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Effective Formation of Stable and Versatile Double-Stranded *β*-Sheets Templated by a Hydrogen-Bonded Duplex

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We report herein an effective approach that is generally applicable for constructing double-stranded β–sheets composed of tetra- and penta-peptides based on a hydrogen-bonded duplex template, regardless of their amino acid sequences and α-helical or β-sheet propensities .

As one of the two major secondary structures, *β*–sheet plays as important a role as *α*-helix in protein folding. The formation and especially, stablization, of discrete *β*–sheet structures, like the stabilization of α-helices, is vital for answering fundamental questions on protein folding[1] and design,[2] and for creating biomimetic foldamers.[3] Since the incorporation of a designed β–hairpin into a monomeric protein structure in 1990[4] and the report of a monomeric *β*–hairpin in 1993,[5] the groups of Gellman,[6] Serrano,[7] Searle,[8] and Balaram[9] have pioneered the creation of discrete *β*–sheet structures,[10] based on which insights into the stabilization of *β*–sheets have been gained. Seminal contributions made by Kelly,[11] Nowick,[12] Hamilton,[13] and Cochran[14] provided various model *β*–sheets. Results from these and recent[15] studies uncovered factors such as amino acid sequence, backbone H-bonding, side-chain interaction, the nature of turns or turn mimetics, and backbone modification for stabilizing β-sheets.

However, the formation of stable and versatile double-stranded β-sheets with a non-covalent approach has few precedence, especially for pairing and aligning various peptides with different secondary structure preferences.

The Gong group has constructed H-bonded duplexes from oligoamide strands that associate via complementary H-bonding sequences, i.e., arrays of H-bonded donors and acceptors.[16] Using a H-bonded duplex as template, short tripeptides were paired into *β*–sheet structures.[17] Given that the energetic contribution made by the formation of the H-bonded duplex should stabilize the hybrid duplex and thus the templated *β*–sheet, we aimed to develop a generally applicable approach for aligning peptide chains of a variety of amino acid compositions into stable *β*–sheets.

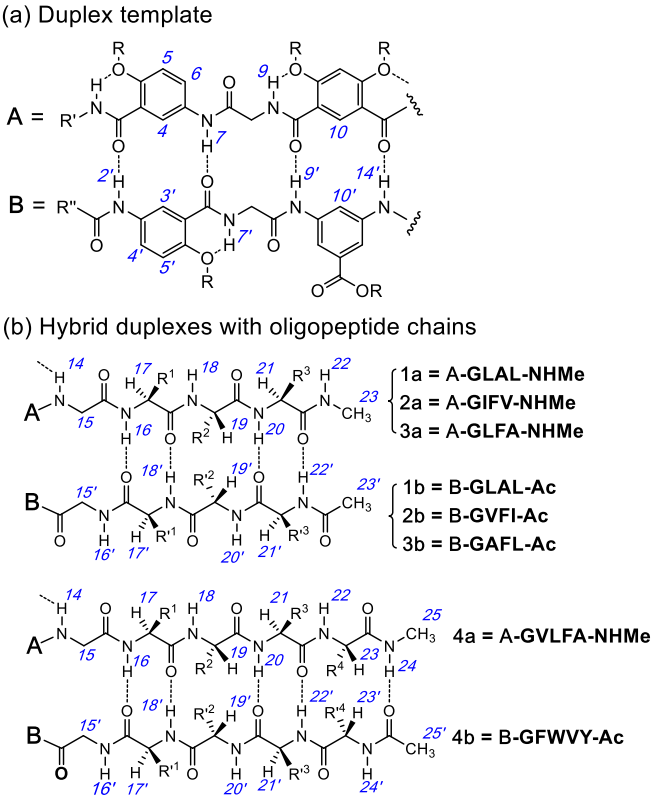

Figure 1. Design of hybrid duplexes. (a) Structures of template **A•B**. (b) Peptide strands attached to **A** and **B**, and the templated *β*-sheets. The aromatic, *α* and NH protons are labelled for the convenience of discussion (R = -$CH_2CH(CH_2CH_3)(CH_2)_3CH_3$ (for **2b**) or *n*-C_8H_{17} (for all others strands), R' = *n*-C_6H_{13}, R'' = *n*-C_5H_{11})

Herein we report the induction and stabilization of double-stranded, antiparallel *β*-sheets by H-bonded duplex template **A•B** that, with the unsymmetrical H-bonding sequences of **A** and **B**, can regiospecifically pair and align peptide chains attached to the same end of the duplex template. Mixing

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strands **1a**, **2a**, **3a**, and **4a** (the "a" strands) resulted from attaching peptide chains to template strand **A**, with another group of hybrid strands **1b**, **2b**, **3b**, and **4b** (the "b" strands) consisting of oligopeptides and template **B** results in the corresponding hybrid duplexes (Figure 1). The oligopeptides are designed to have amino acid residues known to have *β*-sheet (strands **2a**, **2b**) or *α*-helical (strands **1a**, **1b**, **3a**, and **3b**) propensities. Hybrid strands **4a** and **4b**, composed of two peptide strands with amino acid residues of high *β*-sheet propensities, are designed to probe the effect of peptide chain length on *β*-sheet formation.

Pairing strands **1a**, **2a**, and **3a**, with **1b**, **2b**, and **3b** in a 1:1 molar ratio resulted in nine possible combinations, each consisting of two tetra-peptides placed at the same end of template **A•B**. Six of the nine pairs, representing the three possible combinations of *α*-helical and *β*–sheet favouring peptides, i.e., **2a•2b** with both peptide chains favouring *β*-sheet, **1a•1b**, **3a•1b**, and **3a•3b** with peptide chains consisting of amino acid residues favouring *α*-helix, and **2a•1b** and **2a•3b**, each consisting of one peptide chain favouring *β*–sheet and the other preferring *α*-helix, were examined.

The specific pairing of the hybrid strands was first indicated by the drastically different solubilities of the individual strands and the 1:1 mixtures. At room temperature, the individual strands were found to be barely soluble in chloroform. In contrast, each of the six 1:1 mixtures showed noticeably improved, albeit modest solubility (< 2 mM). Adding 5% methanol improved the solubility of both single strands and the hybrid duplexes, with the solubility of the duplexes being much higher than the individual strands (Table S1).

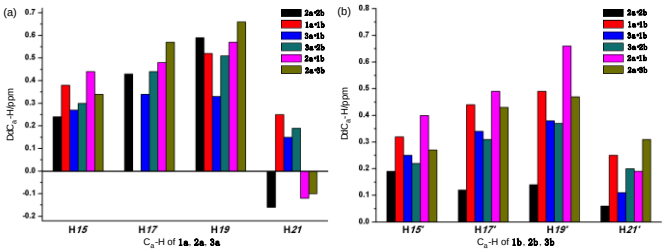

Figure 2. ΔδC^{α}H data [δC^{α}H(duplex) - [δC^{α}H(single strand)] for each C^{α}H in the six hybrid duplexes shown. The ^{1}H NMR spectra (800 MHz) were measured in $CDCl_3$ with 5% CD_3OH (4 mM) at 25 ^{o}C.

Changes in the chemical shifts of the *α* protons (ΔδC^{α}H) of amino acid residues in *α*-helices or *β*–sheets (or in the hybrid duplexes of this work) versus those of the corresponding single strands are known to be indicative of local secondary structures,[18] with upfield shifts being observed for *α*-helical residues, and downfield shifts of δC^{α}H indicating *β*–sheet residues. The δC^{α}H values of nearly all the residues in the hybrid duplexes exhibit downfield shifts relative to those of the single-strands (Figure 2 and Table S2), suggesting that the templated peptide chains, including those consisting of amino acid residues of *α*-helical propensities, adopt an extended conformation typical of *β*–strands. The *α* protons of the two outermost (terminal) cross-strand amino acid residues, i.e., H*21* and H*21'*, in the six hybrid duplexes, show small ΔδC^{α}H values that are either negative (for those of **2a**) or positive (for those of the other strands) (Figure 2), suggesting that the termini of the *β*–sheets are conformationally more flexible than the interiors.

The alignment and H-bonding interaction of the templated peptides was then examined by comparing the chemical shift change (ΔδNH) of the NH proton of each amino acid residue in a 1:1 mixture relative to that of the same residue in the single strand (Table S3). Comparing the ΔδNH values of strands **1a**, **2a**, and **3a** (Figure S1a), and **1b**, **2b**, and **3b** (Figure S1b) reveals that, NH protons *16*, *20*, *18'*, and *22'*, which belong to the H-bonded cross-strand amino acid pairs, exhibit significant downfield shifts, while protons *18*, *22*, *16'* and *20'*, i. e., those of the non-H-bonded cross-strand pairs, give much smaller ΔδNH values. The two distinct trends observed for the NH protons of the H-bonded and non-H-bonded amino acid pairs point to the pairwise H-bonding interaction of amino acid residues as specified by the duplex template, which suggests that the peptides strands indeed align into double-stranded *β*–sheets. The much smaller ΔδNH values of amide protons that belong to the non-H-bonded cross-strand pairs also indicate the weak association between the hybrid duplexes, i.e., these double-stranded structures exist mainly as , discrete and therefore soluble species under the experimental conditions.

Two-dimensional (2D NOESY) NMR studies on hybrid duplexes **2a•2b**, **2a•3b**, and **3a•1b** provided conclusive evidence for the presence of well-defined *β*–sheet structures (Figures S2-S4). Cross-strand NOEs between protons of template strands **A** and **B**, i.e., those involving protons *2'* and *4*, *3'* and *7*, *9'* and *10*, *10* and *10'*, and *10* and *14',* are found with all three duplexes, which demonstrate that template strands **A** and **B** associate sequence-specifically. For each hybrid duplex, in addition to the NOEs involving protons of the peptides (see below), cross-peaks between protons *23* and *23'*, i.e., those of the two terminal methyl groups of the peptide strands, were also observed, which confirms that the ends of the peptide chains are placed into close proximity. Besides, no NOE between the protons of the template and peptide segments was found. These observations are consistent with the expected full alignment of the hybrid strands.

Numerous NOEs were detected for the peptide segment of each hybrid duplex. The NOEs revealed for the peptide segment of **2a•2b** are shown in Figure 3 and S2, which include intra-strand contacts between $C_i^{\alpha}H$ and $N_{i+1}H$, i.e., the *α* protons of the amino acid residues and their adjacent amide protons (Figure S2a), indicating that the peptide strands adopt an extended conformation; cross-stands NOEs involving *α* and NH protons of the peptide backbones (Figures 3 and S2b), along with cross-strand contacts between the side-chain protons of two cross-strand Phe residues proved that the peptide chains are aligned in the way as directed by template **A•B** (Figures S2c).

The same three sets of intra-strand and cross-strand NOEs were also observed for **2a•3b** (Figure S3) and **3a•1b** (Figure S4), which demonstrate that all of the peptide strands adopt extended conformations, align as directed by the duplex template, and have their amino acid residues arranged into H-

bonded and non-H-bonded cross-strand pairs as expected for the templated *β*–sheets. The fact that the two "reluctant" peptide strands of **3a•1b**, which have residues of high *α*-helical propensities and are otherwise unlikely to fit into a model *β*-sheet structure, do align into a *β*-sheet clearly shows the generality of **A•B** in inducing and stabilizing this important secondary structure.

The folding of longer peptides assisted by template **A•B** was investigated by examining the 1:1 mixture of strands **4a** and **4b**. Similar to what is observed with the templated pairing of the other hybrid strands, that **4a** and **4b** undergo template-directed association to form a discrete hybrid duplex is indicated by the considerably improved solubility of the 1:1 mixture of the two strands over either single strand (**4b** is nearly insoluble) in $CHCl_3$ with 5% MeOH. The ^{1}H NMR spectrum of **4a•4b**, with the chosen amino acid residues that give well dispersed peaks (Figure S5), suggests that the penta-peptide chains adopt defined conformations. The NOESY spectrum of **4a•4b**, like those of **2a•2b**, **2a•3b**, and **3a•1b**, contains three sets of NOEs characteristic of a *β*–sheet structure, which include intra-strand NOE interactions between $C_i^{\alpha}H$ and $N_{i+1}H$ indicating the adoption of extended conformations by both peptide chains (Figure S6a). Cross-strand NOEs involving the backbone amide and *α* protons, including that between terminal protons *25* and *25'* (Figure S6b), and those between the protons of the two non-H-bonded cross-strand pairs of amino acid residues (Figure S6c) demonstrate that the penta-peptide chains of **4a•4b** are aligned side-by-side, with their amino acid residues being grouped into the H-bonded and non-H-bonded cross-strand pairs of the expected *β*–sheet.

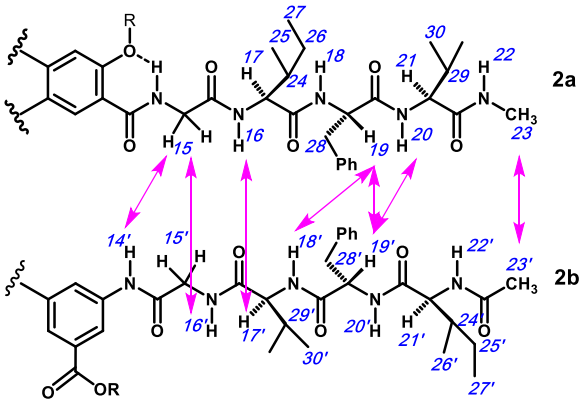

Figure 3. NOEs from the backbone-backbone revealed by the NOESY spectrum (4 mM in $CDCl_3$ with 5% CD_3OH, 800 MHz, mixing time 0.5 s, 27 °C) of the peptide segment of **2a•2b**.

While the reliabilty of **A•B** in templating peptide chains of different sequences into *β*–sheets has been demonstrated, the supramolecular nature of the duplexes allows their formation to be examined with isothermal titration calorimetry (ITC). Titrating an "a" strand into a "b" strand provided the association constants, along with other thermodynamic parameters that otherwise are difficult to collect, of the hybrid duplexes (Table 1), which not only quantify the stabilities of the templated β–sheets, but may also provide additional insights into the assembly and folding of these structures.

The association constants (K_a) of **2a•2b**, **2a•3b** and **3a•1b** are all in the 10^4 M^{-1} range, indicating that their *β*-sheets gain similar level of stabilization. The K_a values, however, do vary depending on the amino acid sequences of the tetra-peptide chains. Duplex **2a•2b**, with its two peptide chains favouring β-sheet, has the largest K_a; duplex **2a•3b**, with one peptide chain favouring *α*-helix and the other for *β*-sheet, has a K_a value in the middle; and **3a•1b** with both of its peptide chains preferring *α*-helix, gives the smallest K_a. The differences in the K_a values are consistent with what is known about the statistical *β*-sheet propensities of amino acids.[19]

ITC also revealed additional details about the assembly and folding of the peptide chains. Although **2a•2b** and **2a•3b** have similar Δ*G* values, the formation of **2a•2b** is mainly driven by enthalpy while that of **2a•3b** involves a reduced enthalpy contribution with an increased and favourable entropy factor. The assembly of **3a** and **1b** has an even smaller enthalpy contribution and is promoted more by entropy. Thus, the association of the peptides of **2a** and **2b** with β-sheet amino acid propensities is driven mainly by enthalpy, which probably results from favourable backbone H-bonding and side-chain interactions. In contrast, the association of peptides with residues that prefer α-helices, i.e., those of **1b**, **3a**, and **3b**, is promoted more by entropy than by enthalpy, which probably reflects the conformational re-adjustment these peptides undergo during their assembly and also the less favourable side-chain interactions after these peptides are "fitted" into β-sheets. Nevertheless, the details and mechanism behind the assembly induced folding of these peptides remain to be elucidated and warrant additional, detailed investigation.

Table 1. Association constants and thermodynamic parameters from ITC measurements in $CHCl_3$ with 5% DMSO.

ITC[a]	ΔH[b]	$T\Delta S$[b]	ΔG[b]	K_a[c]	N[d]
2a to **2b**	-8.9 ± 0.5	-2.16 ± 0.57	-6.74 ± 0.07	8.8 ± 1.1	0.91 ± 0.04
2a to **3b**	-4.2 ± 0.3	2.27 ± 0.45	-6.47 ± 0.15	5.5 ± 1.4	1.16 ± 0.06
3a to **1b**	-2.7 ± 0.1	3.20 ± 0.11	-5.90 ± 0.06	2.1 ± 0.2	1.40 ± 0.02
4a to **4b**	-10.2 ± 0.1	-3.2 ± 0.14	-6.97 ± 0.04	12.9 ± 0.9	1.07 ± 0.01

[a] Concentrations (mM): 1.0 (**2a** and **4a**); 4.0 (**3a**); 0.4 (**1b**), 0.1 (**4b**); 0.075 (**3b**), and 0.05 (**2b**). [b] kcal/mol. [c] 10^4 M^{-1}. [d] Number of binding sites.

Compared with the other three duplexes, **4a•4b**, with one additional pair of amino acid residues and one more cross-strand H-bond, has a K_a that is considerably larger, indicating that the templated *β*-sheet has an enhanced stability as the length of peptide chains extends. Similar to that of **2a•2b**, the formation of **4a•4b**, which involves penta-peptide chains with β-sheet amino acid propensities, is enthapically driven.

For all four hybrid duplexes, the cooperative interaction between the duplex template and peptide segments is apparent. In 5% DMSO/$CHCl_3$, the association constant of H-bonded duplex **A•B** is too low to be measured by ITC;[18] titrating the solution of a penta-peptide into that of another penta-peptide in the absence of template **A•B**, or the solution of **4a** into that of a template-free penta-peptide, failed to

reveal any association between the molecules (Figure S8). These observations clearly demonstrate the measured stabilities of the hybrid duplexes are cooperatively contributed by both the template and the peptide segments.

Results from this work indicate that peptides with low *β*-sheet preferences[19] or an extended strand length are all templated into well-defined *β*-sheets, which clearly demonstrates the effectiveness and generality of this approach. Testing longer peptide chains is a worthy goal of our subsequent study. The non-covalent templation of different peptide chains offers a powerful strategy for not only investigating the formation and stabilization of β-sheets, but also for creating new β-sheet structures consisting of peptide chains that normally are not compatible with this important secondary structure. Such new β-sheet structures may find many possible applications, such as chiral recognition, sensing, and asymmetric catalysis.

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Conflicts of interest

There are no conflicts to declare.

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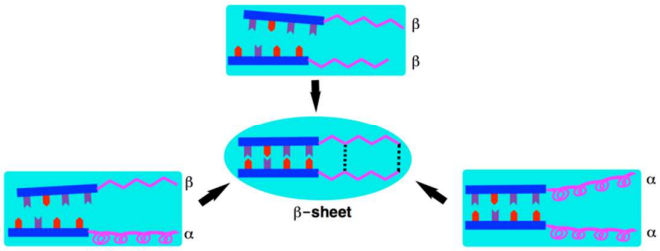

An effective approch to construct the stable and versatile double-stranded β–sheets composed of tetra- and penta-peptides through a hydrogen-bonded duplex template has been explored.