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Sequence-Dependent Effects of Folded Glycopolymer Structures on Their Biomolecular Recognition

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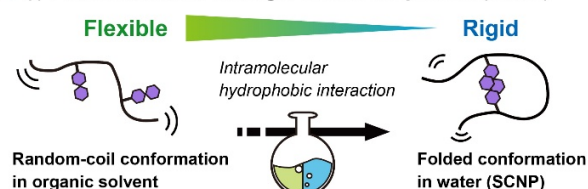
ABSTRACT: We demonstrate that folded polymer structures have distinct effects on their binding affinity to target biomolecules, depending on the polymer sequence pattern. We synthesized random and triblock glycopolymers with either folded or non-folded structures in water. These glycopolymers contained mannose units as biofunctional group, while di(phenylalanine) units were incorporated into the folded glycopolymers as hydrophobic segments to promote self-folding of the polymer chains in aqueous solution. We then evaluated the interactions of these glycopolymers with a model protein, concanavalin A (ConA), using isothermal titration calorimetry. The folded random glycopolymer exhibited a higher binding constant than its non-folded counterpart, whereas the opposite trend was observed in the triblock glycopolymer series. In both sequence patterns, further compaction of the polymer structures led to a decrease in binding affinity toward ConA. These findings highlight the importance of the spatial arrangement of functional groups and polymer-chain flexibility when designing functional polymers inspired by protein behavior.

Dynamic properties such as flexibility of proteins are crucial for their functions, in addition to the static molecular structures.^{1,2} For example, intrinsically disordered proteins (IDPs) regulate their binding behavior by undergoing conformational changes from a disordered (flexible) state to a defined helical (rigid) structure.^{3–6} Biomolecular interactions can be thermodynamically described by Gibbs free energy ($\Delta G = \Delta H - T\Delta S$), where the enthalpy change (ΔH) represents heat released from bond formation between biomolecules, and the entropy change (ΔS) reflects a loss in molecular mobility.^{7–11} The flexibility control of IDPs effectively utilize the entropic term in such interactions. The well-defined sequences and three-dimensional structures of proteins enable precise control over flexibility, thereby contributing to their highly efficient functions.

Replicating the superior functions of biomacromolecules using synthetic polymers remains one of the major challenges in polymer science.^{12–19} To date, sequence-controlled (or -defined) synthetic polymers prepared via living polymerization have enabled specific biomolecular recognition of target molecules.^{20–21} Our group has demonstrated that the precise design of glycopolymers—synthetic polymers displaying carbohydrate units—can enhance the interactions with target proteins by controlling the spatial arrangement of the carbohydrate units.^{22–24} In these systems, multivalent binding of the carbohydrates contributes significantly to the enthalpic gain. However, the entropy change associated with glycopolymer–protein recognition remains difficult to control due to the random-walk behavior of polymer chains in solution, which results in a vast number of possible conformations. This high entropic contribution leads to a

large entropic penalty during complex formation, limiting the overall binding affinity to target biomolecules.^{10,11}

A (i) Folded structure of single-chain nanoparticle (SCNP)



B (ii) Biomolecular interactions of glycopolymers

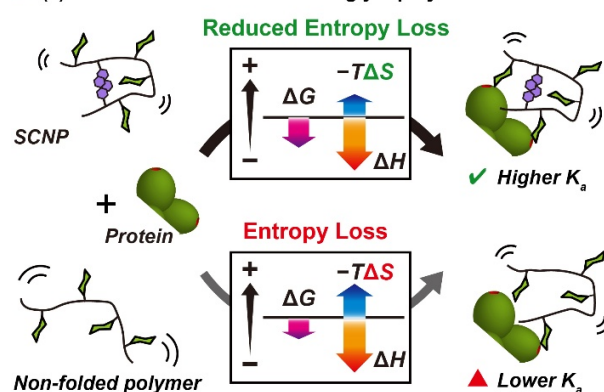


Figure 1. Schematic illustrations of the current study. **(A)** Correlation between a folded polymer structure in water and its flexibility. **(B)** Biomolecular interactions of folded glycopolymer (SCNP) or non-folded glycopolymer with a target protein (concanavalin A).

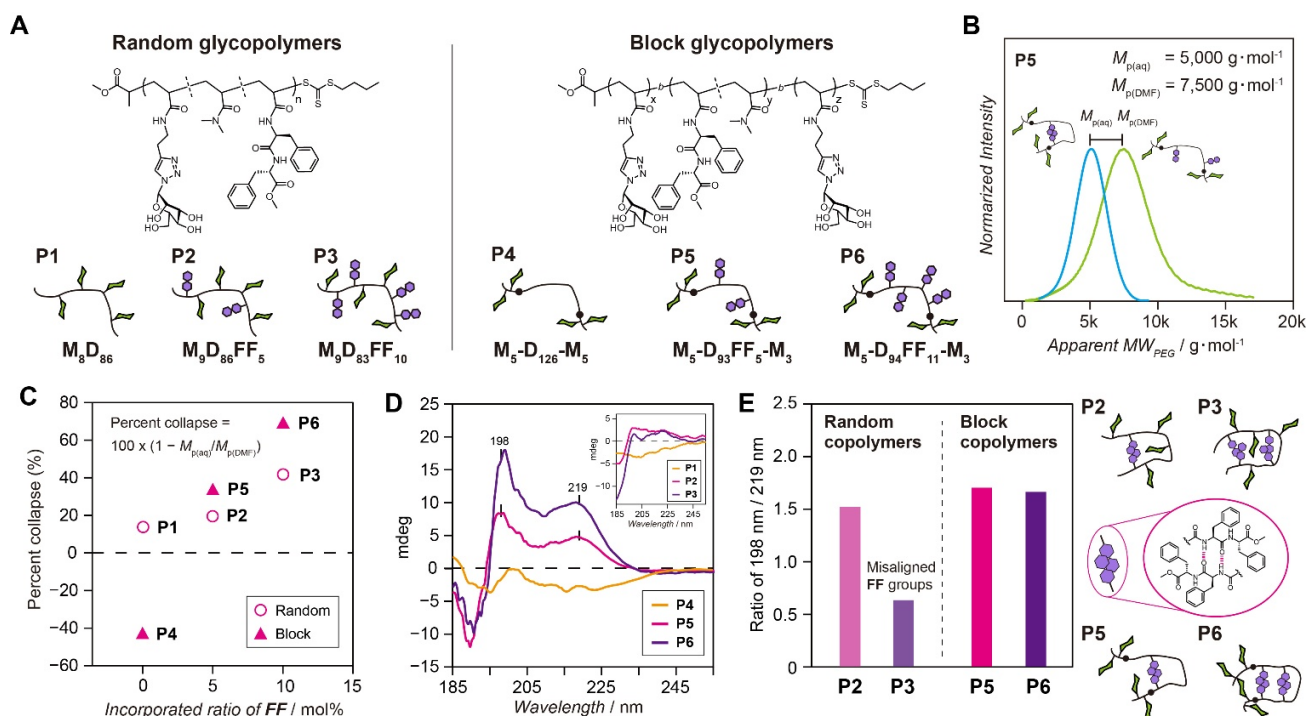


Figure 2. (A) Chemical structures and illustration of random glycopolymers (P1–P3) and triblock glycopolymers (P4–P6). (B) Molecular weight distribution of P5 in distinct SEC systems calibrated using a polyethylene glycol standard. Green and blue lines represent eluent of DMF (10 mM LiBr) and water (100 mM NaNO₃), respectively. (C) Percent collapse plotted as a function of incorporated ratio of di(phenylalanine) (FF) units in the copolymers. Percent collapse was calculated using the following formula: $100\% \times (1 - M_{p(aq)} / M_{p(DMF)})$. Blank circles and solid triangles represent random glycopolymers (P1–P3) and triblock glycopolymers (P4–P6), respectively. (D) Circular dichroism spectra of P4, P5, and P6 at 20 °C. Polymer concentrations were 0.05 g·L⁻¹ in 1 mM phosphate buffer (pH 7). Inset: P1, P2, and P3 (polymer concentrations were 0.1 g·L⁻¹). (E) Ratios of the CD maxima at 198 and 219 nm for random glycopolymers (P2 and P3) and triblock glycopolymers (P5 and P6) with illustrations of the interaction between FF units in the copolymers.

To address this challenge, we focus on single-chain nanoparticles (SCNPs), a class of self-folded polymers formed through covalent or non-covalent intramolecular interactions (Figure 1A).^{25–31} The controlled three-dimensional structures of SCNPs create a confined environment for functional groups within a single polymer chain, which is advantageous for catalytic reactions.^{32–40} Recently, the correlation between the folded structures of SCNPs and their flexibility has been investigated, suggesting that the folded structures of SCNPs are effective for controlling the flexibility of synthetic polymers.^{41,42} While a study by the groups of Palmans and Becer demonstrated that compacted polymer structures of amphiphilic glycopolymers influence their binding affinity to target proteins,⁴³ the effect of regulated polymer-chain flexibility on biomolecular recognition remains unclear.

Herein, we investigate the correlation between the folded structures of SCNPs and their biomolecular recognition. We synthesized SCNPs displaying carbohydrate units, in which self-folding is driven by hydrophobic interaction in water, designed for multivalent carbohydrate–protein interactions (Figure 1B). In this study, the interaction between mannose and concanavalin A (ConA), a tetrameric mannose-binding protein, was used as a model system due to its well-characterized thermodynamic properties.^{44–48} To prepare SCNPs that recognize ConA, we synthesized amphiphilic glycopolymers

via reversible addition-fragmentation chain transfer (RAFT) polymerization using acrylamide-based monomers (Figure 2A). Mannose acrylamide (M), *N,N*-dimethylacrylamide (D), and di(phenylalanine) acrylamide monomer (FF) were randomly incorporated into the polymer structures (Table S1). Inspired by the work of Knight and co-workers,²⁹ we used FF units as hydrophobic moieties to induce polymer self-folding through hydrophobic interactions in water, thereby forming SCNPs. The feed ratio of M was fixed at 10 mol%, while the FF content was varied (0, 5, and 10 mol%) to prepare P1, P2, and P3, respectively. Polymer compositions were determined by proton nuclear magnetic resonance (¹H NMR) spectroscopy, confirming that the incorporated ratios of M, D, and FF were consistent with the feed ratios (Table 1). Analysis of polymer composition across different monomer conversions demonstrated the randomness of the polymer sequences (Figure S11). To investigate the influence of polymer sequence on biomolecular recognition, we also synthesized SCNPs from triblock glycopolymers (Figure 2A). Based on our previous report, in which introducing mannose units at the polymer termini enabled divalent binding to ConA dimers,⁴⁷ triblock glycopolymers were designed with mannose units at the first and third blocks and FF units in the second block. The target degrees of polymerization (DP) were set to 5, 100, and 5 for the first, second, and third blocks, respectively (Table S2). Successful synthesis of the triblock

Table 1. Properties of the prepared glycopolymers.

Polymer	Sequence type	Conformation in water	# M unit ^[a]	# FF unit ^[a]	Total DP ^[a]	$M_n(\text{DMF}) / \text{g mol}^{-1[\text{b}]}$	$M_p(\text{DMF}) / \text{g mol}^{-1[\text{b}]}$	$\bar{D}^{[\text{b}]}$	$M_p(\text{aq}) / \text{g mol}^{-1[\text{c}]}$	% collapse ^[d]	$D_h / \text{nm}^{[\text{e}]}$
P1	Random	Non-folded	8	0	94	5,900	7,400	1.17	6,400	13	5.5 ± 0.3
P2	Random	SCNP	9	5	100	6,900	8,400	1.19	6,700	20	6.8 ± 0.2
P3	Random	SCNP	9	10	102	6,300	7,500	1.11	4,400	42	9.2 ± 0.3
P4	Block	Non-folded	10	0	136	5,200	6,300	1.19	8,900	-40	5.7 ± 0.3
P5	Block	SCNP	8	5	106	5,100	7,500	1.30	5,000	33	6.2 ± 0.1
P6	Block	SCNP	8	11	113	5,200	6,800	1.25	2,100	69	8.0 ± 0.2

[a] The number of **M** and **FF** units, and total degree of polymerization (DP) were determined by ^1H NMR spectroscopy. [b] Molecular weights and dispersity were determined by SEC analysis (DMF with 10 mM LiBr, PEG calibrants). Polymer concentrations were $2 \text{ g}\cdot\text{L}^{-1}$. [c] Molecular weights were determined by SEC analysis (Water with 100 mM NaNO_3 , PEG calibrants). Polymer concentrations were $3 \text{ g}\cdot\text{L}^{-1}$. [d] Percent collapse was calculated using the following formula: $100\% \times (1 - M_p(\text{aq})/M_p(\text{DMF}))$. [e] Hydrodynamic diameters (D_h) were determined by DLS measurement (10 mM phosphate buffer). Sample concentrations were $4 \text{ g}\cdot\text{L}^{-1}$. All the samples were previously incubated at 80°C for 30 min.

glycopolymers **P4**, **P5** and **P6** with **FF** contents of 0, 5 or 10 mol%, respectively, was confirmed by ^1H NMR and size exclusion chromatography (SEC) analysis in *N,N*-dimethylformamide (DMF) (Figure S14–S21, S24).

The self-folding behavior of the synthesized glycopolymers in water was evaluated by SEC analyses. By using the same polyethylene glycol (PEG) standards in both DMF and aqueous SEC systems, the relative molecular weights of the polymers could be compared directly (Figure 2B).^{29,49} The peak apex molecular weights (M_p) of all glycopolymers, except **P4**, were lower in water than in DMF (Figure S23). The percent collapse was calculated using the following formula: $100\% \times (1 - M_p(\text{aq})/M_p(\text{DMF}))$ (Table 1). Although the randomly incorporated **M** units alone induced self-folding in **P1** due to the difference in hydrophilicity between **M** and **D**, the percent collapse increased with higher **FF** content (Figure 2C). Dynamic light scattering (DLS) measurements of these polymers in phosphate buffered saline (PBS) revealed unimodal peaks below 10 nm (Figure S25), indicating that the SCNPs (**P2**, **P3**, **P5**, and **P6**) were folded as single molecules in aqueous solution through intramolecular hydrophobic interactions of **FF** units. We also note that these glycopolymers exhibit reversible aggregation behavior at elevated temperatures, redissolving upon cooling to room temperature (Figure S26).

Circular dichroism (CD) spectroscopy was used to investigate the local structures of the SCNPs. While no observable CD signals were detected for the polymers lacking **FF** units, **P2**, **P3**, **P5**, and **P6** exhibited characteristic peaks corresponding to secondary structures formed by **FF** units in aqueous solution (1 mM phosphate buffer), consistent with previous reports (Figure 2D).²⁹ The maxima at 198 nm and 219 nm are attributed to π - π^* transitions of aromatic interactions and n - π^* transitions of hydrogen bonding in β -sheet-like structures formed by **FF** units, respectively.^{29,50} The ellipticity ratio at 198 and 219 nm for **P3** was lower than that of **P2**, suggesting the formation of a misaligned β -sheet-like structure (Figure 2E). This behavior is likely due to the presence of **M** units, which act as a hydrogen bond donors, within the same polymer chains.²⁹ In contrast, the

ellipticity ratios for **P5** and **P6** were comparable, regardless of their **FF** content (Figure 2E). These results suggest that the spatially localized arrangement of **FF** units in the central block of the triblock structure suppresses undesired interactions between **FF** and the amide groups of **M**.^{30,51} Although β -sheet-like structure formation by **FF** units was influenced by the

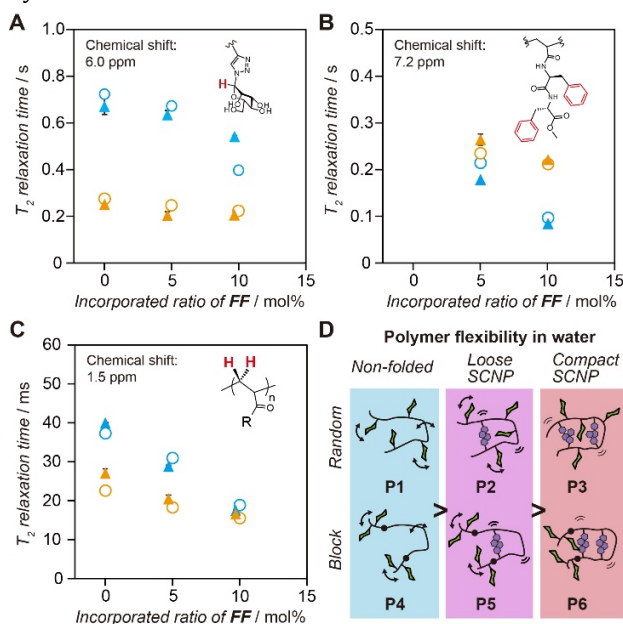


Figure 3. Spin-spin (T_2) relaxation time of the synthesized glycopolymers at (A) 6.0 ppm (anomeric proton of mannose), (B) 7.2 ppm (aromatic proton of diphenylalanine) and (C) 1.5 ppm (methylene proton of polymer main-chain). Blank circles and solid triangles represent random glycopolymers (**P1**–**P3**) and triblock glycopolymers (**P4**–**P6**), respectively. Blue and yellow plots represent the experiments using D_2O and $\text{DMSO}-d_6$ as solvents, respectively. Sample concentrations were $10 \text{ g}\cdot\text{L}^{-1}$. All the samples were previously incubated at 80°C for 30 min. The experiments were triplicated. (D) Illustrations of correlation between the polymer structures and their flexibility.

Table 2. Thermodynamic data for the interaction of glycopolymers with ConA dimer.^[a]

Ligand	$K_d / \mu\text{mol}\cdot\text{L}^{-1}$	$K_a / \text{L}\cdot\text{mol}^{-1}$ [b]	$\Delta G / \text{kJ}\cdot\text{mol}^{-1}$	$\Delta H / \text{kJ}\cdot\text{mol}^{-1}$	$-T\Delta S / \text{kJ}\cdot\text{mol}^{-1}$	N	c [c]
ManAAm (M) [d]	159.3 ± 4.5	$6,280 \pm 180$	-21.3 ± 0.1	-27.5 ± 0.3	6.2 ± 0.4	2 [e]	0.54 ± 0.02
P1	20.9 ± 1.9	$48,200 \pm 4,600$	-26.3 ± 0.2	-123.8 ± 17.4	97.6 ± 17.8	0.34 ± 0.04	0.67 ± 0.15
P2	9.6 ± 0.7	$104,300 \pm 7,500$	-28.2 ± 0.2	-79.6 ± 2.9	51.3 ± 3.1	0.54 ± 0.04	2.20 ± 0.19
P3	22.1 ± 4.2	$47,000 \pm 9,400$	-26.2 ± 0.5	-130.1 ± 16.9	103.8 ± 17.2	0.38 ± 0.03	0.71 ± 0.20
P4	22.1 ± 0.8	$45,300 \pm 1,600$	-26.2 ± 0.2	-68.3 ± 2.1	42.1 ± 2.1	0.63 ± 0.02	1.24 ± 0.08
P5	29.9 ± 0.8	$33,500 \pm 900$	-25.3 ± 0.1	-65.8 ± 0.8	40.5 ± 0.8	0.73 ± 0.01	1.06 ± 0.02
P6	37.8 ± 2.2	$26,500 \pm 1,600$	-24.8 ± 0.2	-79.5 ± 7.1	54.7 ± 7.1	0.61 ± 0.03	0.70 ± 0.08

[a] Data were obtained by ITC measurements at 20 °C (293.15 K). The measurements were repeated three times. [b] Binding constant K_a was calculated as the inverse of the dissociation constant K_d obtained from ITC measurements. [c] The Wiseman constant c was calculated using the following formula: $c = [\text{ConA dimer}] \times K_a \times N$. [d] Data adapted from Ref 47. [e] Stoichiometry N was fixed in fitting the parameters.

polymer sequence pattern, the primary driving force for self-folding in **P2**, **P3**, **P5** and **P6** was the intramolecular hydrophobic interaction of **FF** units in aqueous solution. Differences in the hydrophobicity of the glycopolymers in PBS were evaluated by fluorescence experiments using Nile Red. The maximum emission wavelength ($\lambda_{\text{max, em}}$) of Nile Red was 647 nm for **P1**, **P2**, **P4**, and **P5**, while it was blue-shifted to 622 nm and 627 nm for **P3** and **P6**, respectively (Figure S27). These results indicate that **P3** and **P6** possess more hydrophobic environments within their folded structures in aqueous solution, associated with their higher **FF** contents compared to **P2** and **P4**.⁵² Although the internal environment is not highly hydrophobic, the interaction between **FF** units observed in the CD spectra suggests that **P2** and **P5** adopt loosely cross-linked structures, whereas **P3** and **P6** are more compactly folded SCNPs.

The flexibility of the glycopolymers in solution was evaluated by measuring the spin-spin (T_2) relaxation times. The T_2 relaxation time reflects the decay of free induction decay (FID) signals in NMR measurements; a longer T_2 indicates greater polymer mobility (i.e., flexibility), and vice versa.^{53,54} The T_2 values measured at 1.5, 6.0 and 7.2 ppm—corresponding to the methylene proton of polymer main-chain, anomeric proton of mannose and the aromatic proton of **FF**, respectively—in the ¹H NMR spectra are shown in Figure 3A–C. The T_2 values measured in D₂O decreased more significantly with increasing **FF** content than those measured in DMSO, suggesting that the reduction in T_2 in aqueous solution is caused by the self-folding of the polymer chains. **P3** and **P6**, which contain higher **FF** ratios than **P2** and **P5**, exhibited shorter T_2 values, indicating reduced polymer-chain flexibility. Interestingly, the T_2 value of the mannose proton of **P3** in D₂O was significantly shorter than that of the triblock glycopolymers (**P6**) (Figure 3A). This result indicates that the mannose units located in the terminal blocks of **P6** retain greater mobility than those randomly incorporated into the folded structures of **P3**. These results indicate that we successfully obtained a series of glycopolymers, including non-folded glycopolymers (**P1** and **P4**) and SCNPs displaying mannose units (**P2**, **P3**, **P5**,

and **P6**), in which the polymer-chain flexibility is dependent on their conformation in aqueous solution (Figure 3D).

Subsequently, we evaluated the biomolecular interactions between the synthesized glycopolymers and ConA using isothermal titration calorimetry (ITC). ConA is a tetrameric lectin with four mannose-binding sites, one on each subunit.⁵⁵ An acetate buffer (pH = 5.2) was used to promote ConA dimer formation, preventing its aggregation during measurement.⁴⁴ A representative binding isotherm for **P2** is shown in Figure 4. The binding constants (K_a), enthalpy changes (ΔH), and entropy changes (ΔS) of **P1–P6** at 20 °C are summarized in Table 2, with the raw data provided in Figure S34–S39. Monovalent interaction was assessed using the **M** monomer.⁴⁷ All glycopolymers showed higher K_a values than **M**, indicating the multivalent display of mannose units enhances the interactions. This

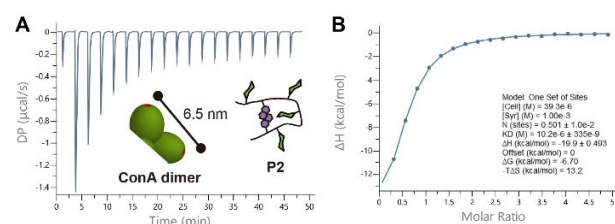


Figure 4. Representative ITC isotherms of **P2** titrated into ConA at 20 °C. **(A)** Raw thermograms after subtracting the control experiment with illustration of **P2** and ConA dimer. **(B)** Binding isotherm, with the solid line representing the fit. The experiment was conducted using 1 mM **P2** in the syringe and 39.3 μM ConA in the cell.

enhancement is attributed to multivalent binding and statistical rebinding of carbohydrate units on the polymers to the binding sites of ConA—known as the cluster glycoside effect.^{47,56–59} The binding constants of **P1** and **P4** were in close agreement with those reported for a multivalent glycoligand that binds to a ConA dimer in our previous work.⁴⁷ Interestingly, **P2**, an SCNP with random sequence pattern, exhibited a two-fold higher K_a than its linear

counterpart **P1**, while **P3**, the more compact SCNP, showed a K_a comparable to **P1**. Given that these glycopolymers have similar mannose contents and DP, this two-fold enhancement in K_a is significant. These results suggest that introducing an optimal amount of hydrophobic **FF** units into a random glycopolymer can favorably modulate biomolecular interactions. This trend is consistent with previous reports.^{43,45} Notably, **P3**, which contains a higher **FF** content than **P2**, did not exhibit stronger interactions than **P1**. Furthermore, a control experiment using a copolymer lacking mannose units confirmed that the **FF** units do not engage in nonspecific interactions with ConA (Figure S40). These results indicate that the high affinity of **P2** is not simply attributed to cooperative hydrophobic interactions between **FF** units and ConA. We propose that the reduced polymer-chain flexibility in **P2** suppressed the entropic penalty upon binding, thereby enhancing its interaction with ConA.^{60,61} However, since **P3** did not show high affinity for ConA, an appropriate degree of polymer flexibility—within a “Goldilocks regime”—is advantageous for achieving strong binding affinity to the target biomolecule.

The effects of the folded polymer structures with block sequence patterns were evaluated comparing **P4**, **P5**, and **P6**. In contrast to the random glycopolymers, increasing **FF** content led to a decreasing trend in K_a in the triblock glycopolymer series (Table 2). Notably, **P5** exhibited a lower K_a than **P2** despite their comparable flexibility (Figure 3). Since **P4**, **P5**, and **P6** have triblock structures with mannose units located in the first and third blocks, these glycopolymers are expected to exhibit divalent binding to a ConA dimer, whose two binding sites are separated by approximately 6.5 nm.⁵⁵ The increased stoichiometry observed for the triblock glycopolymers, compared to the random glycopolymers, supports this divalent binding mode (Table 2). However, in **P5** and **P6**, the self-folding may reduce the distance between the two mannose-bearing blocks, thereby hindering simultaneous binding to both sites and resulting in a lower binding constant.⁴⁷ These results demonstrate that the folded polymer structures distinctly affect biomolecular recognition depending on the polymer sequence pattern, due to differences in the spatial arrangement of functional groups and the polymer-chain flexibility (Figure 5). To maximize interaction strength, an effective molecular design strategy is to maintain a constant number of binding sites while minimizing polymer-chain flexibility.

In conclusion, we investigated the relationship between the conformation of glycopolymers (folded or non-folded) and their biomolecular recognition. Glycopolymers containing di(phenylalanine) units formed β -sheet-like structures in aqueous solution, resulting in non-covalently folded SCNPs. Mannose units were introduced into these SCNPs with either random or block sequences. Interestingly, folded SCNPs with random sequences enhanced binding to a model protein, concanavalin A, whereas the opposite trend was observed for SCNPs with block sequences. These findings demonstrate that both the spatial arrangement of the functional groups and the flexibility of polymer chains are critical factors in the design of biofunctional nanomaterials inspired by protein behavior.

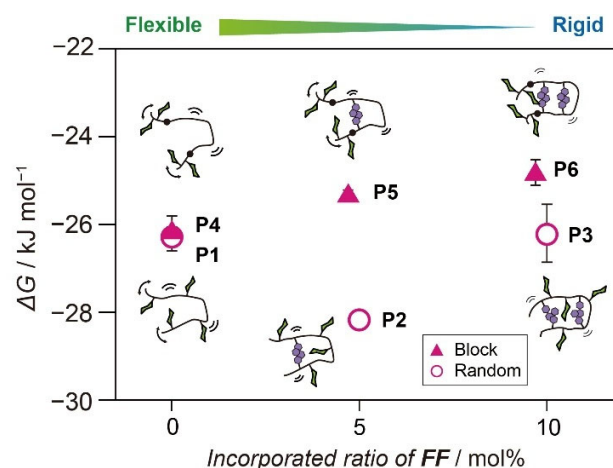


Figure 5. Plots of ΔG for the incorporated ratios of **FF** units in the glycopolymers. Blank circles and solid triangles represent random glycopolymers (**P1–P3**) and triblock glycopolymers (**P4–P6**), respectively. Illustrations represent the glycopolymer conformation in aqueous solution.

ASSOCIATED CONTENT

The Supporting Information is available free of charge via the Internet at <http://pubs.acs.org>.

Experimental procedures, details of synthesis and characterization of polymers, data of T_2 relaxation time measurement and ITC measurement.

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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