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Stabilization of Collagen-Model, Triple-Helical Peptides for In Vitro and In Vivo Applications

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Abstract

The triple-helical structure of collagen has been accurately reproduced in numerous chemical and recombinant model systems. Triple-helical peptides and proteins have found application for dissecting collagen-stabilizing forces, isolating receptor- and protein-binding sites in collagen, mechanistic examination of collagenolytic proteases, and development of novel biomaterials. Introduction of native-like sequences into triple-helical constructs can reduce the thermal stability of the triple-helix to below that of the physiological environment. In turn, incorporation of nonnative amino acids and/or templates can enhance triple-helix stability. We presently describe approaches by which triple-helical structure can be modulated for use under physiological or near-physiological conditions.

Keywords

Collagen; Triple-helix; Template; Peptide-amphiphile; Proline analogs; Disulfide knot

1 Introduction

1.1 Collagen

Collagen is the most abundant protein in animals, and is the major structural protein found in the connective tissues such as basement membranes, tendons, ligaments, cartilage, bone, and skin. The collagen family consists of at least 28 members [1–5]. The most defining feature of collagen is the supersecondary structure, composed of three parallel extended left-handed polyproline type II alpha chains of primarily repeating Gly-Xxx-Yyy triplets. Three left-handed strands intertwine in a right-handed fashion around a common axis to form a triple-helix (Fig. 1). The formation of the triple-helix creates a shallow superhelical pocket inside it. The collagen triple-helix is essential for the integrity of multiple connective tissues.

Depending on the primary structure of the alpha chains [4], collagens have been classified into two main categories, homotrimeric and heterotrimeric. Homotrimeric collagens have three identical alpha chain sequences (designated α1). Examples of such type of collagens are types II and III. Alternatively, heterotrimeric collagens have either three alpha chains of different sequence, designated as α1, α2, and α3 (i.e., type V) or two alpha chains of identical sequence (α1) and third alpha chain of different sequence (α2), such as types I and

VI [6]. Furthermore, based on their quaternary structure, collagens are grouped into subfamilies such as fibrillar, collagen associated with banded fibrils (i.e., fibril-associated collagens with interrupted triple-helices—FACITs), network-forming (i.e., basement membrane and short chain), transmembranous, and membrane associated with interrupted triple helices (MACITs) [6].

1.2 Stability of Collagen-Model Triple-Helical Peptides

For several decades triple-helical peptides (THPs) or "mini-collagens" consisting of collagen-model sequences and their three-dimensional folds have been constructed and studied to fully investigate the structural and biological roles of collagenous proteins [5, 7–13]. A model for the collagen triple-helix was first proposed in the 1950s [14, 15] and subsequently refined over time [16–20]. To understand the stability of the collagen triple-helix, it is important to understand its compositional elements. In the collagen Gly-Xxx-Yyy triplet, the residue in the Xxx position is often L-proline (Pro) and the residue in the Yyy position is often 4(R)-hydroxy-L-proline (Hyp), accounting for 20 % of the total amino acid composition in collagen [21]. The other commonly found amino acids are Ala, Lys, Arg, Leu, Val, Ser, and Thr [21]. The packing of the triple-helical coiled-coil structure requires Gly in every third position. Because of its compact structure, assembly of the triple-helix puts this residue at the interior of the helix and the side chain of the Gly, an H atom, is small enough to fit into the center of the helix.

Several factors influence the stability of triple-helical peptides. The chains are held together by hydrogen bonds that form between the peptide amine of Gly residues and peptide carbonyl groups in an adjacent polypeptide chain (Fig. 2) [9, 18]. Interstrand hydrogen bonds formed between the GlyN-H and XxxC=O are critical for triple-helix stability, as replacement of a central amide bond of (Pro-Pro-Gly)$_{10}$ with either an ester or an (*E*)-alkene, or replacement of the central Pro-Pro with a Pro-*trans*-Pro isostere, substantially destabilizes the THP [22–24].

The role of hydrogen bonding versus inductive effects in triple-helix stabilization is an intensive area of THP research [5]. Incorporation of several nonnative amine acids in the proper position within the peptide sequence often remarkably enhance triple-helix stability. One such example of a stabilizing nonnative amino acid is 4*R*-fluoroproline (4*R*-Flp) (Fig. 3). Incorporation of 4*R*-fluoroproline has been shown to induce hyperstability in the triple-helix of (Pro-4*R*Flp-Gly)$_{10}$ compared to (Pro-4*R*Hyp-Gly)$_{10}$ [25, 26]. Alternatively, 4*S*-Flp destabilizes the triple-helix of (Pro-4*S*Flp-Gly)$_7$ compared to (Pro-4*R*Hyp-Gly)$_7$ [27]. More specifically, substitution of all of the 4*R*-Hyp residues in (Pro-4*R*Hyp-Gly)$_{10}$ or (Pro-4*R*Hyp-Gly)$_7$ by 4*R*-Flp increased T_m by 22 and 9 °C, respectively [25–27]. Conversely, an analogous substitution in (Pro-4*R*Hyp-Gly)$_7$ by 4*S*-Flp dramatically decreased T_m by greater than 26 °C [27]. The relative effects of 4*R*-Hyp, 4*R*-Flp, and 4*S*-Flp coincide with their propensity for forming *trans*-peptide bonds compared to *cis*-peptide bonds [27, 28]. Studies of collagen mimics have indicated that this increased stability arises from the inductive effects of an electronegative substituent in the 4*R* position, as in 4(*R*)-fluoroproline [9, 25, 26].

It has long been noted that the thermal stability of the collagen triple-helix is enhanced by Hyp residues. It was hypothesized that this stability is due to Hyp residues involved in the hydrogen-bonded network mediated by water molecules, which connect the hydroxyl group of Hyp in one strand to the main chain amide carbonyl of another chain [29]. Substituting Pro for Hyp in the chain significantly decreased the T_m in collagen-model peptides [29]. However, collagen peptides containing the nonnative amino acid 4*R*-Flp exhibited higher stability than peptide with Hyp residues [25, 26] and thus the stabilizing effect arising from Hyp might not be due to hydrogen bonding but rather from the electronegative oxygen preorganizing the main chain in the proper conformation for triple-helix formation [30]. X-ray crystallographic analysis indicated that the 4-OH group in Hyp has no effect on the hydration pattern and the resulting molecular structures [31]. The *O*-methylation of hydroxyproline in Yyy stabilizes the triple-helix more than Hyp itself, most likely because the pyrrolidine ring of 4-methoxyproline adopts a Cγ-exo ring pucker. The conformational stability of the triple-helix arising from *O*-methylation provides strong evidence that the hydroxyl group of Hyp acts primarily through stereoelectronic effects [30].

Both the position of the residue containing the electronegative substituent and the stereochemistry of that electronegative substituent play a role in triple-helix stability. The positional effects of γ-substitution are illustrated by the stabilities, relative to $(Pro\text{-}Pro\text{-}Gly)_{10}$, of $(Pro\text{-}4R Hyp\text{-}Gly)_{10}$ (increased triple-helix stability) and $(4R Hyp\text{-}Pro\text{-}Gly)_{10}$ (decreased stability) [32]. For many years, it was believed that peptides containing 4*R*-Hyp in the Xxx position of Gly-Xxx-Yyy repeats do not form collagen-like triple-helices. However, incorporation of 4*R*-Hyp in the Xxx position and Thr or Val in the Yaa position triggers triple-helix formation [33, 34]. For example, acetyl-$(Gly\text{-}4R Hyp\text{-}Thr)_{10}$-$NH_2$ forms a stable triple-helix in water [33]. Both the hydroxyl and methyl groups of Thr with their stereochemical configuration appear to induce the triple-helix stability. Molecular modeling showed that the Thr methyl group shields the interchain hydrogen bond between the carbonyl group of Hyp of the adjacent chain and the amino group of the next Gly residue in the same chain.

Other significant factors that influence the stability of collagen triple helix are the *trans*/*cis* ratio of the Xaa_{i-1}-Pro_i peptide bond and the ring pucker of Pro_i [35, 36]. The *trans*/*cis* ratio is crucial as all of the peptide bonds in the triple-helix prefer the *trans* conformation. Pro in solution can adopt either exo or endo ring puckers (Fig. 3). The first Pro in the Pro-Pro-Gly triplet prefers an endo ring pucker conformation while the second Pro prefers the exo ring pucker conformation [35]. This observation suggested that Pro derivatives preferring the Cγ-endo ring pucker could pre-organize triple-helix formation when in the Xaa position, whereas those that prefer the Cγ-exo ring pucker could preorganize triple-helix formation when in the Yaa position [37].

As discussed earlier, incorporation of 4*R*-Flp (Fig. 3) in the Yyy position of Xxx-Yyy-Gly triplets induces hyperstability in the triple-helix.Thisisbecausethe4*R*-fluorogroupin4*R*-fluoroproline stabilizes the Cγ-exo pucker conformation. These stereoelectronic effects markedly enhance the conformational stability of a collagen triple-helix [38].

Similar to 4*R*-Flp, incorporation of 4*R*-methylproline (4*R*-mep) (Fig. 3) within the peptide sequence significantly increases triple-helix stability. Raines and colleagues studied the effect of 4-methylproline in conformational stability of (Xxx-Yyy-Gly)$_7$. 4*S*-Methylproline (4*S*-Mep) in the Yyy position enhanced triple-helix stability more so than 4*R*-mep in the Xxx position [38]. A (4*R*-mep-4*S*-Mep-Gly)$_7$ triple-helix is more stable than (4*R*-mep-Pro-Gly)$_7$ or (Pro-4*S*-Mep-Gly)$_7$, simply because the steric effects are additive. In contrast, (4*S*-flp-4*R*-Flp-Gly)$_7$ is less stable than (4*S*-flp-Pro-Gly)$_7$ or (Pro-4*R*-Flp-Gly)$_7$ where the stereoelectronic effects induced by the hetero atom are not additive [38].

The combination of stereoelectronic and steric effects can also induce the preorganization of a polypeptide chain. Integration of both effect results in the most stable triple-helix known so far. Among four triple-helices formed from the four different polypeptide chains, (Pro-Pro-Gly)$_7$, (Pro-4*R*-Hyp-Gly)$_7$, (4*S*-flp-4*S*-Mep-Gly)$_7$, and (4*R*-mep-4*R*-Flp-Gly)$_7$, only the latter two showed hyperstability with T_m values of 51 and 58 °C, respectively [37].

The stability of THPs may also be regulated by pH. To create a triple-helix that was pH dependent, Hyp was modified by *O*-alkylation to a carboxylate group [39]. The incorporation of 1 or 3 Hyp(CO_2) residues within a Pro-Hyp-Gly template did not result in a pH-sensitive triple-helix. However, acetyl-[Pro-Hyp(CO_2)-Gly]$_7$-OH formed a triple-helix with a T_m = 17 °C at pH 2.7 but had no triple-helical structure at pH 7.2 [39]. pH-dependent triple-helical stability was also observed for (Pro-4*R*-Amp-Gly)$_6$ sequences, where 4*R*-Amp is (2*S*,4*R*)-4-aminoproline [40, 41].

Self-association of triple-helical structures has been used to “sandwich” a collagen-model sequence between repeats of Gly-Pro-Hyp to obtain THPs of reasonable stability (Fig. 4). Self-associated triple-helical peptides were used to study the structural aspects of collagen via the “host–guest” approach, using sequences such as (Pro-Hyp-Gly)$_n$-Xxx-Yyy-Gly-(Pro-Hyp-Gly)$_n$ [42–44]. Compared with Gly-Pro-Hyp, none of the 20 natural amino acids provides enhanced thermal stability. The most stable residues in the Yyy position were Hyp > Arg> Met, while for the Xxx position Pro > charged residues> Ala> Gln [43–45]. Within a host–guest THP 4*R*-Flp in the Yyy position is slightly destabilizing compared with Hyp, in contrast to (Pro-4*R*-Flp-Gly)$_n$ and (Pro-Hyp-Gly)$_n$ THPs [46]. It has been suggested that different mechanisms are in place when Pro-4*R*-Flp-Gly is inserted within (Pro-Hyp-Gly)$_n$ compared with (Pro-4*R*-Flp-Gly)$_n$ [46].

Electrostatic effects can also contribute favorably to the stability of THPs, as found in host–guest studies [45, 47, 48]. Favorable electrostatic interactions were observed for Gly-Lys-Asp and Gly-Arg-Asp within the acetyl-(Gly-Pro-Hyp)$_3$-Gly-Xxx-Yyy-(Gly-Pro-Hyp)$_4$-Gly-Gly-NH_2 host [49]. Conversely, guests Gly-Arg-Lys, Gly-Lys-Arg, and Gly-Glu-Asp exhibit charge repulsion [49]. The sequence Gly-Pro-Lys-Gly-[Asp/Glu]-Hyp is found to be as stabilizing as (Gly-Pro-Hyp)$_2$ due to interchain axial ion pair interactions [50, 51]. Arg in the Yyy position can offer high stability, possibly based on its ability to form a hydrogen bond with C=O in a neighboring strand [45, 52]. Heterotrimeric host–guest peptide approaches show a decreased thermal stability for an acetyl-(Gly-Pro-Hyp)$_3$-Gly-Pro-Yyy-(Gly-Pro-Hyp)$_4$-Gly-Gly-NH_2 sequence with an increased number of Arg residues [53]. For example, Hyp residues in all Yyy positions results in a THP with a T_m = 47.2 °C compared

to 44.5, 40.8, and 37.5 °C, respectively, when all Hyp is replaced by Arg residues in one chain, two chains, or three chains [53].

1.3 Synthesis of Fmoc-Pro Derivatives

To efficiently incorporate (*2S, 4R*)-4-fluoroproline (Flp) and (*2S, 4R*)-4-methylproline (mep) into peptide sequences, convenient synthetic routes for the preparation of derivatized Flp and Mep residues such as Fmoc-*trans*-Flp **6** and Fmoc-*trans*-Mep **14** are required (Figs. 5 and 6). Several methods have been reported in the literature for the synthesis of *N*-protected-4(*R*)-Flp from *N*-protected-4(*R*)-Hyp derivatives [54–63]. Fields et al. previously reported the synthesis of Fmoc-*trans*-Flp starting from expensive *cis*-4(*S*)-hydroxy-L-proline [64]. The removal of benzyl ester was problematic under standard Pd/C hydrogenation, as the Fmoc group started decomposing upon prolonged exposure to Pd/C-hydrogen [65, 66]. Recently, we developed a more convenient synthetic route for the preparation of Fmoc-*trans*-Flp from readily available (and inexpensive) *trans*-4(*R*)-hydroxy-L-proline (Fig. 5). A one-pot, three-step procedure is deployed involving a bis-deprotection of the *N*- and *C*-termini under catalytic hydrogenation conditions followed by selective capping of the *N*-terminus with an Fmoc group to yield Fmoc-*trans*-Flp.

The strategy for the synthesis of the Fmoc-*trans*-Flp entails initial Cbz-protection of the amine nitrogen followed by benzylation of the carboxylic acid group. Cbz protection of *trans-4*(*R*)-hydroxy-L-proline is performed by using standard protocols to obtain **1** in 85 % of yield (Fig. 5) [67]. The conversion of *N*-Cbz-4(*R*)-Hyp **1** to *N*-Cbz-4(*R*)-Hyp-OBzl **2** is achieved in 80 % yield by reaction with $CsCO_3$ and BnBr [68]. Mitsunobu reaction of *N*-Cbz-4(*R*)-Hyp-OBzl **2** followed by hydrolysis results in the *N*-Cbz-4-Hyp derivative **4** in 42 % (two-step overall yield) yield with inversion of configuration at C-4 position [69]. The next step is the synthesis of diastereomeric fluoroproline by a stereospecific displacement of the hydroxyl group of Hyp by fluorine. Fluorination of *N*-Cbz-4(*S*)-Hyp-OBzl **4** is performed using diethylaminosulfur trifluoride (DAST) as fluorinating agent to yield *N*-Cbz-4(*R*)-Flp-OBzl **5** in 50 % yield [69]. Finally, catalytic hydrogenation of **5** with 5 % Pd/C under hydrogen (atm. pressure) in the presence of Fmoc-OSu affords desired *N*-Fmoc-4-Flp **6** in good yields (Fig. 5) [70]. This one-pot reaction involved the simultaneous removal of the Cbz- and benzyl-protecting groups and re-protection of amine nitrogen with Fmoc group. Optimal yield (~75 %) is observed with a reaction time of 2 h, whereas prolonged exposure of the reaction mixture to hydrogenation resulted in partial Fmoc deprotection.

To synthesize Fmoc-*trans*-mep **14** (Fig. 6), initially *N-Boc-trans*-mep **13** is synthesized by following a straightforward method reported by Goodman et al. [71]. The reaction starts with initial Boc protection of commercially available *trans*-4(*R*)-hydroxy-L-proline followed by carboxylic acid group reduction to provide Boc-protected alcohol **7**. Selective protection of the primary alcohol with TBDMSCl gave compound **8**, which is then oxidized with trichlorocyanuric acid and catalytic TEMPO to afford pyrrolidinone **9** in 78 % yields. Reaction of pyrrolidinone **9** with methyltriphenylphosphonium bromide gave olefin **10** in 75 % yields. Silyl-ether deprotection of **10** was first carried out to unmask the hydroxyl directing groups. Treatment of the resulting olefin **11** with 3 mol% of the Crabtree catalyst,

under H_2 atm, gave excellent selectivity of *trans*-4-methyl pyrrolidine **12** in 85 % yields. Oxidation of the indolylprolinol derivative **12** in the presence of TEMPO, bleach, and sodium chlorite provided *N*-Boc-*trans*-mep **13**. Finally, deprotection of Boc group with 4 N HCl followed by Fmoc installation using Fmoc-OSu and aqueous $NaHCO_3$ in dioxane provided Fmoc-*trans*-mep **14** in 85 % yield [38].

1.4 Covalent Modification of Triple-Helical Peptides

Several covalent modification strategies have been employed to induce triple-helix structure formation and/or enhance their stability. Substantial stabilization can be achieved by use of a self-assembly approach where alignment of amphiphilic compounds at the lipid–solvent interface facilitates peptide alignment and structure initiation and propagation. Addition of lipophilic molecules at the *N*-terminus of the peptide, known as the “peptide-amphiphile” (PA) approach, often stabilizes self-associated peptides. The term peptide-amphiphile was first used in 1984, when an alanine residue was interposed between a charged head group and a double-chain pseudo-lipid tail [72]. In this approach pseudo-lipids such as mono- and di-alkyl chains are covalently attached to peptides to create peptide-amphiphiles that then associate via hydrophobic interactions (Fig. 4) [73–77]. Peptide-amphiphiles are advantageous in that they present a multivalent ligand [78] that is chemically well defined, avoiding loss of activity that can occur during nonspecific coupling of peptides to lipids [79]. The amphiphilic character of peptide-amphiphiles allows for the control of assembled structures by manipulating their molecular composition [80]. The thermal stability of triple-helical peptide-amphiphile head groups can be modulated by the length of the lipophilic moiety [73, 74, 76, 81, 82]. The thermal stability of the triple-helical structure in the peptide-amphiphile was found to increase as the monoalkyl tail chain length was increased over a range of C_6–C_{16} [74]. Conversely, alterations in the pseudo-lipid tail composition affect peptide-amphiphile aggregate structures [76, 80]. Desirable peptide head group melting temperature values can be achieved for in vivo use, as triple-helical PAs have been constructed with T_m values ranging from 30 to 70 °C [64, 73, 74, 76, 81–85].

The stability of associated THPs may also be regulated by photolysis. Modification of the sequence (Gly-Pro-Hyp)$_3$-Gly-Cys-Hyp-Gly-Pro-Hyp-Gly-Pro-Cys-(Gly-Pro-Hyp)$_5$-Gly-Gly-NH_2 with an azobenzene bridge between the Cys residues resulted in a THP whose stability decreased up irradiation at λ =330 nm at 27 °C [86]. Unfortunately, the poor solubility of this peptide prohibited quantitative analysis of its stability [86].

The use of template-assisted approaches is another important strategy to create stable triple-helical conformation. In recent years much progress has been made in template-assisted synthetic methodologies. These include orthogonal protection schemes, chemoselective ligation, and the design of novel and increasingly flexible templates. Many templates have been used to create stable THPs. The main categories of template strategies involve a scaffold to which the peptides are covalently attached or the use of metal ions for non-covalent association. The covalent templates include (1) multi-Lys branching [87–91], (2) a double-disulfide “knot” (cystine knots) [53, 92–96], (3) *cis*,*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid (Kemp triacid; KTA) [97–101], (4) tris(2-aminoethyl)amine (TREN) [102], (5) cyclotriveratrylene (CVT) [103], (6) macrocyclic

scaffold [104], or (7) carbohydrates (Fig. 7) [105, 106]. Alternatively, the metal ions Ca^{2+}, Ru^{2+}, Ni^{2+}, Fe^{2+}, Cd^{2+}, and Hg^{2+} have been used as templates in conjugation with amino acids or *N*-terminal pyridyl or bipyridyl functionalities to create multistrand linkages [107–109].

A significant strategy to stabilize THPs is the covalent attachment of three strands via a *C*-terminal branch. The *C*-terminal branch is expected to align and entropically stabilize the *C*-terminus of the THP and thus enhance triple-helical thermal stability [90] and to provide a model of the disulfide-linked *C*-terminus of type III collagen [110, 111]. Branching can be achieved by selective deprotection of Lys N^{α}- and N^{ε}-amino groups.

Solid-phase assembly of triple-helical collagen-model peptides using a Lys-Lys C-terminal branch requires three different protecting group strategies (Fig. 8): N^{α}-amino protection (**A**); Lys N^{ε}-amino side-chain protection (**B**), which must be stable to the N^{α}-amino group removal conditions; and C^{α}-carboxyl protection (*linker*), which must be stable to both the N^{α}- and N^{ε}-amino-protecting group removal conditions. Based on the three different protecting group strategies, four different branching combinations have been developed [90, 112]. Branching is achieved by synthesizing **A**-[Lys(**B**)]$_2$-Tyr(**C**)-Gly-*linker* resin and deprotecting the N^{α}- and N^{ε}-amino groups. Tyr was incorporated prior to branching to provide a convenient chromophore for eventual concentration determination. Incorporation of Ahx (6-aminohexanoic acid) onto the *N*-termini of all three strands provided a flexible spacer. All solid-phase methods to prepare THPs were based on Fmoc N^{α}-amino group protection. For example, the type (IV) collagen-derived α(IV)1263-1277 branch was assembled by a three-dimensional orthogonal strategy, where **B** was *tert*-butyloxycarbonyl (Boc) and *linker* was 4-trityloxy-*Z*-but-2-enyloxyacetic acid [cleaved by $(Ph_3P)_4Pd$-catalyzed nucleophilic transfer] [91]. A $(Gly\text{-}Pro\text{-}Hyp)_8$ branch has also been assembled by using the same orthogonal strategy, where **B** was allyloxycarbonyl (Aloc) [cleaved by $(Ph_3P)_4Pd$-catalyzed nucleophilic transfer] and linker was hydroxymethylphenoxy (HMP; cleaved by TFA). The third three-dimensional orthogonal strategy was developed using the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) group as **B** (cleaved by hydrazine/DMF) and the linker was HMP [90]. Due to the mild conditions the Fmoc/Dde combination (with ivDde replacing Dde) is now the most preferred. For example, Farndale, Barnes, and colleagues reported the synthesis of THPs, based on the bovine α_1(III)CB4 fragment, via this *C*-terminal covalent attachment strategy [113–115].

A double-disulfide “knot” (cystine knots) either at the *C*-terminal region or at the *N*-terminal region of three peptide strands is another frequently used strategy to stabilize THPs [3, 92, 94–96]. One representative example of a *C*-terminal cysteine knot is construction of branched heterotrimeric THP **15** (Fig. 9). For this peptide all three individual chains are constructed by solid-phase methodology using Fmoc chemistry. The α1 chain contained either 3 or 5 Gly-Pro-Hyp repeats on the *N*-terminus of Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Cys(Acm)-Gly-Gly-OH, where Acm was acetamidomethyl, the α2 chain contained 3 or 5 Gly-Pro-Hyp repeats on the *N*-terminus of Gly-Pro-Gln-Gly-Leu-Leu-Gly-Ala-Hyp-Gly-Ile-Leu-Gly-Cys(Acm)-Cys(S*t*Bu)-Gly-Gly-OH, and the α1’ chain contained either 3 or 5 Gly-Pro-Hyp repeats on the *N*-terminus of Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Cys(S*t*Bu)-Gly-Gly-OH. The three chains are

assembled into the heterotrimer by stepwise regioselective cross-linking. Peptide α1 is treated with 3-nitro-2-pyridine-sulfenyl chloride (Npys-Cl) to convert Cys(Acm) to Cys(Npys). Peptide α2 is treated with $P(C_4H_9)_3$ to remove the S*t*Bu group. Peptides α1 and α2 are reacted at pH 4.5 to form a dimer. The dimer is treated with Npys-Cl to convert the α2 chain Cys(Acm) to Cys(Npys). The α1' chain is treated with $P(C_4H_9)_3$ to remove the S*t*Bu group. Peptide α1' and the dimer are reacted at pH 4.5 to form a trimer.

Another example of an *N*-terminal cysteine knot is construction of branched THPs **16**–**19** (Fig. 10) using the same protocol described for the *C*-terminally branched cysteine knot heterotri-meric THP [53]. Heterologous trimerization is achieved by stepwise disulfide bond-forming reactions (Fig. 9). First, the free thiol groups of the biscysteinyl peptides (**20a**, **20b**) are converted to 2-pyridylthio (PyS) groups by treatment with PySSPy. The purified PyS-activated peptides (**21a**, **21b**) are then mixed with monocys-teinyl peptides (**22a**, **22b**) to yield corresponding heterodimeric peptides (**23a**, **23b**). Formation of small amounts of homodimers is also observed in this reaction. The S-Acm groups in the heterodimers are converted to S-3-nitro-2-pyridinesulfenyl (Npys) groups by treating them with NpysCl. Even under optimized conditions, this reaction generated some by-products, including homodimers. Finally, the Npys-activated dimers (**24a**, **24b**) were mixed with monocysteinyl peptides (**22a**, **22b**) to yield desired heterotrimers.

Stabilization of THPs can also be achieved by cross-linking both the *C*- and *N*-terminal ends of three peptide chains. This strategy required synthesis of three different fragments (Fig. 11) [88]. The first synthesis was of linear peptides of the sequence Cys-Gly-$(Gly\text{-}Pro\text{-}Hyp)_n$-Lys(Ser)-$NH_2$ (*n* =3–10) **25** using Fmoc chemistry, starting from Fmoc-Lys[Boc-Ser(*t*Bu)]-OH, which in turn was prepared from reaction of Boc-Ser(*t*Bu)-OSu with Fmoc-Lys-OH. The second synthesis was of two different trivalent anchor molecules: tris-bromoacetylated Lys-Lys dimer **26** and tris-aminooxyacetylated branch peptide **27** (synthesized from Bocaminooxyacetic acid). Chemoselective ligation of **25** and **26** followed by oxidation of the Lys(Ser) residue created an aldehyde group for the next ligation site. Final ligation of the *N*-terminus-coupled peptide with the tris-aminooxyacetyl group of anchor molecule **27** in aq. buffer produced di-cross-linked peptide **30**.

Use of the conformationally constrained organic template *cis*,*cis*-1,3,5-trimethylcyclohexane 1,3,5-tricarboxylic acid (Kemp triacid, KTA, Fig. 7) is another strategy to induce or stabilize triple-helical structures [97–101]. This template possesses three carboxyl groups which can be coupled to the *N*-termini of three peptide chains. Attachment of a Gly residue as a spacer between each peptide chain and each carboxyl group on KTA is vital to compensate for the difference in diameters between the KTA and the collagen triple-helix and to facilitate the synthesis [97]. Two synthetic routes have been used to prepare KTA template-assembled collagen-based structures. The first method exclusively utilized solid-phase methodology [101], whereas in the second method formation of the peptide bond between KTA and peptide–peptoid chains was in solution [99]. In the solid-phase method Boc-$(Gly\text{-}Pro\text{-}Hyp)_n$ MBHA resin is prepared and then the Boc group is removed. KTA-$(Gly\text{-}OH)_3$ is then coupled to the *N*-termini of peptide chains using DIPCDI and HOBt. In the second method peptide–peptoid chains are synthesized by solid-phase methods and cleaved from the resin.

The free amine peptide–peptoid chains are then coupled with KTA-(Gly-OH)$_3$ in solution using EDC and HOBt.

TREN (Fig. 7) is another beneficial scaffold for the synthesis of template THPs [102]. The TREN molecule is a flexible tripodal structure with three aminoethyl groups. TREN-(suc-OH)$_3$ template is prepared by coupling TREN to monobenzylated succinate, and removing the benzyl ester groups by hydrogenation. The succinic acid groups extend the flexibility of the scaffold and provide terminal carboxylates for attachment of peptide chains. The succinic acid spacers release any steric hindrance by extending the reactive sites. TREN-(suc-OH)$_3$ is acylated to three peptide strands simultaneously on the solid phase using DIC and HOBt.

Another synthetic scaffold molecule, which can be attached to both model and native collagen sequences, is cone-shaped CVT (Fig. 7) [103]. Attachment to the *N*-terminus of the collagen peptides with CVT is done in solution using BOP as a coupling reagent.

Stable collagen triple-helices are also synthesized by using a macrocyclic scaffold [104]. This scaffold belongs to a class of 18-membered cyclic hydropyran oligolides with alternating ester and ether linkages. In this scaffold, three ligand attachment sites form an equilateral triangle on one face. Macrocyclic template is coupled to the *N*-terminus of collagen-model peptides in solution using PyBOP and DIEA.

The use of carbohydrates as potential templates is another strategy for the *de novo* design of triple-helical peptides (Fig. 7) [105, 106]. Carbohydrates are multifunctional molecules having comparative rigidity of ring forms, simplicity in regioselective manipulation of functional groups, and access to mono- and di-saccharide stereoisomers. The primary and secondary hydroxyl of mono- and di-saccharides provide a more flexible control of the directionality and distances among attachment points of the peptide chains. This carbopeptide strategy starts with aminooxy-acetyl (Aoa) functionalization of the methyl α-D-galactopyranose (d-Gal*p*) derivative to obtain template molecules followed by *C*-terminal peptide aldehyde coupling to the template via oxime formation.

The simplified *N*-terminal di-Glu template Gly-Phe-Gly-Glu-Glu-Gly was assembled by solid-phase Fmoc methodology, isolated as Fmoc-Gly-Phe-Gly-Glu-Glu-Gly, and acylated to three peptide strands simultaneously on the solid phase using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) [89].

As discussed earlier, heterotrimeric triple-helical peptides have usually been prepared using (1) disulfide-mediated cross-linking or (2) electrostatic-assisted self-assembly. More recently, “2 + 1 strand click synthesis” has been utilized to synthesize discrete homo- and heterotrimeric triple-helical peptides whereby the *C*-termini of the peptides are chemically stapled using the Huisgen Cu(I)-catalyzed azide-alkyne cycloaddition reaction [116]. The “2 + 1 strand click synthesis” requires assembly of a two-stranded branched peptide possessing a *C*-terminal 6-azidelysine residue, which has the capacity to “click” to a single-stranded peptide bearing a (2S)-propargylglycinamide at the *C*-terminus.

Metal chelation also plays an important role in the construction of templated triple-helices [107–109]. As an example the addition of a dopamine residue on the *N*-terminus of (Gly-Nleu-Pro)$_6$ via a flexible succinic acid linker followed by incubation with Fe^{3+} converted a non-triple-helical peptide to a triple-helical peptide with a T_m value of 28 °C [107]. In similar fashion, addition of hydroxamic acid on the *C*-terminus of Boc-β-Ala-TRIS-[(Gly-Pro-Nleu)$_6$]$_3$, followed by incubation with Fe3+, resulted in a stabilized triple-helical structure (7 °C increase in T_m) [108]. Based on metal ion chelation, Chiemelewski et al. reported the self-assembly of THPs to form microflorettes [109]. To obtain collagen-based macromolecular assemblies with temporal control, the peptide design consisted of a central collagen-based core composed of nine repeating units of the tripeptide Pro-Hyp-Gly and distinct metal-binding ligands at each termini (**NCoH**, Fig. 12). The metal-binding ligands were a His$_2$ moiety on the *C*-terminus and a nitrilotriacetic acid unit on the *N*-terminus. The core triple-helical unit has been used in the formation of collagen-like peptide fibers in solution upon the introduction of Zn^{2+}, Cu^{2+}, Ni^{2+}, or Co^{2+}. This assembly process was found to be fully reversible using EDTA as a metal ion chelator. The assembly process proceeds under mild conditions using neutrally buffered aqueous solution at room temperature.

The effect of templates is often to enhance the thermal stability of collagen-like sequences. For example, the THP acetyl-Gly-Gly-(Pro-Hyp-Gly)$_5$-NH$_2$ has a T_m value of 9.2 °C, while template versions of the same sequence, using either (+)CTV or KTA, have T_m values of 58 and 62 °C, respectively [103]. Similarly, (Gly-Pro-Hyp)$_6$ has a T_m =25.4 °C, (Pro-Hyp-Gly)$_6$ with a *C*-terminal branch has a T_m =39.4 °C, (Gly-Pro-Hyp)$_6$ with an *N*-terminal branch has a T_m = 56.2 °C, and (Gly-Pro-Hyp)$_6$ with both an *N*- and *C*-terminal branch has a T_m =69.7 °C [88, 117]. An *N*-terminal macrocyclic template induced triple-helical structure of the sequence Gly-(Pro-Pro-Gly)$_7$-NH$_2$ (T_m = 39.9 °C), whereas the non-templated sequence was not triple-helical [104]. Triple-helices containing peptoid residues, such as *N*-isobutylglycine (Nleu), have been stabilized by templates. Acetyl-(Gly-Nleu-Pro)$_6$-NH$_2$ had a T_m =26 °C, while KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$, TREN-[suc-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$, and Boc-β-Ala-TRIS-[(Gly-Nleu-Pro)$_6$-OCH$_3$]$_3$ had T_m values of 36, 46, and 33 °C, respectively [100, 102, 118]. The thermal stability of the α2β1 integrin-binding sequence (Gly-Pro-Hyp)$_3$-Gly-Phe-Hyp-Gly-Glu-Arg-(Gly-Pro-Hyp)$_3$ was enhanced by an *N*-terminal Gly-Phe-Gly-Glu-Glu-Gly template, resulting in an increase in T_m from 25 to 44 °C [89]. Acetyl-(Pro-Hyp-Gly)$_5$-Pro-Cys(S*t*Bu)-Cys(S*t*Bu)-Gly-Gly-Gly-NH$_2$ had a T_m = 20.3 °C, while the double-disulfide-linked [acetyl-(Pro-Hyp-Gly)$_5$-Pro-Cys-Cys-Gly-Gly-Gly-NH$_2$]$_3$ had a T_m =68.1 °C [119]. Formation of the cysteine knot in (Gly-Pro-Pro)$_3$-Gly-Pro-Arg-Gly-Glu-Lys-Gly-Glu-Arg-Gly-Pro-Arg-(Gly-Pro-Pro)$_3$-Gly-Pro-Cys-Cys-Gly increased T_m from 35 to 43 °C [120].

2 Materials

1. Dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), *N*,*N*-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA, peptide synthesis grade), methyl-*tert*-butyl ether (MTBE), 1,8-diaza-bicyclo[5.40.]undec-7-ene (DBU), dimethyl sulfoxide (DMSO), acetonitrile (peptide synthesis or HPLC grade), sodium acetate, ammonium acetate, NH_4HCO_3, isopropanol (Optima/HPLC

grade), β-mercaptoethanol (molecular biology grade), thioanisole, ethanedithiol, phenol, Tris–HCl, NaCl, $CaCl_2$, and brij-35 are purchased from Fisher Scientific (Atlanta, GA).

2. Piperidine is purchased from AnaSpec (San Jose, CA).

3. Fmoc-amino acids, *N*-[(1H-benzotriazol-1-yl)(dimethyl-amino)methylene]-*N*-methylmethanaminium hexafluoro-phosphate *N*-oxide (HBTU), 1-H-benzotriazolium-1-[*bis* (dimethylamino)methylene]-5-chloro-hexafluorophos-phate-(1-), 3-oxide (HCTU), 1-hydroxybenzotriazole (HOBt), Fmoc-Gly-Sasrin resin, Fmoc-Rink Amide 4-methyl-benzhydrylamine (MBHA) resin, and NovaSyn TGR PEG resin (*see* Note 1) are purchased from EMD Biosciences (San Diego, CA).

4. 5,5'-Dithiobis(2-nitrobenzoic acid), α-cyano-4-hydroxycinnamic acid (MS grade), and 2-hydroxyethyl disulfide are purchased from Sigma-Aldrich (St. Louis, MO).

5. Alamar Blue is obtained from BioSource International (Camarillo, CA).

6. The monoalkyl chains hexanoic acid [CH_3–$(CH_2)_4$–CO_2H, designated C_6], octanoic acid [CH_3–$(CH_2)_6$–CO_2H, designated C_8], decanoic acid [CH_3–$(CH_2)_8$–CO_2H, designated C_{10}], dodecanoic acid [CH_3–$(CH_2)_{10}$–CO_2H, designated C_{12}], tetradecanoic acid [CH_3–$(CH_2)_{12}$–CO_2H, designated C_{14}], palmitic acid [CH_3–$(CH_2)_{14}$–CO_2H, designated C_{16}], and stearic acid [CH_3–$(CH_2)_{16}$–CO_2H, designated C_{18}] are purchased from Sigma-Aldrich.

3 Methods

3.1 Synthesis and Characterization of Peptide-Amphiphiles

1. Incorporation of individual amino acids is by Fmoc solid-phase methodology using cycles described previously [90, 91].

2. For Fmoc removal, a solution of DBU–piperidine–NMP (1:5:44) is used for 5–10 and 10–15 min.

3. All couplings are performed with HCTU/HOBt.

4. Acylation of the peptide–resin involves condensing a fourfold molar excess of alkyl acid to the N^{α}-deprotected resin, with a 3.8-fold molar excess each of HBTU and HOBt in DMF for 2 h. The reaction is initiated by the addition of an eightfold molar excess of DIEA and proceeds for 1 h.

5. Cleavage and side-chain deprotection of the lipidated peptide–resins is accomplished by treating the resin for 1–2 h with either H_2O–TFA (1:19) or ethanedithiol–thioanisole–phenol–H_2O–TFA (2.5:5:5:5:82.5) [121, 122].

[1]By utilizing the array of readily available orthogonally protected Lys derivatives, including, but not limited to, Fmoc-Lys(Dde), Dde-Lys(Fmoc), Fmoc-Lys(Fmoc), Fmoc-Lys(Mmt), Fmoc-Lys(Mtt), or Fmoc-Lys(ivDde), an array of topological designs can be created. The dilute acid utilized for side-chain deprotection of the Mmt and Mtt groups will cleave the template from the Gly-SASRIN resin. Thus, a more acid-stable resin such as Rink amide MBHA should be utilized in combination with Fmoc-Lys(Mmt) and Fmoc-Lys(Mtt).

6. Resins are filtered and rinsed with TFA, and the combined filtrate and wash reduced under vacuum at room temperature to ~0.5 mL and precipitated with MTBE.

7. The precipitate is centrifuged and washed three times with MTBE, dissolved in acetonitrile–H_2O (1:9–1:4), and purified by preparative reversed-phase HPLC.

8. Preparative reversed-phase HPLC is performed on a Rainin AutoPrep System with a Vydac 218TP152022 C_{18} or 214TP152022 C_4 column (15–20 μm particle size, 300 Å pore size, 250 mm × 22 mm) at a flow rate of 10 mL/min with 0.1 % TFA in H_2O (A) and 0.1 % TFA in acetonitrile (B), and detection at λ = 220 nm. For very hydrophobic samples, it is advantageous to use 0.1 % TFA in isopropanol instead of acetonitrile [73, 74]. Analytical HPLC is performed on a Hewlett-Packard 1100 Liquid Chromatograph equipped with an ODS Hypersil C-18 column (5 μm particle size, 100 mm × 2.1 mm).

9. PA composition can be determined by MALDI-TOF mass spectrometry (MS) [64, 73, 84, 85, 123–125]. PA homogeneity can also be evaluated by diphenyl and nonporous C18 reversed-phase HPLC [124] and/or hydrophobic interaction HPLC.

10. Triple-helicity is monitored by circular dichroism (CD) spectroscopy in the far UV wavelengths [64, 73, 74, 83–85, 90, 91, 125]. Peptides are dissolved in the appropriate buffer at concentrations of 2–500 μM. Conditions should reflect final relevant assay conditions as much as possible based on limitations imposed by sample availability and/or instrumentation. For example, if the peptides are to be used in a cell-binding assay at 10 μM in PBS, then the CD spectra should be acquired at that peptide concentration in a similar buffer. Since chloride ions interfere with the acquisition of data, a phosphate buffer can be substituted for PBS. A typical spectrum for a triple-helix shows a positive molar ellipticity at λ ~ 225 nm and a negative molar ellipticity at λ ~ 205 nm. In addition to the far UV wavelength scan of λ ~ 195–250 nm, one can determine the melting temperature by monitoring the change in molar ellipticity at λ = 225 nm with a constant change in temperature from 5 to 85 °C. For samples exhibiting sigmoidal melting curves, the inflection point in the transition region (first derivative) is defined as T_m. Alternatively, T_m is evaluated from the midpoint of the transition.

11. NMR spectroscopy can also be used to study the relative thermal stability, alignment, and flexibility (backbone mobilities) of triple-helical PAs [73, 74, 126].

3.2 Template-Assembled Synthetic THP Methods

3.2.1 Preparation of [N-tris(Fmoc-Ahx)-Lys-Lys]-Tyr-Gly Branched Template

1. Deprotect 1 g of Fmoc-Gly-Sasrin resin with 20 % piperidine in DMF for 30 min followed by a second 5-min treatment.

2. Wash the resin three times with DMF. If the substitution level is higher than 0.5 mmol/g, add 0.2 mmol benzoic anhydride dissolved in 25 ml DMF and 0.4 mmol DIEA, mix for 1 h, and wash the resin three times with DMF.

3. Dissolve 3 eq of Fmoc-Tyr(*t*Bu) in approximately 10 mL DMF. Add 2.7 eq of HBTU and 3 eq of HOBt, and then add the mixture to resin. Add 6 eq of DIEA, mix for 30–60 min, and wash the resin with DMF three times.
4. Remove Fmoc as indicated above and wash the resin three times with DMF.
5. Dissolve 3 eq of Fmoc-Lys(Dde) (*see* Note 1) in approximately 10 mL DMF. Add 2.7 eq of HBTU and 3 eq of HOBt and then add the mixture to the resin. Add 6 eq of DIEA, shake for 30–60 min, and wash with DMF three times.
6. Repeat the Fmoc removal and Fmoc-Lys(Dde) coupling steps such that the resin contains two adjacent Lys(Dde) residues (*see* Note 2).
7. If the desired construct is homotrimeric (*see* Note 3), the Dde and Fmoc groups can be simultaneously removed by treatment with 2 % hydrazine in DMF for 30 min followed by three rinses with DMF. An optional spacer of Fmoc-Ahx acid can be added to reduce the steric hindrance of the branch.
8. Keeping in mind that the chain is now trimeric, dissolve 3 eq of Fmoc-Ahx in approximately 10 mL DMF, add 2.7 eq of HBTU and 3 eq of HOBt, and add the mixture to the resin. Add 6 eq of DIEA, mix for 60 min, and wash three times with DMF.
9. A small volume of template is cleaved from the resin using H_2O–TFA (5:95) for 1 h and analyzed by MALDI-TOF mass spectrometry to confirm proper branch assembly. This scale of template is sufficient for three 0.25 mmol syntheses.

3.2.2 Peptide Synthesis, Side-Chain Deprotection, and Peptide–Resin Cleavage

1. Incorporation of individual amino acids is by Fmoc solid-phase methodology [90, 91].
2. For Fmoc removal, a solution of DBU–piperidine–NMP (1:5:44) is used for 5–10 and 10–15 min.
3. All couplings are performed with HCTU/HOBt.

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[2]One can create a variety of templates based on the simple Lys-branching method. The template can accommodate 2–8 branches with ease and can also create a number of homo- or hetero-multimeric sequences. However, as the number of branching points increases care must be taken during *N*-*α*-amino or *N*-ε-amino deprotection and subsequent couplings. The resin becomes increasingly “sticky” as the number of free amino groups increases, which can complicate subsequent deprotection and coupling reactions. Ensure that a proper solution volume or resin mixing action is taken to avoid deletions during peptide chain assembly.

[3]One can also utilize the branching strategy to construct heterotrimeric triple-helical peptides [112]. The synthesis of triple-helical peptides with one chain of different sequence from the other two requires a four-dimensional orthogonal scheme, where HMP is the linker and Dde or 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) [127, 128] side-chain protection of Fmoc-Lys is used. Synthesis by Fmoc strategy of one chain (i.e., the α2 collagen chain sequence) proceeds through the α-amino of Lys; thus, the Dde/ivDde and Fmoc groups are *not* removed simultaneously. The α2 chain sequence is assembled by Fmoc chemistry as described above. After incorporation of the α2 chain sequence, the peptide chain is “capped” by using allyloxycarbonyl (Aloc) chloride or Aloc-Gly, creating an *N*-terminal Aloc group. The Lys α-amino Dde or ivDde groups are then removed with hydrazine (see above), which does not remove Aloc, and synthesis by Fmoc strategy proceeds for the other two chains (i.e., α1 collagen chain sequence). Once all three chains are equivalent in terms of number of residues incorporated, the Aloc group is removed by treatment with $(Ph_3P)_4Pd$ in $CHCl_3$–acetic acid–*N*-methyl morpholine (20:1:0.5) [129] and the Fmoc groups removed as described above. Finally, Fmoc-Gly, -Pro, and -Hyp(*t*Bu) are incorporated into all three chains. Cleavage and side-chain deprotection are by TFA as described above.

4. Peptide–resins are cleaved and side-chain deprotected by treatment with H_2O–TFA (5:95), H_2O–thioanisole–TFA (5:5:90), orethanedithiol–H_2O–thioanisole–phenol–TFA(2.5:5:5:5:82.5) for ≥2 h depending upon the peptide sequence [121, 122].
5. Resins are filtered and rinsed with TFA, and the combined filtrate and wash reduced under vacuum at room temperature to ~0.5 mL and precipitated with MTBE.
6. The precipitate is centrifuged, washed three times with MTBE, dissolved in acetonitrile–H_2O (1:9–1:4), and purified by preparative reversed-phase HPLC.

3.2.3 Purification and Characterization of Peptide-Template

1. Preparative reversed-phase HPLC is performed with a Vydac 218TP152022 C18 column (15–20 μm particle size, 300 Å pore size, 250 mm × 22 mm) at a flow rate of 10 mL/min with 0.1 % TFA in H_2O (A) and 0.1 % TFA in acetonitrile (B), and detection at λ=220 nm.
2. The eluent is lyophilized to a powder and repurified using a semipreparative Vydac 219TP54 diphenyl column (5 μm particle size, 300 Å pore size, 250 mm × 4.6 mm) at a flow rate of 2 mL/min with detection at λ=220 nm.
3. Analytical HPLC is performed with an ODS Hypersil C-18 column (5 μm particle size, 100 mm × 2.1 mm).
4. Chain assembly is confirmed by Edman degradation and MALDI-TOF mass spectrometry using a specialized matrix mixture containing 2,5-dihydroxybenzoic acid/2-hydroxy-5-methoxybenzoic acid (9:1, v/v) [84, 117].

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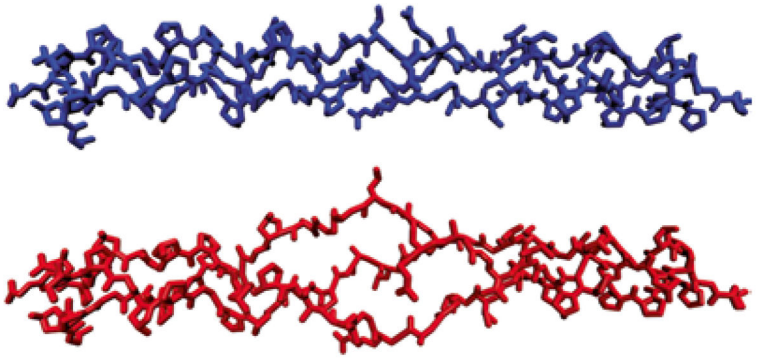

Fig 1.

"Ball and stick" computer-generated models of (*top*) a continuous collagen triple-helix (peptide T3-785, 3[(Pro-Hyp-Gly)$_3$-Ile-Thr-Gly-Ala-Arg-Gly-Leu-Ala-Gly-(Pro-Hyp-Gly)$_4$]) and (*bottom*) an unwound (heat denatured) version of the same sequence

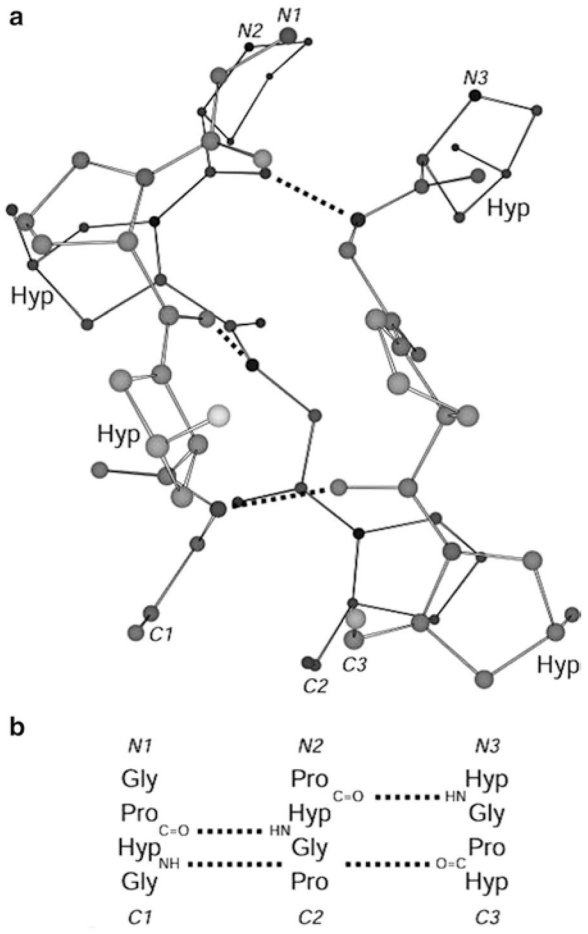

Fig 2.

Segment of a (Pro-Hyp-Gly)$_n$ triple-helix. (**a**) Ball-and-stick representation indicating 4-hydroxy-L-proline residues and XaaC=O–H–NGly hydrogen bonds. (**b**) Register of the residues in the three strands of panel (**a**). Atomic coordinates are from *Science* 1994, 266:75 (PDB entry 1CAG)

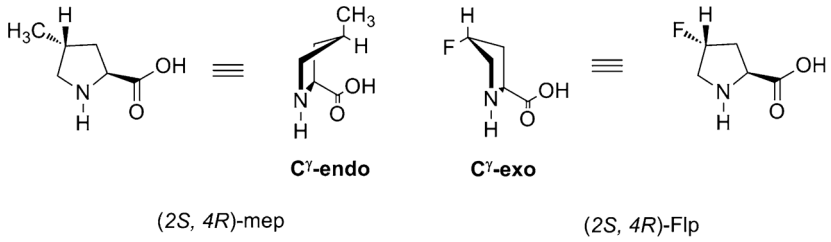

Fig 3.
Ring conformations of 4-substituted L -prolines

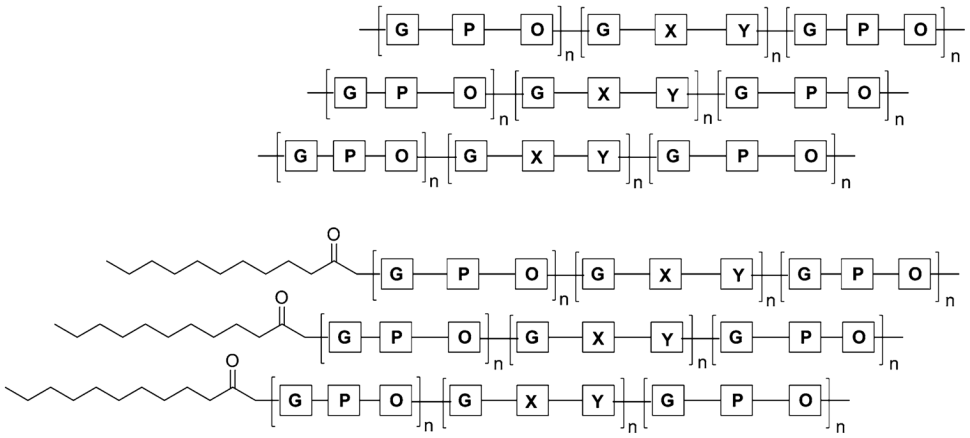

Fig 4.

Modular structures of (*top*) sandwiched associated triple-helical peptide and (*bottom*) sandwiched associated triple-helical peptide-amphiphile. The associated THP features repeats of Gly-Pro-Hyp [$(GPO)_n$] on both the *N*- and *C*-termini to induce or stabilize triple-helical structure, and a diverse collagen-like sequence [$(GXY)_n$] in the middle for structural and/or biological studies. The peptide-amphiphile additionally possesses a pseudo-lipid attached to the *N*-terminus to further enhance triple-helical stability via hydrophobic interactions

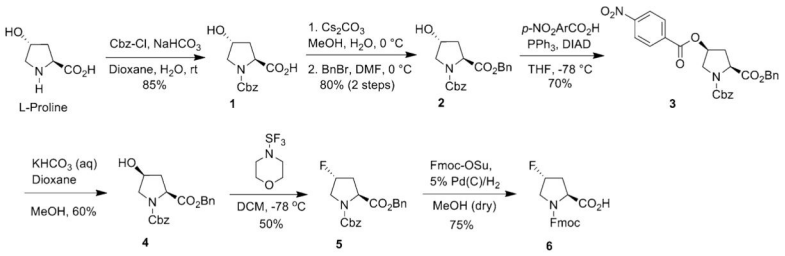

Fig 5.

Synthesis of Fmoc-(2*S*,4*R*)-4-fluoroproline (Flp)

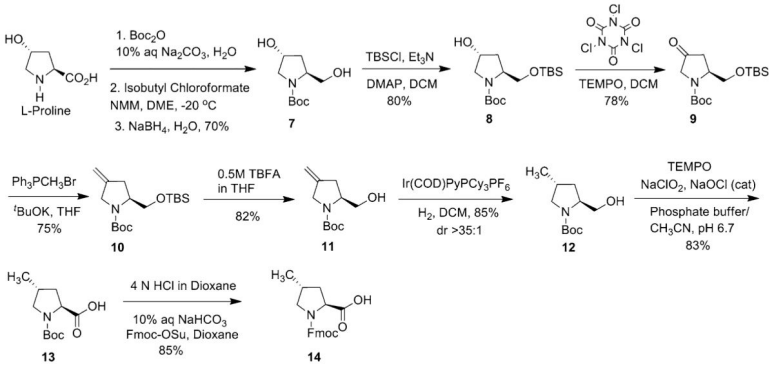

Fig 6.

Synthesis of Fmoc-(2*S*,4*R*)-4-methylproline (mep)

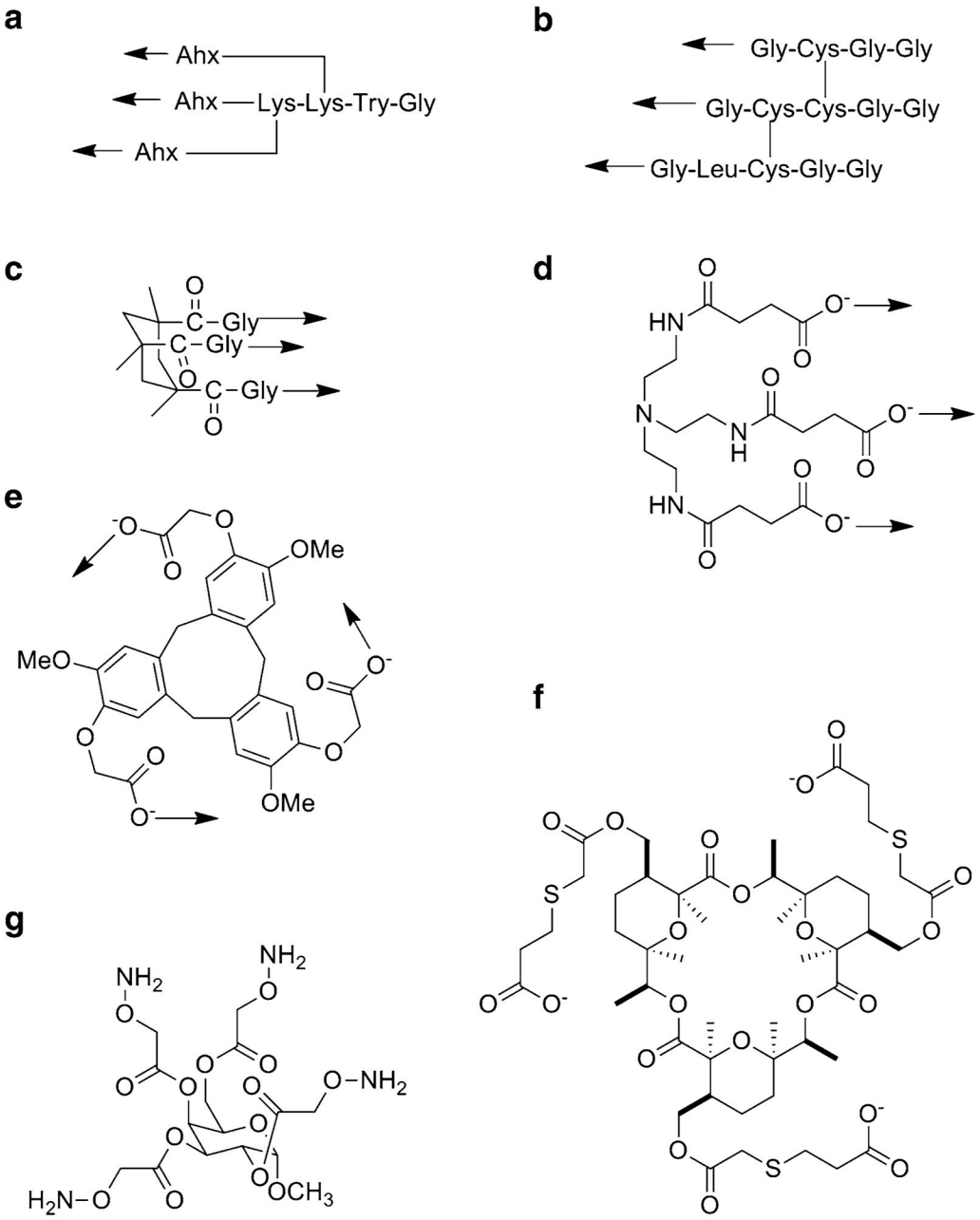

Fig 7.

Templates used for the chemical synthesis of triple-helical peptides: (**a**) di-Lys branch after coupling to 6-aminohexanoic acid (Ahx); (**b**) disulfide bridge (cystine knot); (**c**) *cis*,*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid (KTA) after coupling to Gly; (**d**) tris(2-aminoethyl)amine (TREN) after coupling to succinic acid; (**e**) cyclotriveratrylene (CTV) after coupling to a bromoethanoic acid; (**f**) macrocyclic; and (**g**) methyl 2,3,4,6-tetra-O-Aoa-α-D-Galp. The *arrows* indicate the direction of collagen-like sequence incorporation

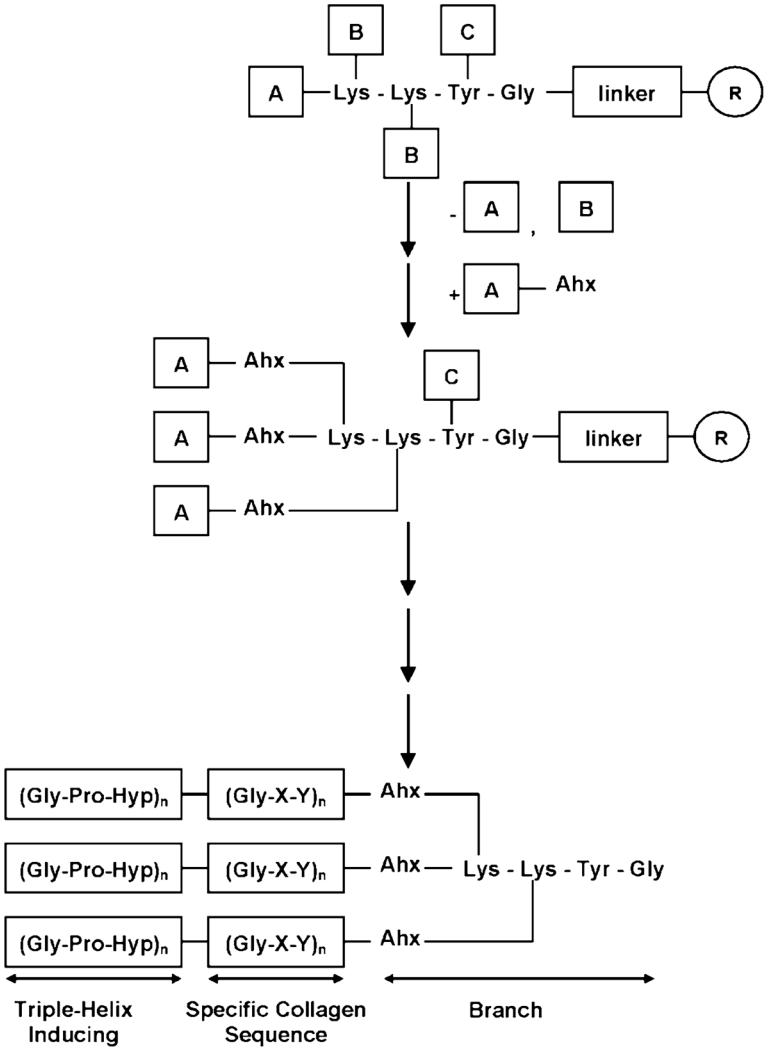

Fig 8.

General scheme for the synthesis of branched, triple-helical peptides. Ahx is 6-aminohexanoic acid. Reprinted with permission from *Biopolymers*, copyright 1993, Wiley and Sons

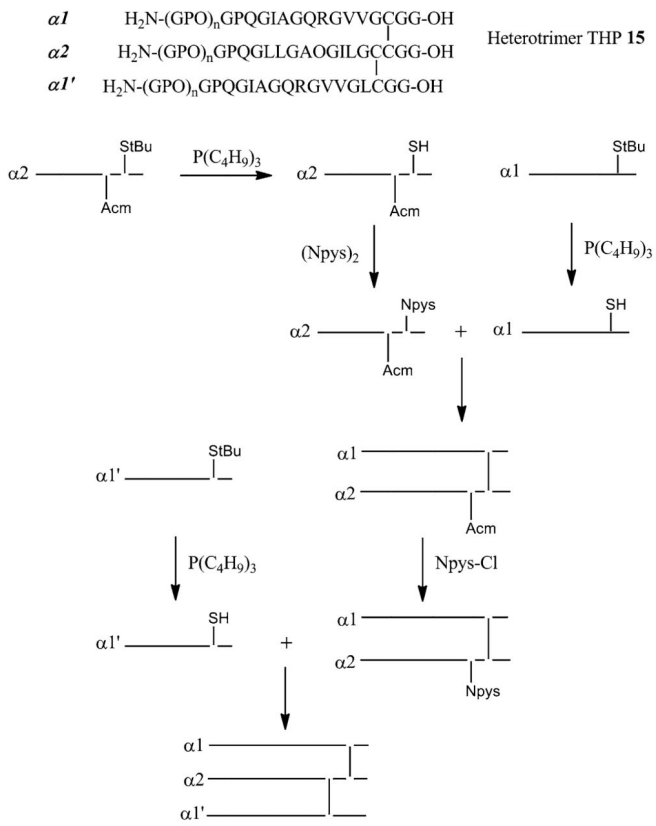

Fig 9.

Heterotrimer THP **15** and general scheme for regioselective assembly of the α1, α2, and α1′ cysteine peptides into heterotrimers with the α1α2α1′ register

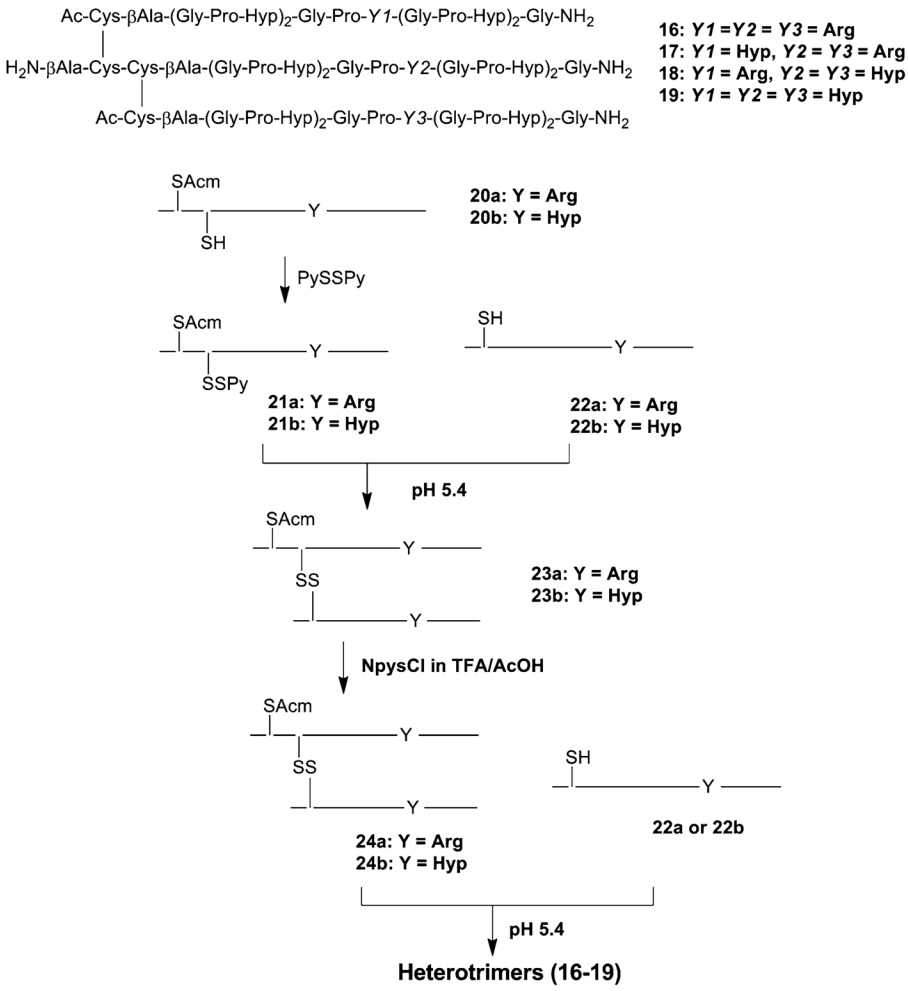

Fig 10.

Synthesis of heterotimeric THPs utilizing an *N*-terminal cystine knot

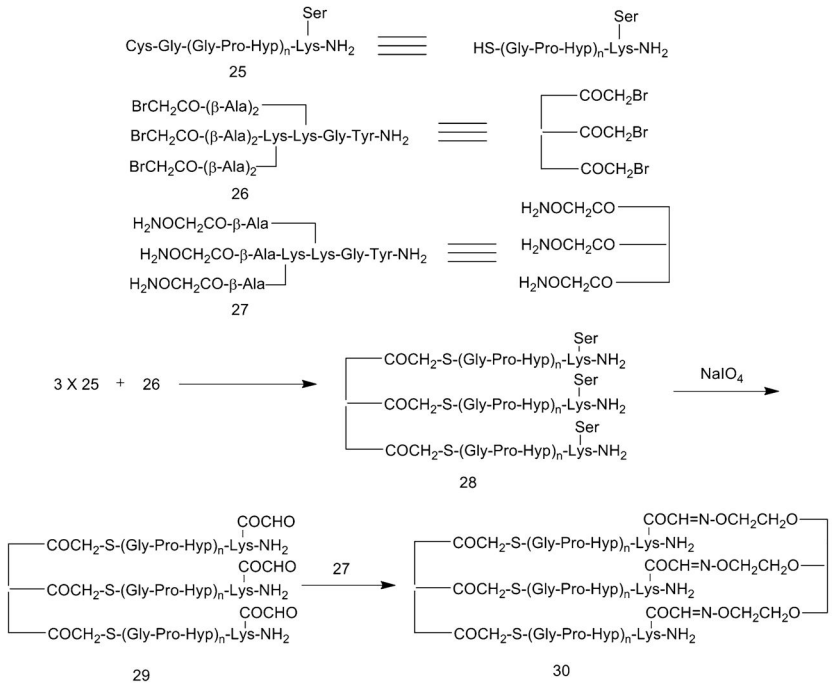

Fig 11.

Structures of the collagen-model peptide **25** and the linker peptides **26** and **27**, and the synthetic scheme for the assembly of cross-linked THP **30**

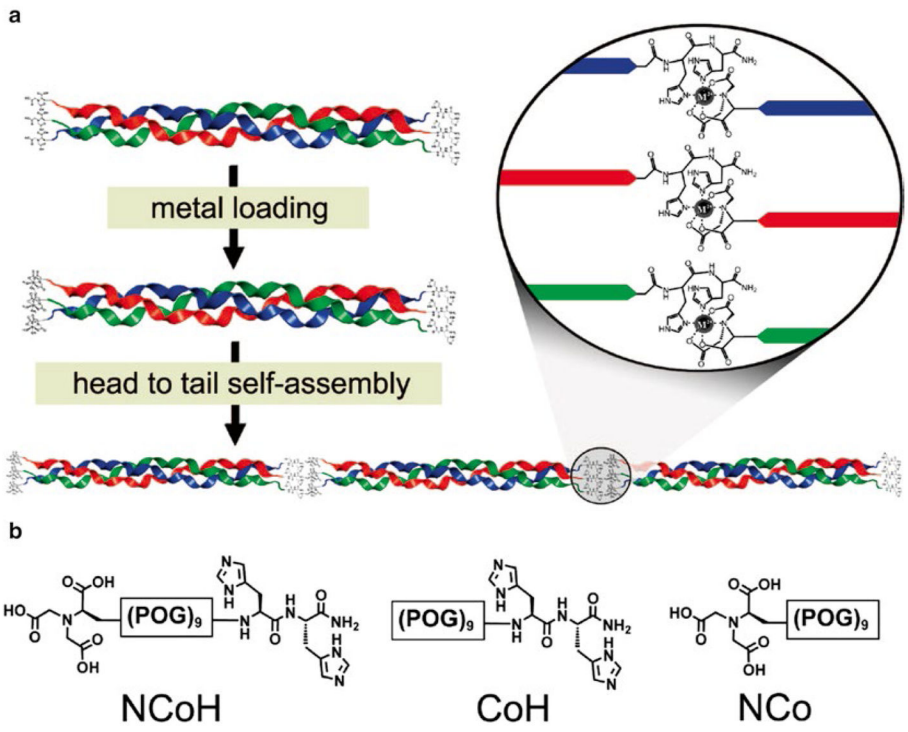

Fig 12.

(**a**) Schematic representation of the design of the **NCoH** THP and assembly into higher order structures. Following triple-helix formation, the addition of metal ions triggers an initial assembly directed by the NTA and His_2 moieties. (**b**) Structures of peptides **NCoH**, **CoH**, and **NCo**. Reprinted with permission from J Am Chem Soc, copyright 2009, American Chemical Society