**) with various glycosylation patterns were prepared, including those containing readily available D-glucose (Glc), D-mannose (Man), and D-xylose (Xyl). In its most stable chair conformation, galactose has an axial 4-hydroxyl. Glc and Xyl however have no axial hydroxyls, and Man possesses an axial 2-hydroxyl group (**Figure 2b**). Following the hydrogel formation procedure, it was observed that with the exception of α-galactose- and α-mannose-modified peptides (**11**, **15** respectively), all these dimeric glycopeptides form white opaque hydrogels (**Figure 2a**). It appears that the stereochemistry of the glycosidic linkage influences the self-assembling capability when the glycan contains an axial -OH group. However, in the cases of monosaccharides that have all equatorial hydroxyl groups, both α- and β-anomers were capable of gelation.

To evaluate the gelating abilities of glycopeptides bearing more diverse glycans with varied stereochemistry, including D-N-Acetylgalactosamine (D-GalNAc), L-Arabinose (L-Ara), D- Fucose (D-Fuc), L-Rhamnose (L-Rha) and L-Fucose (L-Fuc), compounds **18-27** were synthesized. In all these cases only the β-anomer-modified peptide formed hydrogel under the gelation conditions. These results further demonstrate the impacts generated from the axial -OH group in the sugar moiety. Moreover, the example of GalNAcylated peptide proved that the 2-acetylamino group does not affect the hydrogel formation.

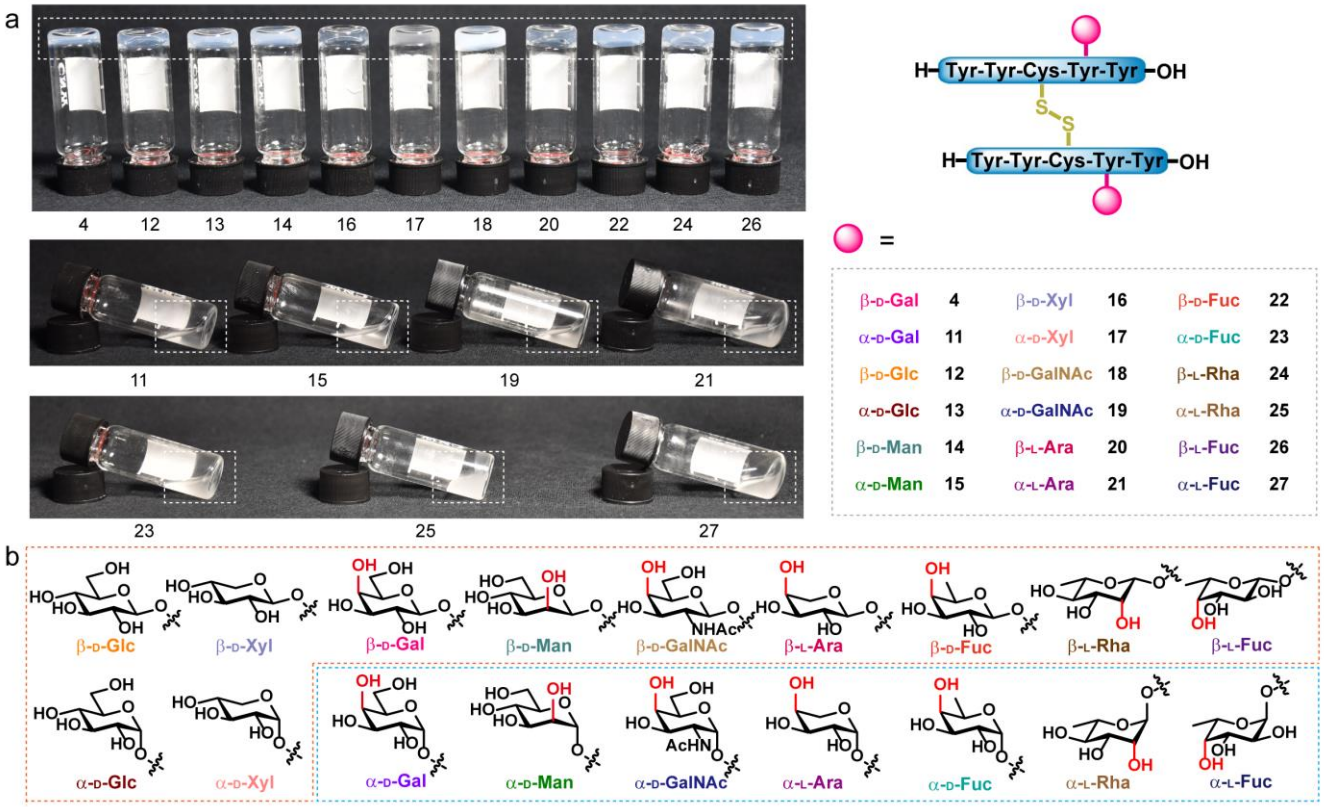


Figure 2. Gelation ability of glycopeptides with different glycan modifications. (a) Macroscopic images of hydrogels formed from self-assembling glycopeptides (top left) and precipitation (bottom left) in phosphate buffer (0.25%, *w*/*w*), and schematic representation of glycopeptide sequences evaluated (right). (b) Chemical structures of monosaccharides in evaluated glycopeptides that either form hydrogels (displayed in the orange box), or precipitate from the solution (displayed in the blue box). The axial hydroxyls are highlighted in red.

The morphology of hydrogels formed in this way was examined using transmission electron microscopy (TEM). As shown in **Figure 3**, under aqueous conditions glycopeptides **4**, **12**, **13**, **14**, **16**, and **17** assembled to nanofibers that intertwined to form nanobundles, which trap water inside a 3D network thus generating viscous gels. Interestingly, the nanobundles derived from **13** displayed a very organized bamboo-like morphology (**Figure 3d**), but in contrast, glycopeptides **11** and **15** only produced irregular aggregates in phosphate buffer, and no self-assembled nanostructures were observed. Similar results could also be observed for glycopeptides **18-27** (**Figure S3**). It is noteworthy that some short nanofibers could be observed for the sample prepared from glycopepitde **23** with α-D-Fuc modification, but no hydrogel was formed (**Figure S3f**).

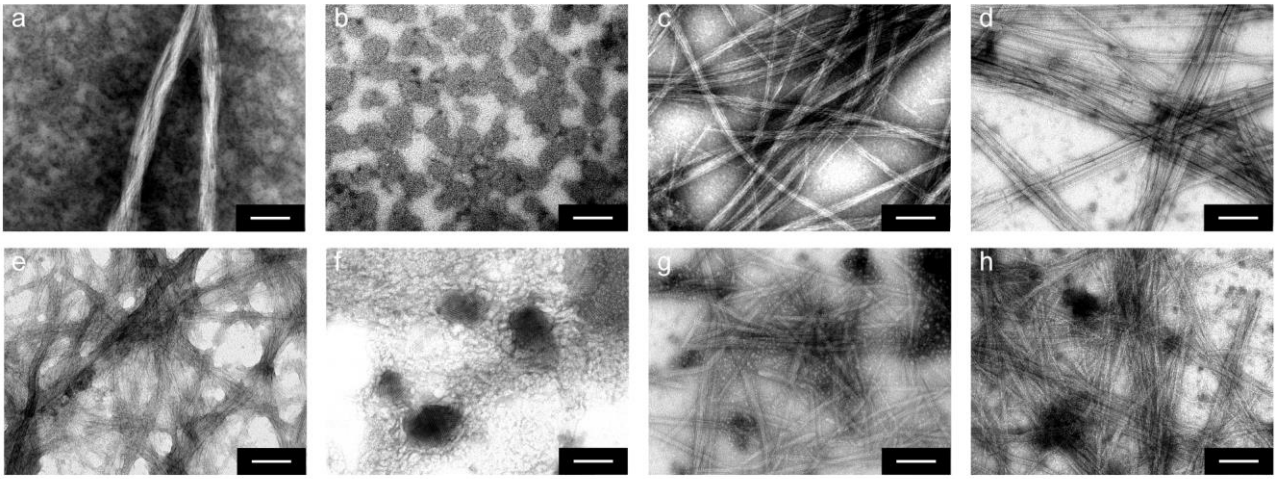


Figure 3. TEM images of glycopeptides **4** (a), **11** (b), **12** (c), **13** (d), **14** (e), **15** (f), **16** (g), and **17** (h) in phosphate buffer after a heating-cooling procedure. (a), (c), (d), (e), (g) and (h) show the nanofiber structures in consistence with their gel-forming ability. (b) and (f) show the irregular aggregates generated from glycopeptide **11** and **15**. Scale bars: 100 nm

Mechanistic investigations. In an attempt to rationalize the self-assembly process, a series of measurements were made of the representative glycopeptide **4** to obtain its structural information. ThT stain experiments showed that the fluorescence intensity increased when the sample became more concentrated in solution (**Figure 4a**), indicating that the peptides in the assembly may adopt a β-sheet structure.[33] The FTIR spectra of **4** (**Figure 4b**) display a shift of Amide I absorbance from 1645 cm^{-1} (dry powder) to 1628 cm^{-1} (D_2O hydrogel), suggesting the presence of β-sheet structures, possibly promoted by water,[34] in contrast to a reported case in which the addition of water results in a weakened signal of the β-sheet.[35] Unfortunately, neither the variable temperature CD measurements (**Figure S4**), nor the NMR study could provide more detailed structural information of the assembly in solution, but a close proximity between carbohydrates and two tyrosines, Tyr_1 and Tyr_2, as suggested by the NOESY experiments using deuterated DMSO as the solvent (**Figures S5-S11**), was observed.

In light of the previous reports using single-crystal X-ray diffraction to probe the mechanism of self-assembly,[36, 37, 38] and in particular, studying the ability of protein low-complexity domains (LCDs) to form gel-like networks based on a potential correlation between crystalline LCD segments and the primary fibrils formed in their assembly,[39, 40] we attempted to grow crystals of glycopeptide **4**. Despite the challenges in obtaining suitable crystals of glycoconjugates due to the flexible carbohydrate moiety,[41] we tested a large number of conditions commonly used in protein crystallization. Fortunately, we were able to obtain a single-crystal of glycopeptide **4**, and subsequently solved its X-ray structure in a high resolution (1.6 Å). The crystallographic data (see **Table S1** in the Supplementary Information; PDB code: 7CoN) shows that the dimeric molecule adopts a C-2 symmetric conformation and the two β-sheet pentapeptides adopt an anti-parallel conformation (**Figure 4c**). By examining the structure seen through the disulfide bonds, it could be noticed that the side chains of four tyrosines (Tyr_1, Tyr_1', Tyr_5 and Tyr_5') at both peptide termini are on one side, and the aromatic rings of Tyr_4 and Tyr_4' with the glycans attached are positioned on the opposite side. Such an amphiphilic arrangement represents the basic unit of the assembly. Moreover, these glycopeptides stack in a left-hand helical direction to form cross-β amyloid-like structure, and the rotation of two adjacent molecules leads to a ~45° angle (**Figure 4d**). Notably, the diameter of the helical assembly in the crystal structure (ca. 2.49 nm) corresponds well with the average width of a single fibril in the hydrogel derived from **4** which, according to the TEM data is 2.31 ± 0.34 nm (**Figure S12, Table S2**). This suggests that the self-assembling mechanism for generating nanofibers in hydrogel may be similar to that observed in the crystal structure.

Further examination of both the intra- and inter-molecular interactions between each glycosylated pentapeptide in the crystal structure, showed that not only the hydrogen bonds between the β-sheet peptides are crucial for the self-assembling process (**Figure 4e**), but the galactose units in each assembling unit are in close proximity to the aromatic

rings in the adjacent glycopeptide dimer (**Figure 4d**). On the contrary, no obvious aromatic-aromatic interactions were observed in this system. When a three-parameter operational definition was used to characterize the potential interactions,[42, 43, 44] the C1 axial hydrogen (H_1) located on the α-face of Gal lies in the range where interaction with the aromatic π system (**Figure 4f**) is possible. Based on these observations, as well as the distinct gelating properties of glycopeptide **4** compared to the non-glycosylated dimeric peptide reported previously,[31] we suspected that the carbohydrate C-H/aromatic π bonds might play essential roles in stabilization of the cross-β fibrils. This prompted us to further investigate the potential impacts of such interactions on gel formation.

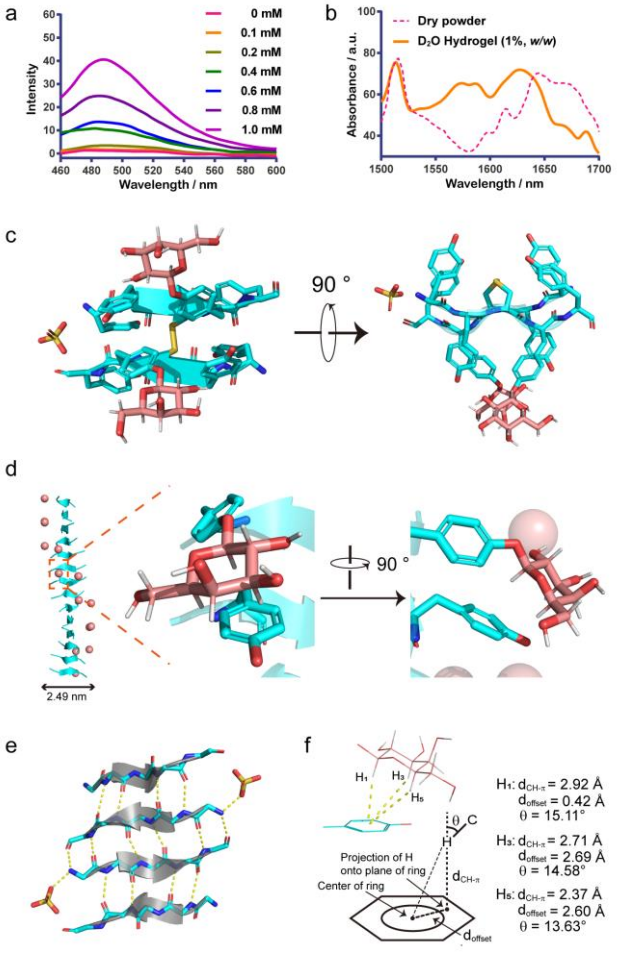


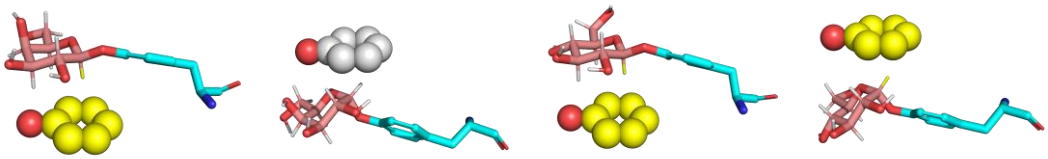
Figure 4. Representative spectroscopic data of self-assembling glycopeptide **4** and mechanistic insights related to CH-π interactions. (a) Emission intensities of ThT (20 μM, λ_{ex} = 440 nm) for **4** at various concentrations. (b) FT-IR spectra of **4** in dry powder (dotted line) and hydrogel states (solid line). (c) Crystal structure of **4** in assembly viewed perpendicular to the fibril axes (left) and viewed down the fibril axes (right). (d) Cartoons showing an elongated structure of **4** assembling to form a fibril viewed perpendicular to the axes (left), and the enlarged views rendering the close proximity of carbohydrate and tyrosine residues between two molecules of **4** (right) in the crystal structure. (e) Cartoons showing the β sheet peptides of **4** (carbohydrate omitted) side-by-side stacking through intra- or intermolecular backbone hydrogen bonding in the crystal structure. (f) Calculated data based on crystal structure of **4**, characterizing H_1, H_3 and H_5 of galactose that interact with the aryl ring. Parameters used to identify CH–π interactions[43, 44] include CH–π angle (θ, ⩽ 40°), CH–π distance ($d_{CH-\pi}$, ⩽ 4.0 Å), C-projection distance (d_{offset} ⩽ 2.0 Å for Tyr).

Carbohydrate-aromatic interactions and the influence from the glycan stereochemistry on the properties of hydrogels. Besides the frequently observed π-π interactions, aromatic rings can also participate in other types of non-covalent forces, such as CH-π interactions, which in the carbohydrate-aromatic system often exist between two or three methine hydrogens of the pyranose and the π electrons of the aromatic ring.[45] Although CH-π interactions are considered less common than hydroxyl-based hydrogen bonding in carbohydrates, studies have shown that such non-covalent bonds exist in many related protein-carbohydrate systems. The preference of forming CH-π bonds is associated with the stereochemistry of carbohydrates, concerning the distribution of axial C-Hs and orientation of glycosidic bonds.[46] For instance, Kiessling and Woolfson *et al*. discovered that the existed CH-π interactions between glycans and proximate aromatics favored the α-faces of Gal and Man more than their β-faces regardless of the anomeric structures, while for Glc or Xyl, both faces could form CH-π bonds depending on the appropriate anomeric configuration.[43] In our study, the gelating abilities of glycopeptides seemed to be correlated well with such preferences. Specifically, in the cases of the galactosylated peptides **4** and **11**, only the former with fully exposed α-faces of Gals could effectively form bonds with tyrosine side chains, significantly stabilizing the self-assembly. In contrast, **11** could not produce the crucial CH-π bonds from the disfavored β-faces of Gals, because the α-glycosidic linkages block the preferred bonding sides (**Table 2, Figures S13**). In comparison, the glucosylated peptides **12** and **13** could both generate stable CH-π bonds from the monosaccharides regardless of the configurations of glycosidic bonds, as in either case there is a suitable open face with axial C–H bonds able to interact with the aromatic rings (**Table 2**). The performance of other glycopeptides **14-27** in hydrogel formation experiments can also be rationalized in terms of the CH-π interactions.

To further prove the hypothesis that strong CH-π interactions in this glycopeptide system could stabilize the nanofibril formation and promote the self-assembly, we modified the substituents on the side chains of involved tyrosines, and evaluated the properties of hydrogels produced from the resulting galactosylated peptides. Four glycopeptides analogous to **4** were prepared, with –H, –F, –CN, and $–NO_2$ replacing the –OH on the aryl rings of Tyr_2 and $Tyr_{2'}$. Under the gelating conditions, all four glycopeptides were able to form hydrogels with a storage modulus (G′) that could be measured using rheology experiments. A negative correlation was found to exist between G′ and the electrostatic surface potential values,[47] indicating that the stor-

age modulus of hydrogels decreases as the side chain aromatics of Tyr_2 and $Tyr_{2'}$ become more electron-poor (**Figure S14**). Since CH-π interactions are weaker with more electron-poor aromatics,[43] the obtained results support the idea that the non-covalent CH-π bonds may play essential stabilizing roles in this glycosylated pentapeptide dimer-derived self-assembly.

Table 2. Explanation of the relationship between glycan stereochemistry and glycopeptide self-assembly.

Glycoform	β-Gal	α-Gal	β-Glu	α-Glu
Model[a]				
Preferred face for CH-π interaction[43]	α	α	α	β
Exposed face	α	β	α	β

[a] Cartoons rendering the interactions between the aromatic ring and β-Gal at the α-face of glycan based on crystal structure of **4**, and working models in the cases of α-Gal **11**, β-Glc **12**, and α-Glc **13** representing preferred CH–π interactions (highlighted in yellow) and interaction unlikely to be formed (highlighted in grey) with the aromatic rings according to literature precedent[43] and the exposed faces of monosaccharides opposite to the ones with glycosidic linkages to aromatics. Carbon atoms are colored cyan, nitrogen is blue, oxygen is red, sulfur is yellow orange, hydrogen is white, and the pyranose rings are highlighted in salmon, unless otherwise noted.

Next, we further investigated whether different monosaccharides in the pentapeptide dimer would influence the properties of the formed hydrogels. The rheological experiments indicated that the storage modulus at angular frequency (ω=1) of six hydrogel samples were ranging from ~10 Pa to >1000 Pa, depending on different types of monosaccharide (**Figures S15, S16a**). The melting points of the hydrogels (**Figures S16b**), as well as the vertical and horizontal distances (**Figures S18**) of the formed nanobundles determined by atomic force microscopy (AFM), (**Figures S17**), showed that measurable changes could result from adjustment of the stereochemistry of glycans in the self-assembling glycopeptides. However, no obvious trends could be found to quantitatively correlate the glycoforms and their physical properties, further underscoring the complexity of this supramolecular system. Nevertheless, our data demonstrate that the stereochemistry of carbohydrates could be an important factor in modulation of glycopeptide-derived self-assembly.

Development of an enzyme-resistant hydrogel from a self-assembling glycosylated D-peptide. While peptide hydrogels have shown significant potential in the development of biomaterials, a major problem is that peptides derived from natural amino acids are susceptible to enzymatic degradation. In the cases of materials requiring durability under physiological conditions, the short half-life of L-peptides becomes a serious issue and the use of mirrored D-peptides has been shown to be a viable solution.[48] However for glycopeptides, the approach of mirroring both the peptide and the glycan may result in difficulties to obtain the requisite compounds, since the enantiomers of common D-glucose, D-mannose, D-galactose, etc. might not be readily available. In addition, it has been difficult to predict whether the combination of unnatural D-peptides and common sugars would result structures with self-assembling ability similar to those with known peptide motifs. Thus, what we have discovered in the study of glycopeptide systems may provide a feasible solution. For example, to predict the self-assembling ability of [yycy(β-D-Glc)y]$_2$ (**28**), its mirror-image form, [YYCY(β-L-Glc)Y]$_2$ (**28'**), could be of reference (**Figure 5a**) as they should have identical physical properties. Because β-L-Glc possesses three axial C–Hs on its α face that may facilitate effective CH-π hydrogen bonds in the [YYCYY]$_2$ system (**Figure 5b**), it would be anticipated that glycopeptide **28'** should be a hydrogelator. Therefore, D-peptide-derived sequence **28** should have the ability to self-assemble and form a hydrogel.

Experimentally, the synthesized β-D-Glc-D-peptide **28** successfully forms a hydrogel at a concentration of 0.5 % (*w*/*w*), as expected, and the TEM image clearly indicates that **28** self-assembles to nanofibers (**Figure 5c**). A protease assay was conducted to further evaluate the properties of two hydrogels prepared from glycopeptides **12** and **28**, and demonstrated that the D-peptide-derived hydrogel was much more resistant to proteinase K, no obvious degradation being observed after five days. In contrast, severe

decomposition was seen for the other hydrogel that were produced from glycopeptide **12** after two days (**Figure S19**). These findings also suggest that in the supramolecular systems involving essential carbohydrate-aromatic interactions, poorly available and rare sugars may be replaced by more easily accessible carbohydrates that can meet the requirements for generating CH-π bonds.

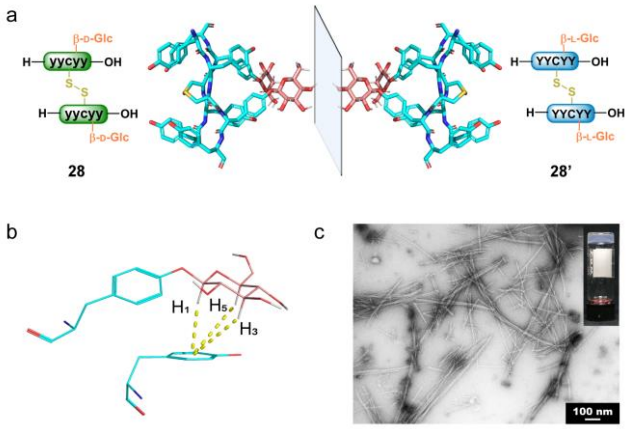


Figure 5. Design of D-glucosyl-D-peptide hydrogel. (a) Graphical presentation of mirroring glycopeptide **28**. (b) A plausible working model showing the interaction between L-glucose on Tyr_4 and the Tyr_2 residue, where three axial hydrogens at the exposed α-face of glucose may facilitate the CH-π interactions. (c) TEM image and gelating test of hydrogel prepared from the glycosylated D-peptide **28** in 10 mM phosphate buffer (0.5%, *w*/*w*).

3. CONCLUSIONS

Carbohydrates usually possess complicated stereochemical information originating from the chiral centers in each monosaccharide unit. These stereogenic centers not only provide various chiral alcohols for hydrogen bonding, which has been extensively studied, but also afford methines that may engage interactions with aromatics *via* CH-π hydrogen bonding.[46] Although a number of studies have revealed the critical roles of carbohydate-aromatic CH-π bonds in biological processes including protein folding[49] and protein-glycan recognition,[43] very few mechanistic investigations have been conducted on carbohydrate-related self-assembling systems, perhaps due to the challenges in finding convincing structural evidences. Based on a fluorenylmethyloxycarbonyl (Fmoc)-protected amino sugar system, Ulijn *et al.* pioneered the study of CH-π interactions as a key driving force in the self-assembly utilizing spectroscopic methods and molecular simulations.[50] However, such non-covalent bonding in supramolecular assembly involving more complexed glycopeptide structures still needed to be discovered and understood. Our X-ray diffraction data of a single crystal from the tyrosine-rich glycopeptide **4** has provided information demonstrating that CH-π interactions may be a potential link between the stereochemistry of glycans and its impact on glycopeptide self-assembly.

In this supramolecular system, hydrogen bonding and solvophobic effects are certainly among the major driving forces for the self-assembly. Surprisingly, π–π interactions that are often found to be crucial in many other self-assembling structures were not obvious in the crystal structure that was obtained, although the sequence contains a high content of tyrosine residues. A rational explanation would be that the CH-π bonds afford essential stabilization to the nanostructures. This is supported by experiments involving different monosaccharides that apparently possess varied CH-π bond-forming capability, and also electronic variations of the aromatic π bond donors. Furthermore, the experimental results indicate that tuning the strength of CH-π interactions may contribute to the change of glycopeptide assemblies in terms of micromorphology, as well as the mechanical property and thermostability of the hydrogels that are formed.

In conclusion, the self-assembling tyrosine-rich glycopeptide reported here represents a novel example of amphiphilic structure that contains aromatics closely interacting with the carbohydrate moiety. This is distinct from previous strategies that recommended more distant separation of hydrophobic and hydrophilic termini.[16, 17] On the basis of these results, several new pointers for designing self-assembling glycopeptides could be added to the existing empirical rules. A carbohydrate could be used as a CH-π hydrogen bond donor, which may play crucial roles beyond the commonly observed O-H hydrogen bonding. By changing the stereochemistry in the carbohydrate motif, the strength of CH-π bond could be finely tuned, which may influence the self-assembling process and the properties of the formed nanostructure. We have also demonstrated that such glycopeptide self-assembly could be expanded to D-peptide-derived structures, and along this line, we believe that more gelators and applications may be discovered using properly designed glycopeptides.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Supporting tables and graphics as well as experimental procedures and spectroscopic data for all reactions and compounds (PDF)

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Author Contributions

C.H. designed the research, performed the synthetic experiments, carried out characterization of hydrogels, analyzed data and wrote the manuscript, S.W. performed the synthetic experiments and carried out crystallization experiment, D.L. performed the synthetic experiments, C.C. analyzed the crystallization data, W.Z. performed the NMR simulation experi-

ments, M.M supervised the research, L.L supervised the research, S.D. designed and supervised the research, discussed the results and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Whitesides, G. M.; Grzybowski, B. Self-assembly at all scales. *Science* **2002**, *295*, 2418-2421.

(2) Goodson, H. V.; Jonasson, E. M. Microtubules and microtubule-associated proteins. *Cold Spring Harb. Perspect. Biol.* **2018**, *10*, a022608.

(3) Ulijn, R. V.; Jerala, R. Peptide and protein nanotechnology into the 2020s: beyond biology. *Chem. Soc. Rev.* **2018**, *47*, 3391-3394.

(4) Raymond, D. M.; Nilsson, B. L. Multicomponent peptide assemblies. *Chem. Soc. Rev.* **2018**, *47*, 3659-3720.

(5) De, Santis E.; Ryadnov, M. G. Peptide self-assembly for nanomaterials: the old new kid on the block. *Chem. Soc. Rev.* **2015**, *44*, 8288-8300.

(6) Sawada, T.; Mihara, H.; Serizawa, T. Peptides as new smart bionanomaterials: molecular-recognition and self-assembly capabilities. *Chem. Rec.* **2013**, *13*, 172-186.

(7) Zhou, J.; Xu, B. Enzyme-instructed self-assembly: a multistep process for potential cancer therapy. *Bioconjug. Chem.* **2015**, *26*, 987-999.

(8) Alakpa, E. V.; Jayawarna, V.; Lampel, A.; Burgess, K. V.; West, C. C.; Bakker, S. C. J.; Roy, S.; Javid, N.; Fleming, S.; Lamprou, D. A.; Yang, J.; Miller, A.; Urquhart, A. J.; Frederix, P. W J. M.; Hunt, N. T.; Péault, B.; Ulijn, R. V.; Dalby, M. J. Tunable Supramolecular hydrogels for selection of lineage-guiding metabolites in stem cell cultures. *Chem* **2016**, *1*, 298-319.

(9) Cai, Y.; Shen, H.; Zhan, J.; Lin, M.; Dai, L.; Ren, C.; Shi, Y.; Liu, J.; Gao, J.; Yang, Z. Supramolecular "Trojan Horse" for nuclear delivery of dual anticancer drugs. *J. Am. Chem. Soc.* **2017**, *139*, 2876-2879.

(10) Zaramella, D.; Scrimin, P.; Prins, L. J. Self-assembly of a catalytic multivalent peptide-nanoparticle complex. *J. Am. Chem. Soc.* **2012**, *134*, 8396-8399.

(11) Lampel, A.; McPhee, S. A.; Park, H.-A.; Scott, G. G.; Humagain, S.; Hekstra, D. R.; Yoo, B.; Frederix, P.; Li, T. D.; Abzalimov, R. R.; Greenbaum, S. G.; Tuttle, T.; Hu, C.; Bettinger, C. J.; Ulijn, R. V. Polymeric peptide pigments with sequence-encoded properties. *Science* **2017**, *356*, 1064-1068.

(12) Tao, K.; Makam, P.; Aizen, R.; Gazit E. Self-assembling peptide semiconductors. *Science* **2017**, *358*, eaam9756.

(13) Lowik, D. W.; Leunissen, E. H.; van den Heuvel, M.; Hansen, M.B.; van Hest, J. C. Stimulus responsive peptide based materials. *Chem. Soc. Rev.* **2010**, *39*, 3394-3412.

(14) Zozulia, O.; Dolan, M. A.; Korendovych, I. V. Catalytic peptide assemblies. *Chem. Soc. Rev.* **2018**, *47*, 3621-3639.

(15) Lampel, A.; Ulijn, R. V.; Tuttle, T. Guiding principles for peptide nanotechnology through directed discovery. *Chem. Soc. Rev.* **2018**, *47*, 3737-3758.

(16) Datta, S.; Bhattacharya, S. (2015) Multifarious facets of sugar-derived molecular gels: molecular features, mechanisms of self-assembly and emerging applications. *Chem. Soc. Rev.* **2015**, *44*, 5596-5637.

(17) Delbianco, M.; Bharate, P.; Varela-Aramburu, S.; Seeberger, P. H. Carbohydrates in supramolecular chemistry. *Chem. Rev.* **2016**, *116*, 1693-1752.

(18) Roytman, R.; Adler-Abramovich, L.; Kumar, K. S.; Kuan, T. C.; Lin, C. C.; Gazit, E.; Brik, A. Exploring the self-assembly of glycopeptides using a diphenylalanine scaffold. *Org. Biomol. Chem.* **2011**, *9*, 5755-5761.

(19) Tsuzuki, T.; Kabumoto, M.; Arakawa, H.; Ikeda, M. The effect of carbohydrate structures on the hydrogelation ability and morphology of self-assembled structures of peptide-carbohydrate conjugates in water. *Org. Biomol. Chem.* **2017**, *15*, 4595-4600.

(20) Kawano, S.; Tamaru, S.; Fujita, N.; Shinkai, S. Sol-gel polycondensation of tetraethyl orthosilicate (TEOS) in sugar-based porphyrin or-ganogels: inorganic conversion of a sugar-directed porphyrinic fiber library through sol-gel transcription processes. *Chemistry* **2004**, *10*, 343-351.

(21) Wang, K. R.; Han, D.; Cao, G. J.; Li, X. L. Synthesis and predetermined supramolecular chirality of carbohydrate - functionalized perylene bisimide derivatives. *Chem. Asian. J.* **2015**, *10*, 1204-1214.

(22) Kobayashi, H.; Friggeri, A.; Koumoto, K.; Amaike, M.; Shinkai, S.; Reinhoudt, D. N. Molecular design of "super" hydrogelators: understanding the gelation process of azobenzene-based sugar derivatives in water. *Org. Lett.* **2002**, *4*, 1423-1426.

(23) Yang, G.; Zheng, W.; Tao, G.; Wu, L.; Zhou, Q.-F.; Kochovski, Z.; Ji, T.; Chen, H.; Li, X.; Lu, Y. Diversiform and transformable glyco-nanostructures constructed from amphiphilic supramolecular metallocarbohydrates through hierarchical self-assembly: the balance between metallacycles and saccharides. *ACS nano* **2019**, *13*, 13474-13485.

(24) Liu, J.; Sun, Z.; Yuan, Y.; Tian, X.; Liu, X.; Duan, G.; Yang, Y.; Yuan, L.; Lin, H. C.; Li, X. Peptide glycosylation generates supramolecular assemblies from glycopeptides as biomimetic scaffolds for cell adhesion and proliferation. *ACS Appl. Mater. Interfaces* **2016**, *8*, 6917-6924.

(25) Talloj, S. K.; Cheng, B.; Weng, J. P.; Lin, H. C. Glucosamine-based supramolecular nanotubes for human mesenchymal cell therapy. *ACS Appl. Mater. Interfaces* **2018**, *10*, 15079-15087.

(26) Zhao, F.; Heesters, B. A.; Chiu, I.; Gao, Y.; Shi, J.; Zhou, N.; Carroll, M. C.; Xu, B. L-Rhamnose-containing supramolecular nanofibrils as potential immuno-suppressive materials. *Org. Biomol. Chem.* **2014**, *12*, 6816-6819.

(27) Li, X.; Kuang, Y.; Shi, J.; Gao, Y.; Lin, H. C.; Xu, B. Multifunctional, biocompatible supramolecular hydrogelators consist only of nucleobase, amino acid, and glycoside. *J. Am. Chem. Soc.* **2011**, *133*, 17513-17518.

(28) Lee, J.; Ju, M.; Cho, O. H.; Kim, Y.; Nam, K. T. Tyrosine-rich peptides as a platform for assembly and material synthesis. *Adv. Sci. (Weinh)* **2019**, *6*, 1801255.

(29) Halim, A.; Brinkmalm, G.; Ruetschi, U.; Westman-Brinkmalm, A.; Portelius, E.; Zetterberg, H.; Blennow, K.; Larson, G.; Nilsson, J. (2011) Site-specific characterization of threonine, serine, and tyrosine glycosylations of amyloid precursor protein/amyloid beta-peptides in human cerebrospinal fluid. *Proc. Natl. Acad. Sci. USA.* **2011**, *108*, 11848-11853.

(30) Min, K. I.; Yun, G.; Jang, Y.; Kim, K. R.; Ko, Y. H.; Jang, H. S.; Lee, Y. S.; Kim, K.; Kim, D. P. Covalent Self-Assembly and One-

Step Photocrosslinking of Tyrosine-rich oligopeptides to form diverse nanostructures. *Angew. Chem. Int. Ed. Engl.* **2016**, *55*, 6925-6928.

(31) Jang, H. S.; Lee, J. H.; Park, Y. S.; Kim, Y. O.; Park, J.; Yang, T. Y.; Jin, K.; Lee, J.; Park, S.; You, J. M.; Jeong, K.-W.; Shin, A.; Oh, I.-S.; Kwon, M.-K.; Kim, Y.-I.; Cho, H.-H.; Han, H. N.; Kim, Y.; Chang, Y. H.; Paik, S. R.; Nam, K. T.; Lee, Y.-S. Tyrosine-mediated two-dimensional peptide assembly and its role as a bio-inspired catalytic scaffold. *Nat. Commun.* **2014**, *5*, 3665-3675.

(32) Li, J.; Xing, R.; Bai, S.; Yan, X. (2019) Recent advances of self-assembling peptide-based hydrogels for biomedical applications. *Soft Matter* **2019**, *15*, 1704-1715.

(33) Biancalana, M.; Koide, S. Molecular mechanism of Thioflavin-T binding to amyloid fibrils. *Biochim. Biophys. Acta.* **2010**, *1804*, 1405-1412.

(34) Byler, D. M.; Susi, H. Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers* **1986**, *25*, 469-487.

(35) Krysmann, M. J.; Castelletto, V.; Kelarakis, A.; Hamley, I. W.; Hule, R. A.; Pochan, D. J. Self-assembly and hydrogelation of an amyloid peptide fragment. *Biochemistry* **2008**, *47*, 4597-4605.

(36) Garcia, A. M.; Iglesias, D.; Parisi, E.; Styan, K. E.; Waddington, L. J.; Deganutti, C.; De Zorzi, R.; Grassi, M.; Melchionna, M.; Vargiu, A.V.; Marchesan, S. Chirality effects on peptide self-assembly unraveled from molecules to materials. *Chem* **2018**, *4*, 1862-1876.

(37) Zhang, S.-Q.; Huang, H.; Yang, J.; Kratochvil, H. T.; Lolicato, M.; Liu, Y.; Shu, X.; Liu, L.; DeGrado, W. F. Designed peptides that assemble into cross-α amyloid-like structures. *Nat. Chem. Biol.* **2018**, *14*, 870-875.

(38) Bera, S.; Mondal, S.; Xue, B.; Shimon, L. J. W.; Cao, Y.; Gazit, E. Rigid helical-like assemblies from a self-aggregating tripeptide. *Nat. Mater.* **2019**, *18*, 503-509.

(39) Hughes, M. P.; Sawaya, M. R.; Boyer, D. R.; Goldschmidt, L.; Rodriguez, J. A.; Cascio, D.; Chong, L.; Gonen, T.; Eisenberg, D. S. Atomic structures of low-complexity protein segments reveal kinked β sheets that assemble networks. *Science* **2018**, *359*, 698-701.

(40) Yuan, C.; Ji, W.; Xing, R.; Li, J.; Gazit, E.; Yan, X. Hierarchically oriented organization in supramolecular peptide crystals. *Nat. Rev. Chem.* **2019**, *3*, 567-588.

(41) Okamoto, R.; Mandal, K.; Sawaya, M. R.; Kajihara, Y.; Yeates, T. O.; Kent, S. B. (Quasi-)racemic X-ray structures of glycosylated and non-glycosylated forms of the chemokine Ser-CCL1 prepared by total chemical synthesis. *Angew. Chem. Int. Ed. Engl.* **2014**, *53*, 5194-5198.

(42) Brandl, M.; Weiss, M. S.; Jabs, A.; Sühnel, J.; Hilgenfeld, R. CH··· π-interactions in proteins. *J. Mol. Biol.* **2001**, *307*, 357-377.

(43) Hudson, K. L.; Bartlett, G. J.; Diehl, R. C.; Agirre, J.; Gallagher, T.; Kiessling, L. L.; Woolfson, D. N. Carbohydrate-Aromatic Interactions in Proteins. *J. Am. Chem. Soc.* **2015**, *137*, 15152-15160.

(44) Hsu, C. H.; Park, S.; Mortenson, D. E.; Foley, B. L.; Wang, X.; Woods, R. J.; Case, D. A.; Powers, E. T.; Wong, C. H.; Dyson, H. J.; Kelly, J. W. The Dependence of Carbohydrate-Aromatic Interaction Strengths on the Structure of the Carbohydrate. *J. Am. Chem. Soc.* **2016**, *138*, 7636-7648.

(45) Laughrey, Z. R.; Kiehna, S. E.; Riemen, A. J.; Waters, M. L. Carbohydrate– π interactions: What are they worth? *J. Am. Chem. Soc.* **2008**, *130*, 14625-14633.

(46) Asensio, J. L.; Arda, A.; Canada, F. J.; Jimenez-Barbero, J. Carbohydrate-Aromatic Interactions. *Acc. Chem. Res.* **2013**, *46*, 946-954.

(47) Jimenez-Moreno, E.; Jimenez-Oses, G.; Gomez, A. M.; Santana, A. G.; Corzana, F.; Bastida, A.; Jimenez-Barbero, J.; Asensio, J. L. A thorough experimental study of CH/pi interactions in water: quantitative structure-stability relationships for carbohydrate/aromatic complexes. *Chem. Sci.* **2015**, *6*, 6076-6085.

(48) Wang, H.; Feng, Z.; Xu, B. D-amino acid-containing supramolecular nanofibers for potential cancer therapeutics. *Adv. Drug Deliv. Rev.* **2017**, *110-111*, 102-111.

(49) Culyba, E. K.; Price, J. L.; Hanson, S. R.; Dhar, A.; Wong, C. H.; Gruebele, M., Powers, E. T.; Kelly, J. W. Protein native-state stabilization by placing aromatic side chains in N-glycosylated reverse turns. *Science* **2011**, *331*, 571-575.

(50) Birchall, L. S., Roy, S., Jayawarna, V., Hughes, M., Irvine, E., Okorogheye, G. T., Saudi, N., De Santis, E., Tuttle, T., Edwards, A. A., Ulijn, R. V. Exploiting CH-π interactions in supramolecular hydro-gels of aromatic carbohydrate amphiphiles. *Chem. Sci.* **2011**, *2*, 1349-1355.

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