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Bidirectional solid phase synthesis of a model oligoglycine bolaamphiphile and purification by rapid self-assembly

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We utilised a simple bidirectional (N→C and C→N) solid phase synthesis strategy entailing conventional solid phase peptide synthesis and fragment condensation with a water-soluble carbodiimide to synthesise a model anionic glycylglycine bolaamphiphile containing a suberic acid linker moiety, namely *N*,*N*′-suberoyldiglycylglycine. The synthetic suberoyldiglycylglycine was purified using its inherent ability to rapidly self-assemble in an aqueous acidic solution (0.1% trifluoroacetic acid). Monitoring of the rapid assembly process corroborated our visual observation and confirmed packing-directed self-assembly rather than non-specific aggregation or precipitation. The progress of suberoyldiglycylglycine self-assembly was observed to be via the formation of oligomers in the solution, which then self-assembled to form layered β-sheet type macrostructures. Within 24 h, nanotubes grew from these macrostructures and eventually combined to formed microtubes, which we isolated after 5–7 days. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: bolaamphiphile; solid phase synthesis; purification; rapid self-assembly

Introduction

Fuoss and Edelson [1] first introduced the term 'bolaform electrolyte' in 1951 for a hydrophobic chain connecting two ionic moieties. Essentially, a bolaamphiphile is a molecule consisting of two hydrophilic species connected by a hydrophobic tether. Since its discovery, many bio-inspired peptidolipidic bolaamphiphiles have been synthesised and characterised [2–9]. These bolaamphiphiles display two very unique properties, namely, if designed in a particular way, they tend to have biological activity as well as the propensity to self-assemble into a variety of nanostructure, microstructure and macrostructure. Bolaamphiphiles have also been the subject of much attention because of their application in functionalised nanomaterials, as nanotubes, as nanovesicles, as templates to cast nanowires, as nanocarriers of drugs and many other bionanotechnological applications. For selected reviews on bolaamphiphiles and their applications, refer to References [2–9].

Oligoglycine bolaamphiphiles, introduced by Grigoryan *et al*. [10] as possible antistaphylococcic agents, have gained much prominence in the field of self-assembly [11–20]. In 1996, Shimizu *et al*. [11] observed vesicle assembly in microtubular structures made up of synthetic oligoglycine-based bolaamphiphiles. These dicarboxylic glycylglycine bolaamphiphiles self-assemble in a variety of ways such as the formation of vesicles, fibres, tubes and spheroids [12]. The formation of these assembly patterns is pH dependent in the aqueous dispersions [13]. Matsui *et al*. [15,16], using glycylglycine bolaamphiphiles, indicated the possibility of organising peptide nanotubes into bundles through the use of metal coordination bridges, as well as creating nanowires.

Taking into account that many peptides have a natural tendency to self-assemble, we investigated the application of SPPS-derived bolaamphiphiles. To achieve this, we have adapted a solid phase synthetic route to synthesise a model bolaamphiphile, bis(N^{α}-amido-glycylglycine)-1,8-octane dicarboxylate or *N*,*N*′-suberoyldiglycylglycine (Figure 1), one of the bolaamphiphiles created by Grigoryan *et al*. [10] using solution phase synthesis. Solution phase methods depend heavily on the unpredictable solubility properties of the peptide intermediates, whereas solid phase synthesis offers the ease of intermediate purification from starting materials and by-products by simple filtration and washing steps of resin-bound intermediates. Furthermore, the ease of synthesising very complex peptides, selectively protected on their functional side chains, and even large peptide libraries on solid phase makes it a methodology of choice in peptide library synthesis.

Our synthetic strategy was based on the SPPS protocol developed by Atherton and Sheppard [21,22] where the peptide is anchored with an acid-labile ester bond and chain elongation proceeds from the C-terminus towards the N-terminus. The SPPS protocol employed a protection scheme in which a base-labile Fmoc group, requiring only mild removal conditions [23,24], is used to protect the α-amino group of the incoming amino acid. The solid phase synthesis of a bolaamphiphile with dicarboxylic acid linker requires an adaptation of conventional SPPS synthetic protocol to a bidirectional synthetic route

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***Abbreviations used**: E-SEM, environmental scanning electron microscopy; ESMS, electrospray ionisation mass spectrometry; FTIR, Fourier transform infrared spectroscopy.*

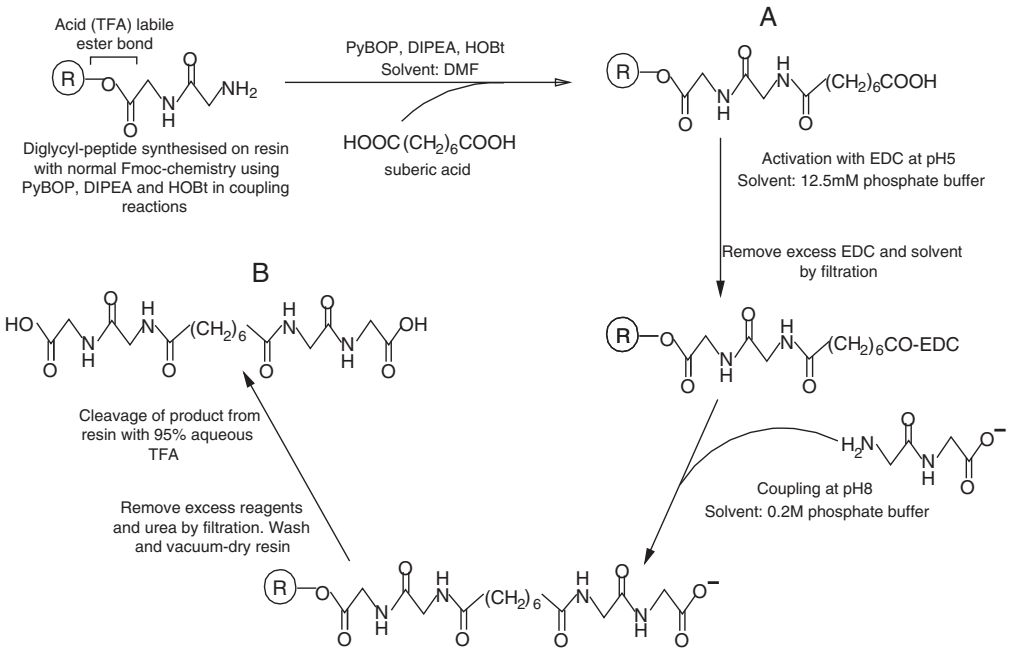

Figure 1. A scheme depicting the general solid phase synthesis strategy employed used for the synthesis from the resin-bound diglycyl moiety to resin-bound diglycyl suberic acid moiety (A) and finally to *N*,*N*′-suberoyldiglycylglycine (B).

(Figure 1). The first part of the synthesis is in the C→N direction leaving the second carboxyl group of dicarboxylic acid linker open for the following part of the synthesis in the N→C direction. The reversal of the synthesis direction to the N→C direction poses some problems, and early studies in peptide synthesis chemistry showed that most of the N→C synthesis strategies resulted in optically impure peptides [25]. Numerous synthetic strategies [26–32] have since been introduced to address this anomaly. We report here on the use of one of the strategies [32] in combination with SPPS to synthesise peptide bolaamphiphiles. The choice of method depended on the solubility of the peptide fragment and the identity of the N-terminal residue being attached in the N→C direction.

To synthesise in the N→C direction, the resin-bound carboxyl group must be activated and the carboxyl group of the incoming amino acid must be reversibly protected to prevent polymerisation. *In situ* activation of the carboxyl group of the resin-bound linker by, for example, methods using phosphonium/uronium/immonium or carbodiimide activation reagents yields high coupling efficiencies if the α-carboxyl group of the incoming amino acid is reversibly protected (reviewed in [33]). Phosphonium/uronium/immonium-based reagents react with the α-carboxyl groups in the presence of a base to form the highly active benzotriazolyl ester or analogous ester [33,34]. Combining the catalyst HOBt with carbodiimides would also lead to an extremely active benzotriazolyl ester of the carboxyl group [33,34]. HOBt and a number of its analogues are also the so-called configuration-trapping agents limiting racemisation to less than 0.4% for most amino acids [33,35].

The reversible protection of the α-carboxyl group of the incoming amino acid could be quite complex if introduced into the base-labile Fmoc-synthesis strategy [23,24]. Pre-activation of the carboxyl group by forming a more stable active ester, such as an OPfp [36] should eliminate the necessity of protecting the α-carboxyl group of the incoming amino acid. Alternatively, the use of the water-soluble and stable carbodiimide, EDC, offers the possibility of pH control of the activation and coupling reactions, thus also eliminating the necessity of protecting the incoming carboxyl group [32].

With the diglycine bolaamphiphile, *N*,*N*′-suberoyldiglycylglycine (Figure 1), serving as model, we report here a new solid phase synthesis strategy, ideal solvent conditions for rapid self-assembly and purification using its self-assembly character. To confirm that purification indeed occurred via self-assembly, we also assessed the rapid self-assembly with electrospray mass spectrometry (ESMS) and environmental scanning electron microscopy (E-SEM).

Materials and Methods

Materials

NovaSyn® KA (0.15 mEq/g), NovaSyn® TGT (0.2–0.26 mEq/g), HOBt and PyBOP® were from Novabiochem Co. (Luzern, Switzerland). Fmoc-Gly-OH was from Advanced ChemTech (Louisville, USA). DMF (99.5%), glacial acetic acid, diethyl ether (99.5%), DCM (99%), methanol (99%), glycylglycine (>98%), sodium dihydrogen phosphate dihydrate (99%) and disodium hydrogen phosphate dihydrate (99%) were from Merck (Darmstadt, Germany). 2-Methylbutan-2-ol (*t*-amyl alcohol; 98%) was from BDH Chemicals (Poole, UK). DIPEA, TFA (>98%), piperidine (98%), suberic acid (>98%), pyridine (98%), EDC and aluminium oxide (>98%) were from Sigma Chemical Co. (St. Louis, MI, USA). Acetonitrile (HPLC-grade, UV cut-off 190 nm) was from Romil LTD (Cambridge, UK). Analytical grade water was prepared by filtering glass distilled water through a Millipore Milli-Q® water purification system (Millipore Headquarters 290 Concord Road, Billerica, MA 01821 USA).

Bolaamphiphile Synthesis

In our synthesis, *in situ* activation of the carboxyl groups of Fmoc-Gly-OH and suberic acid linker was accomplished using PyBOP®,

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a relatively low-cost reagent, HOBt as catalyst and DIPEA as base [25].

Part A: synthesis of R-O-Gly$_2$-CO(CH$_2$)$_6$COOH (Figure 1A)

The synthesis was accomplished on a polydimethylacrylamide resin encapsulated in Kieselguhr (R). Normal SPPS, based on the Fmoc chemistry [23,24], was used with freshly distilled amine-free DMF as solvent and 20% high quality distilled piperidine in DMF as base for removal of the Fmoc group after each coupling step. The first Gly residue was coupled to the resin with an acid-labile ester bond. Activation of the incoming carboxyl group was accomplished by using PyBOP®, as activation agent, with freshly distilled DIPEA as base and HOBt as catalyst. The PyBOP® reagent was used as follows: a fivefold molar equivalent (in terms of resin capacity) of both Fmoc-Gly-OH (or suberic acid) and HOBt were dissolved in a minimum amount of DMF, fivefold molar equivalent PyBOP® was dissolved separately in a minimum of DMF and mixed with a tenfold molar equivalent DIPEA and both were mixed thoroughly with the resin. In our shake flask method, the total volume of DMF was limited to $<$1.5 ml/g of resin. The reaction time of the coupling steps is approximately 60 min for coupling of the Gly residues and overnight for the dicarboxylic acid linker. The coupling time depended on the completeness of acylation as determined with the Kaiser [37] and Fmoc tests [24] after the coupling/deblocking of the Gly residues and a picric acid test [38] after the coupling of the dicarboxylic acid linker. After the coupling of the linker, the resin was washed with DMF and diethyl ether then thoroughly dried under vacuum.

Part B: synthesis of N,N′-suberoyldiglycylglycine (Figure 1B)

The unprotected glycylglycine fragment was attached to R-O-Gly$_2$-CO(CH$_2$)$_6$COOH (Figure 1A) using a two-step carbodiimide method [33]. A ten times molar equivalent of EDC was dissolved in 250 µl analytical grade water and added to the resin swollen in 250 µl of 25 mM phosphate buffer (pH 5.0). After 15 min of activation, the excess EDC was removed from the resin and the suspension of activated resin was added dropwise to 1.5 ml glycylglycine in 0.2 M phosphate buffer (pH 8.0). The resin mixture was gently shaken for 24–36 h, where after it was washed on a sintered glass filter with water, *t*-amyl alcohol, acetic acid, again *t*-amyl alcohol and finally with peroxide-free diethyl ether and then thoroughly dried under vacuum. To monitor the coupling reaction, a sample of the resin (~5–10 mg) was taken after 24 and 36 h, washed and treated with TFA (as described later). The TFA-product mixture was then removed and evaporated under nitrogen. The residual product was made up in 50% acetonitrile and analysed using ESMS.

Removal of peptides from the solid phase resin

The peptides were cleaved from the resin with 95% TFA and 5% H_2O as scavenger. The peptide resin was treated for 3–6 h with four-bed volumes 5% H_2O in TFA. After cleavage, the resin was removed by filtration and washed with 5% H_2O in TFA, acetic acid and analytical grade water. The combined filtrate containing the bolaamphiphile was dried under high vacuum on a BÜCHI Rotavapor (BÜCHI Labortechnik AG, Switzerland) at 40–50 °C, resuspended in 50% acetonitrile and lyophilised.

Purification of the Synthesis Product

The synthesis product was purified using the unique self-assembly properties of the bolaamphiphile product. A number of ±10 mM solutions were prepared in 0.1% TFA and left unperturbed for 1–7 days. The self-assembled solution was centrifuged using a bench top PicoFuge® Microcentrifuge (Stratagene, La Jolla, CA, USA) for 5 min and the supernatant carefully removed. The resulting pellet was washed with cold analytical quality water using the centrifugation procedure for further analysis or dissolved in 50% acetonitrile and lyophilised.

Analysis of Synthesis and Self-assembled Products

Electrospray ionisation MS was performed using a Micromass triple quadrupole mass spectrometer fitted with an electrospray ionisation source (Micromass UK Ltd., Manchester, UK). The carrier solvent was 50% (v/v) acetonitrile in analytical grade water delivered at a flow rate of 20 µl/min during each analysis using a Pharmacia LKB 2249 pump (Pharmacia, Freiburg, Germany). Ten microlitre of the sample solution (0.2 mg in 1.0 ml of a 50% CH_3CN : 0.05% aqueous TEA solution or 50% CH_3CN) was introduced into the ionisation source using a Rheodyne injector (Rheodyne/IDEX, Rohnert Park, CA, USA). A capillary voltage of 3.5 kV was applied throughout, and the source temperature was set at 80 °C. The skimmer lens offset was 5 V, and the cone voltage was varied between 50 and 60 V. Data acquisition was in the negative or positive mode, scanning the first analyser (MS_1) through $m/z = 100$ to 1500 at a scan rate of 675 amu/s. Averaging spectra across the elution peak and subtracting the background produced representative spectra. The instrument was calibrated using the ion spectrum of polyethylene glycol acquired under similar conditions.

To obtain the fragment patterns, the $[M+H]^-$ molecular species was selected in MS_1 and subjected to collisionally induced decomposition. These experiments were conducted using the same ionisation parameters as before. Argon was introduced into the collision cell at $(1.8 \pm 0.2) \times 10^{-3}$ mb, and the collision energy setting was 30 eV. Data acquisition was in the negative mode, scanning the second analyser (MS_2) through $m/z = 10$ to 450 at a scan rate of 220 amu/s.

NMR of crude preparation and purified bolaamphiphile in DMSO-d_6 were recorded on a Varian VXR 300-MHz instrument (Agilent Technologies, Inc. Santa Clara, CA, USA). Assignments were made empirically.

Environmental Scanning Electron Microscopy

For imaging with E-SEM, a 5 µl sample of the self-assembled stock solution of the bolaamphiphile was placed on a support film coated with silver and mounted on a specimen stub and then air dried and viewed in E-SEM mode using a Peltier cooling stage. The stage was cooled to 5 °C at a pressure of 5–6.5 Torr, at a relative humidity of between 75 and 100%. By adjusting the water vapour pressure in the chamber and the temperature of the cooling stage, it was possible to increase or decrease the water level in and on the surface of the sample. Images were obtained using the gaseous secondary electron detector. All samples were viewed using a Philips XL 30 FEG-SEM (FEI, Hillsboro, Oregon, USA) at 15 kV accelerating voltage. The peptide fibres isolated from 7-day or older assembled samples were washed thoroughly with deionised water, and excess water was blotted off with filter paper. A 4-nm layer of Au/Pd alloy was deposited onto the samples using an E5100 Polaron sputter coater (Polaron Equipment Ltd. Hertfordshire, UK). The samples were viewed in the high vacuum mode at 10–15 kV accelerating voltage.

Fourier Transform Infrared Spectroscopy

Self-assembled samples were carefully isolated and washed with analytical quality water by centrifugation to remove traces of TFA as confirmed with IR (absence of TFA bands that interferes with amide I signals). A lyophilised self-assembly sample was analysed by means of photoacoustic Fourier transform IR (FTIR) spectroscopy. The photoacoustic detector used was an MTEC model 300 unit (MTec Photoacoustics, Inc, Ames, Iowa, USA) that was coupled to a Perkin Elmer Paragon 1000 (Perkin Elmer, Waltham, MA, USA). The following parameters were used for the determination of each spectrum: mirror velocity, 0.1 cm/s; resolution, 8 cm^{-1}; source aperture, maximum; number of scans, 128; sample reference, carbon black; and detector gas atmosphere, helium. A typical scan required 15 min. The IR spectrum was scanned from wavenumber 4000 to 450 cm^{-1} and was mathematically adjusted to compensate for the photoacoustic effect.

Results and Discussion

A number of groups [10–16] have synthesised either the model bolaamphiphile, *N*,*N*′-suberoyldiglycylglycine, or oligoglycine analogues using solution phase synthesis. However, this synthetic strategy has many limitations especially in terms of versatility of peptide design specifically related to asymmetric bolaamphiphiles. The synthesis of the model bolaamphiphile required an adaptation of conventional SPPS synthetic protocol to a bidirectional synthetic route (Figure 1). In our synthesis of the symmetric *N*,*N*′-suberoyldiglycylglycine (Figure 1), the coupling of the first amino acid residue, Gly, with an acid-labile ester bond to the resin was >99% according to an analytical Fmoc test [24]. The elongation of each of the peptide chains in the N→C direction was successful as monitored by the Kaiser test [37]. The overnight coupling of the dicarboxylic acid linkers led to >99% coupling efficiency in each case as determined by an analytical picric acid test [38].

With the activation of the free carboxyl group of the linker on the resin, it was possible to synthesise the second peptide unit in the normal C→N direction and couple it in our solid phase method as protected or unprotected peptide fragment in the N→C direction. Although we were able to pre-activate the free carboxyl group of the resin-bound linker by forming its Pfp ester, the unpredictable solubility of some of the incoming peptides, such as glycylglycine in suitable organic solvents, made this a less attractive option. Using PyBOP® as activation agent in this step would necessitate the incoming peptide fragment to be protected in order to limit polymerisation. Unprotected glycylglycine dipeptide was readily available, but it was highly soluble in water and poorly soluble in most organic solvents. By using Gly as N-terminal residue of the peptide unit coupled in the N→C direction, we avoided the problem of racemisation and increased our choice of activation agents. Having investigated the phosphonium, carbodiimide and active ester activation procedures for the N→C direction synthesis step, we decided on a modified two-step carbodiimide method, using the water-soluble carbodiimide, EDC (Figure 1). The choice of method hinged solely on the solubility of the peptide fragment being attached in the N→C direction, as well as the presence/absence of a chiral N-terminal residue. The EDC method, commonly used in peptide fragment condensation reactions [32], utilises the water-soluble properties of the carbodiimide with activation and coupling proceeding at different pH values (pH 5 and 8) in order to limit unwanted side reactions and polymerisation. The first step at pH 5, together with low phosphate buffer concentration, favours the activation of the free carboxyl group of the linker and minimises phosphate competition for EDC. Adjustment to pH 8 by addition of the excess phosphate at the time of the addition of glycylglycine peptide fragment prevents the activation of the incoming carboxyl groups on the incoming peptide by the excess carbodiimide. This allows the preferential formation of the peptide bond between the activated carboxyl groups of the acid and the amino group of the incoming peptide fragment. To limit the activation of the carboxyl group of the incoming peptide, the excess EDC was removed by repeated washing of the resin after the initial activation step. The high concentration of 0.2 M phosphate in the second step favours the reaction of EDC with phosphate, resulting in the formation of a urea derivative. Phosphate thus quenches any residual EDC further minimising activation of the carboxyl group of the incoming peptide.

The crude and purified peptide bolaamphiphile was characterised by ESMS (Figures 2 and 3). ESMS of the crude product showed limited contamination by HO-Gly_2-$CO(CH_2)_6COOH$ at $m/z = 286$, indicating incomplete coupling of the second Gly-Gly unit (Figure 2A). ^{1}H-NMR of the crude synthetic compound also indicated that there was incomplete coupling in the last step of the original synthetic sequences. Signals observed at 2.0–2.2 p.p.m. in the α-CH_2 of the diacid linker were diagnostic for indicating the incomplete coupling of the peptide fragment.

Purification of *N*,*N*′-suberoyldiglycylglycine

For the purification of *N*,*N*′-suberoyldiglycylglycine from the crude synthetic product, we exploited its inherent ability to self-assemble. A number of solvents were assessed for self-assembly, but diluted aqueous TFA proved to be exceptional in the promotion of rapid self-assembly. A solution of the crude preparation of approximately 10 mM, based on the critical micellular concentration *N*,*N*′-suberoyldiglycylglycine [12], was prepared in 0.1% TFA and was left undisturbed at room temperature for up to 7 days. Rapid visual self-assembly took place, and we were able to isolate self-assembled purified product via centrifugation within a day, but the best overall yields were obtained after 5 days.

NMR of the self-assembled fraction of the *N*,*N*′-suberoyldiglycylglycine synthesis product confirmed that the self-assembled fraction contained both glycylglycine moieties in the major compound. ESMS analysis of the self-assembled product and the supernatant from the purification verified the efficiency of this extremely simple purification protocol. Refer to Figure 2A and B and the following discussion for more detail on the ESMS analyses. We confirmed that the major contaminant, diglycylsuberic acid or HO-Gly_2-$CO(CH_2)_6COOH$ at $m/z = 286$, remained in the solution and could thus easily be removed (Figure 2A). The purified mass yield using the rapid self-assembly was 88%, indicating a high yield synthesis and successful purification protocol. In our group, we were also able to successfully synthesise Leu-containing and Lys-containing asymmetric analogues of the model bolaamphiphile, all with >70% yields, using our novel bidirectional solid phase methodology to couple the diglycyl unit (results not shown). However, only the anionic analogues and bolaamphiphiles with aliphatic residues responded well to self-assembly dependent purification in 0.1% TFA.

Characterisation of Purified *N*,*N*′-suberoyldiglycylglycine

The high purity and correct molecular mass of *N*,*N*′-suberoyldiglycylglycine was confirmed with ESMS as we observed $[M-H]^-$ with $m/z = 401$ ($C_{16}H_{26}N_4O_8$ requires 401.3967) and a TFA adduct

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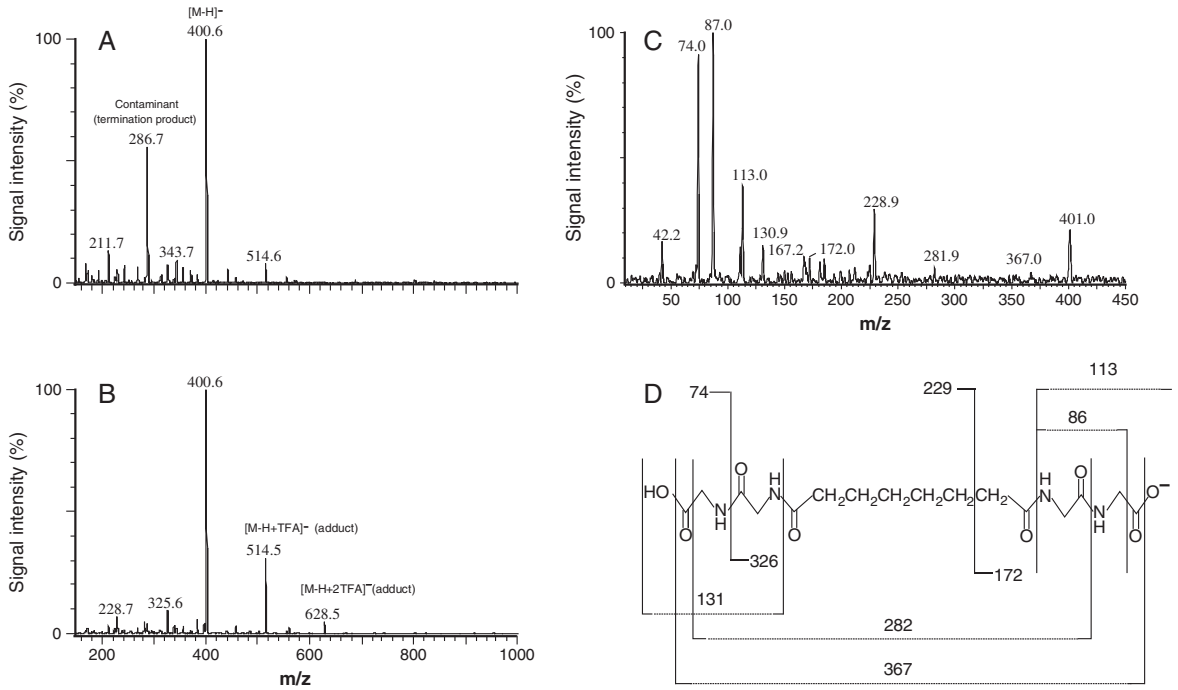

Figure 2. Negative mode ESMS spectra of the products from the self-assembly purification step: supernatant (A) and self-assembled product in pellet (B). The spectrum in C depicts the CID fragmentation pattern of $[M-H]^-$ after selection in MS_1. Figure 2D shows the proposed fragment identification.

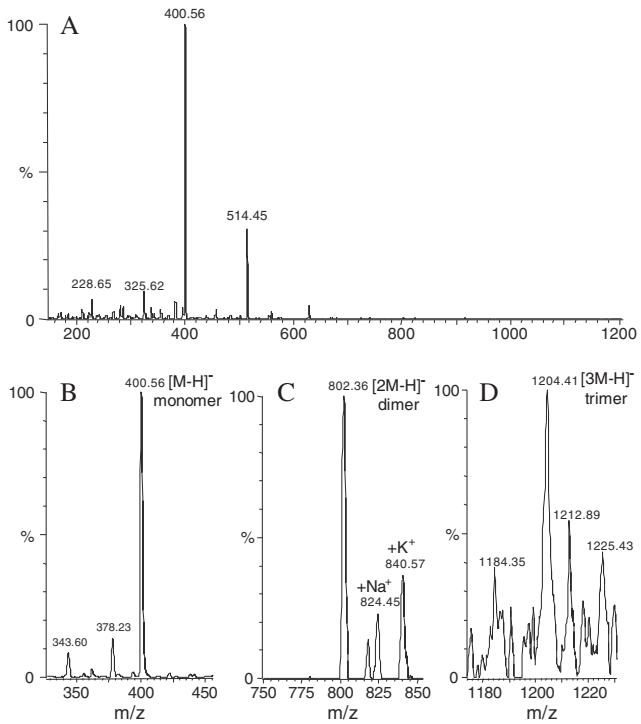

Figure 3. Negative mode ESMS spectra of *N,N′*-suberoyldiglycylglycineat at initiation of self-assembly (A) and after 7 h, showing the monomers remaining in solution (B) and detection of ESMS stable dimers (C) and trimers (D) in solution.

($m/z = 514.5$) (compare Figure 2B with 2A). Collisionally induced fragmentation of the $m/z = 401$ molecular ion in the self-assembled fraction gave the expected fragmentation pattern, namely fission at the amide bonds and other product ions from the loss of a carboxyl group or an OH group (Figure 2C and D). The product ion with $m/z = 228.7$ was attributed to the fission of the C–C bond neighbouring the amide carbonyl of the linker.

The NMR analysis of the purified *N,N′*-suberoyldiglycylglycine also indicated a very high purity and was consistent with the structure of this compound and gave the following results: δ_H(300 MHz; DMSO-d_6; TMS) 1.21–1.29 (4H, m, $2 \times \gamma$-CH_2), 1.43–1.55 (4H, m, $2 \times \beta$-CH_2), 2.13 (4H, t, $J = 7.4$ Hz, $2 \times \alpha$-CH_2), 3.45 (4H, d, $J = 5.5$ Hz, $5_G \times 2$), 3.51 (4H, d, $J = 5.5$ Hz, $2_G \times 2$); 8.13 (4H, t × 2, $J = 5.4$ Hz, $2 \times 3_G$ and $2 \times 6_G$); δ_C (75 MHz; DMSO-d_6; TMS). 24.97 (suberic $\beta \times 2$) β 28.40 (suberic $\beta \times 2$); 35.17 suberic $\gamma \times 2$); 41.84 ($CH_2COO \times 2$); 40.93 ($CONHCH_2CO \times 2$); 169.81 ($CH_2CH_2CONH \times 2$); 171.68 ($CH_2COO \times 2$); 173.09 ($CONHCH_2CO \times 2$).

Probing the Rapid Self-assembly of Purified *N,N′*-suberoyldiglycylglycine

During the self-assembly dependent purification of the anionic bolaamphiphile, we found that self-assembly was rapidly initiated in the 0.1% TFA solution. To eliminate the probability of non-specific precipitation or aggregation in the presence of the TFA, we decided to probe and revisit the self-assembly process and macro-assemblies of this model bolaamphiphile.

ESMS was conducted over a period of 7 h on the purified bolaamphiphile dissolved in 0.1% TFA. The results revealed the formation of ESMS stable dimers and trimers in the solution, indicating higher-order oligomeric structures and the onset of self-assembly (Figure 3). We confirmed the rapid self-assembly after 7 h by using E-SEM (Figure 4A). According to earlier reports [11,12,14], the time required for self-assembly was 7 to 14 days, however, we observed macro-assemblies and nanostructures within a day. A distinct feature of bolaamphiphiles is that the initiation of self-assembly is usually marked by the formation of monolayers [39]. Layering was evident from the electron

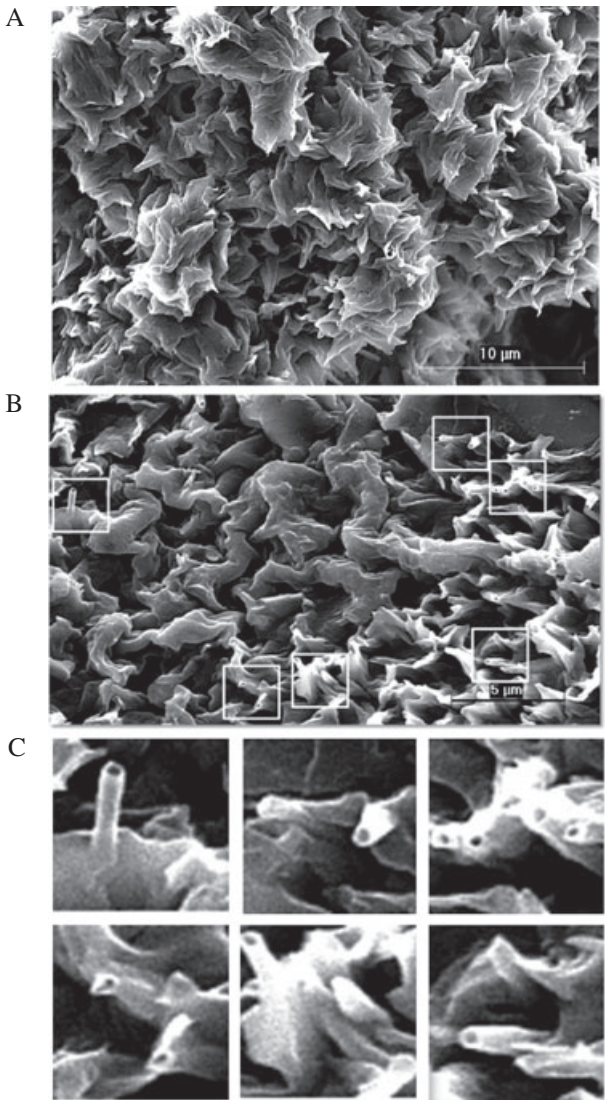

Figure 4. Environmental scanning electron microscopy images of the self-assembly process of *N,N′*-suberoyldiglycylglycine over 24 h with (A) self-assembly into monolayer and multilayers after 7 h; (B) self-assembly into nanotubes after 24 h; (C) magnified images of areas in white squares (2.5 × 2.5 µm) in B showing the nanotubes.

micrographs obtained after 7 h (Figure 4A). Apart from layering, the self-assembling bolaamphiphile folded leading to protrusions into the solvent environment (Figure 4A). This rapid self-assembly is attributed to the promotion of strong intralayer hydrogen bonding through the use of 0.1% TFA. The solvent also promotes more effective hydrophobic interaction of the hexamethylene chain by 'drying' out the flanking peptide backbone, i.e. it outcompetes the water molecules attached to the peptide backbone [40]. Kogiso *et al.* [14] showed with X-ray diffraction that the amides and two carboxylic acid groups of an analogous diglycyl bolaamphiphile with a dodecadiacid linker formed a β-sheet layered assembly via eight amide–amide hydrogen bonds and two acid–acid hydrogen bonds.

After 24 h, E-SEM imaging revealed the growth of multiple nanotubes, from the surface of the layered macrostructure. These nanotubes, with outer and outer and inner diameters of 235 ± 9 and 135 ± 6 nm, respectively, probably assembled from the protrusions formed by the folded monolayers. They typically also

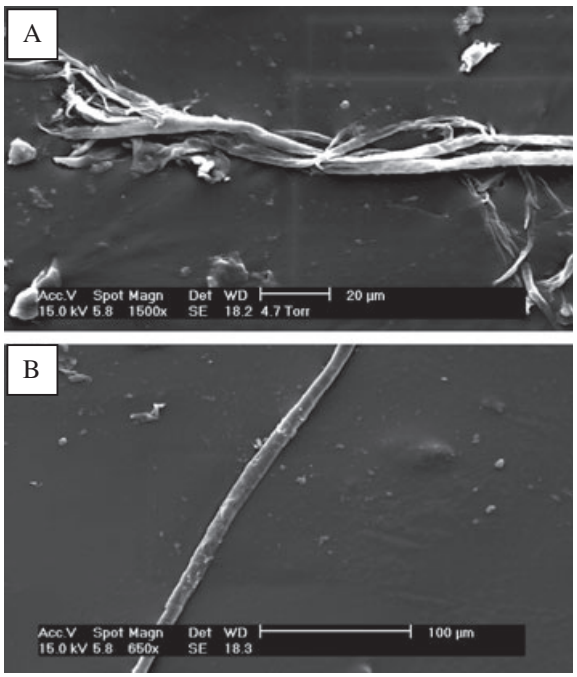

Figure 5. Scanning electron microscopy images of the self-assembly structures of *N,N′*-suberoyldiglycylglycine after 5–7 days with (A) self-assembly structure with multiple elongated microtubes and (B) larger microtubular structure. Samples were sputter-coated Au/Pd alloy.

formed protrusions into the solvent away from the layered structure (Figure 4B and C). The formation of the curved tubular structures from monolayers could be due to strain and tilts induced by the hydrophobic interactions and the hydrogen-bonded network. The tight hydrophobic interaction of the hexamethylene chain promoted by the 'dehydration' effect of the TFA [40] of the flanking peptide moieties and low pH of the solution probably resulted in the shortening of the intralayer hydrogen bonds, and this, in turn, induced strain and a tilt in the molecular arrangement making the β-sheet network more convex [16]. This tilt could lead the β-sheet structures to fold and form nanotubes [16].

After 7 days in the acidic bolaamphiphile solution, we observed, with the use of E-SEM, assemblies of elongated microtubes with approximately 3.7 µm outer diameter (Figure 5A).

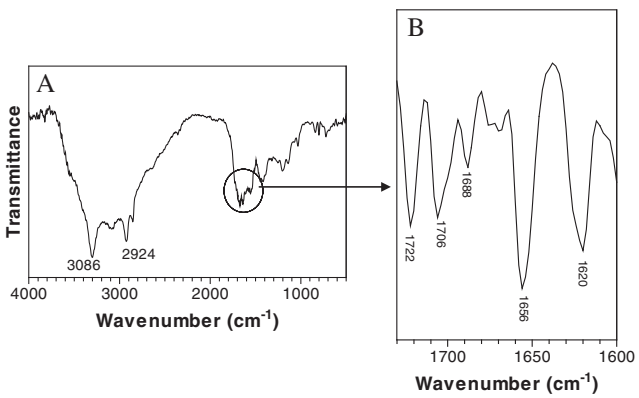

Figure 6. FTIR of the *N,N′*-suberoyldiglycylglycine microtubular structures and fibres isolated after 5 days (A) and second derivative IR spectra of the bolaamphiphile in the amide I region (B).

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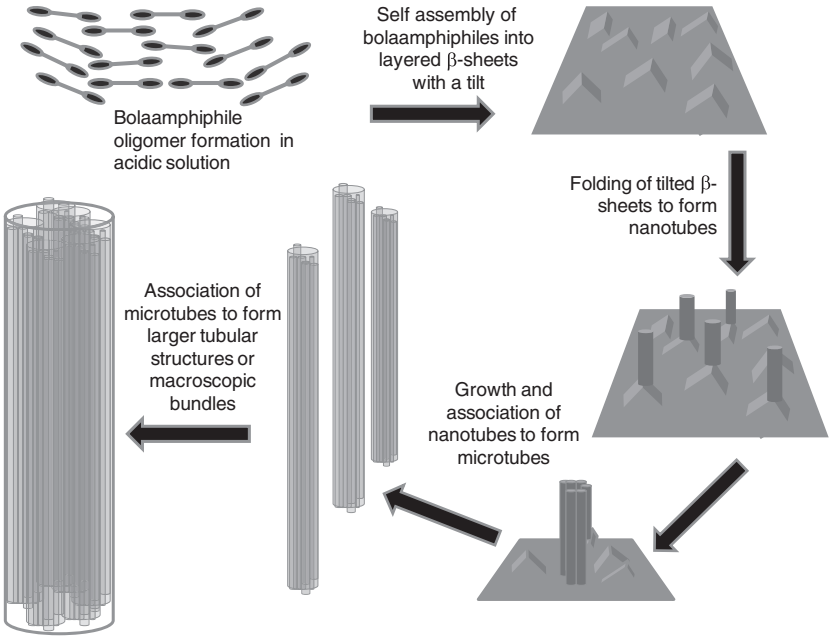

Figure 7. A schematic representation of the major steps in self-assembly of *N,N'*-suberoyldiglycylglycine.

These microtubes could assemble to form the larger microtubes ($10.5 \pm 1\ \mu m$ outer diameter) we also observed in the self-assembly fraction (Figure 5B). More interlayer and intralayer hydrogen bonding would result in the extension of the β-sheet network, and this translates into longer and wider tubes [41]. Alternatively, the assembly of multiple nanotubes via intertubular hydrogen bonds into macroscopic bundles would also lead to microtubular structures with wider diameters [16].

FTIR spectroscopy of the microtubes showed a strong CH_2 antisymmetric and weaker symmetric stretching band at 2925 and 2852 cm^{-1}, respectively (Figure 6A). This may suggest a gauche-including conformation rather than an all *trans* zigzag conformation [42–44] of the hexamethylene spacer and 'bended' spacer in the tubular structures. However, highly similar CH_2 stretching bands in this model bolaamphiphile were interpreted by Kogiso *et al.* [12] as an indication of high *trans* conformational populations. Strong amide I bands at 1656 and 1620 cm^{-1} combined with the weaker band at 1688 cm^{-1} indicate a hydrogen-bonded network, possibly a mixture of parallel and antiparallel β-sheet type network [41,45–48] (Figure 6B). Furthermore, N–H stretching bands at 3301 cm^{-1} (amide stretch A) and 3083 cm^{-1} (amide stretch B) together with the amide II bands at 1554 and 1576 cm^{-1} indicate strong intermolecular amide–amide bonds (Figure 6A) [11–13,42,46–49]. The detected amide stretch A band was comparable with that for polyglycine I and the amide stretch B band to that of the acid crystal of this model bolaamphiphile as observed by Kogiso *et al.* [12]. The significant red-shifted carboxyl acid bands at 1706 and 1722 cm^{-1} (Figure 6B) also corroborated observations that the terminal glycine α-COOH groups participate in bifurcated and lateral acid–acid hydrogen bonds in the intermolecular network that leads to the formation of tubes [11–13,41,49]. These bands were also highly comparable with those found by Kogiso *et al.* [12] for the acid crystal of this model bolaamphiphile. Our FTIR results substantiated previous findings that self-assembly takes place via the formation of β-sheet type network [11,13,41] and that layer formation is a result of intralayer hydrogen bonding via the terminal α-carboxyl groups [41].

In general, the observed tubular structures of *N,N'*-suberoyldiglycylglycine (bis(N^{α}-amido-glycylglycine)-1,8-octane dicarboxylate) that formed in the acidic environment correlated with crystalline tubules that the group of Matsui [13,16] found for the self-assembly of a closely related bolaamphiphile, bis(N^{α}-amido-glycylglycine)-1,7-heptane dicarboxylate, at an acidic pH. However, the organisation into macroscopic bundles [16] took place in the absence of metal ions and may be dependent on the TFA, although we were unable to find any IR bands indicating that TFA may be acting as cross-linking agent.

Conclusions

The SPPS method combined with fragment condensation using water-soluble EDC not only represents a very simple, cheap efficient way to attach peptide fragments in the N→C direction but also addresses the issue of solubility of the peptide fragments for use in the synthesis of large bolaamphiphilic peptide libraries. The advantages of our bidirectional solid phase synthesis method are its flexibility, versatility in terms of bolaamphiphile design (i.e. to synthesise both symmetrical and asymmetrical peptide bolaamphiphiles) and the possibility to apply it to a convergent synthesis protocol. Such an approach provides countless possibilities for the synthesis of 'peptido'-organic compounds and even compounds with multiple peptide arms in which the direction of the peptide bond is reversed in a part of the compound.

An important finding in this study is that the inherent ability of some small molecules to self-assemble can be used as a purification method for such compounds. Crystallisation has long been an established purification method in organic chemistry. Some organic components, however, are difficult to crystallise, especially larger molecules such as peptides and polypeptides. However, many bio-inspired organic compounds and peptides have been observed to self-assemble [2–20,41,44,50–61], but the possibility of rapid self-assembly as purification method for peptides and analogous compounds has gone, until now, unrecognised.

The choice of dilute aqueous TFA as the acidic solvent medium for the self-assembly of the model anionic bolaamphiphile significantly reduced self-assembly time and allowed the purification of the compound of interest from a crude mixture. Monitoring of the rapid assembly process corroborated our visual observation and confirmed programmed self-assembly rather than non-specific aggregation or precipitation. Also, we obtained a better understanding of self-assembly in 0.1% TFA and found that there is a progression from oligomers in the solution to layered β-sheet structures from which nanotubes and finally microtubes, consisting of a multitude of elongated smaller microtubes, grew over the self-assembly process (Figure 7).

To our knowledge, this is the first study using rapid self-assembly induced by TFA for purification of an anionic oligoglycine bolaamphiphile from a crude synthetic mixture. These results indicated that the acidic environment combined with the dehydration effect induced by TFA lead to highly specific self-assembly that can be compared with crystallisation and can therefore function as a purification method for analogous anionic peptides and bolaamphiphiles.

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References

1 Fuoss RM, Edelson DJ. Bolaform electrolytes. I. Di-(β-trimethylammonium ethyl) succinate dibromide and related compounds. *J. Am. Chem. Soc.* 1951; **73**: 269–273. DOI:10.1021/ja01145a090
2 Zhao X. Design of self-assembling surfactant-like peptides and their applications. *Curr. Opin. Colloid In.* 2009; **14**: 340–348. DOI:10.1016/j.cocis.2009.07.002
3 Shimizu T. Molecular self-assembly into one-dimensional nanotube architectures and exploitation of their functions. *Bull. Chem. Soc. Jpn.* 2008; **81**: 1554–1566. DOI:10.1246/bcsj.81.1554
4 Meister A, Blume A. Self-assembly of bipolar amphiphiles. *Curr. Opin. Colloid In.* 2007; **12**: 138–147. DOI:10.1016/j.cocis.2007.05.003
5 Gao BX, Matsui H. Peptide-based nanotubes and their applications in bionanotechnology. *Adv. Mater.* 2005; **17**: 2037–2050. DOI:10.1002/adma.200401849
6 Meister A, Blume A. Self-assembly of bipolar amphiphiles. *Curr. Opin. Colloid In.* 2007; **12**: 138–147. DOI:10.1016/j.cocis.2007.05.003
7 Shimizu T. Bottom-up synthesis and morphological control of high-axial-ratio nanostructures through molecular self-assembly. *Polym. J.* 2003; **35**: 1–22. DOI:10.1295/polymj.35.1
8 Zhang S, Marini DM, Hwang W, Santoso S. Design of nanostructured biological materials through self-assembly of peptides and proteins. *Curr. Opin. Chem. Biol.* 2002; **6**: 865–871. DOI:10.1016/S1367-5931(02)00391-5
9 Luk Y-Y, Abbott NL. Applications of functional surfactants. *Curr. Opin. Colloid In.* 2002; **7**: 276–275. DOI:10.1016/S1359-0294(02)00067-5
10 Grigoryan NA, Mndzhoyan ZO, Kazaryan EV, Ter-Zakharyan YZ, Mndzhoyan OL. Synthesis and antistaphylococcic activity of dicarboxylic acid derivatives having an amino acid fragment. *Pharm. Chem. J.* 1992; **26**: 153–157. DOI:10.1007/BF00766456
11 Shimizu T, Kogiso M, Masuda M. Vesicle assembly into microtubes. *Nature* 1996; **383**: 487–488.
12 Kogiso M, Ohnishi S, Yase K, Masuda M, Shimizu T. Dicarboxylic oligopeptide bolaamphiphiles: proton-triggered self-assembly of microtubes with loose solid surfaces. *Langmuir* 1998; **14**: 4978–4986. DOI:10.1021/la9802419
13 Matsui H, Gologan B. Crystalline glycylglycine bolaamphiphile tubules and their pH-sensitive structural transformation. *J. Phys. Chem. B* 2000; **104**: 3383–3386. DOI:10.1021/jp994117p
14 Kogiso M, Masuda M, Shimizu T. Supramolecular polyglycine II-type structure of glycylglycine bolaamphiphile. *Supramol. Chem.*1998; **9**: 183–189.
15 Matsui H, Pan S, Gologan B, Jonas SH. Bolaamphiphile nanotube-templated metallized wires. *J. Phys. Chem. B*, 2000; **104**: 9576–9579.
16 Matsui H, Douberly GE. Organization of peptide nanotubes into macroscopic bundles. *Langmuir* 2001; **17**: 7918–7922. DOI:10.1021/la010910+
17 Gao BX, Djalali R, Haboosheh A. Peptide nanotubes: simple separation using size-exclusion columns and use as templates for fabricating one-dimensional single chains of Au nanoparticles. *Adv. Mater.* 2005; **17**: 1753–1757. DOI:10.1002/adma.200500357
18 Kameta N, Mizuno G, Masuda M, Minamikawa H, Kogiso M, Shimizu T. Molecular monolayer nanotubes having 7–9 nm inner diameters covered with different inner and outer surfaces. *Chem. Lett.* 2007; **36**: 896–897. DOI:10.1246/cl.2007.896
19 Kameta N, Masuda M, Mizuno G, Morii N, Shimizu T. Supramolecular nanotube endo sensing for a guest protein. *Small* 2008; **5**: 561–565. DOI:10.1002/smll.200700710
20 Kameta N, Yoshida K, Masuda M, Shimizu T. Supramolecular nanotube hydrogels: remarkable resistance effect of confined proteins to denaturants. *Chem. Mater.* 2009; **21**: 5892–5898. DOI:10.1021/cm903108h
21 Atherton E, Gait MJ, Sheppard RC, Williams BJ. The polyamide method of solid-phase peptide and oligonucleotide synthesis. *Bioorg. Chem.* 1979; **8**: 351–370.
22 Atherton E, Sheppard RC. Solid phase synthesis: a practical approach. In *The Practical Approach Series*, IRL Press, Oxford University Press: Oxford, 1989.
23 Carpino LA, Han GY. The 9-fluorenylmethoxycarbonyl function, a new base sensitive amino-protecting group. *J. Am. Chem. Soc.* 1970; **92**: 5748–5749.
24 Carpino LA, Han GY. The 9-fluorenylmethoxycarbonyl amino-protecting group. *J. Org. Chem.* 1972; **37**: 3404–3409.
25 Bodansky M, In *Principles of Peptide Synthesis* . Hafner K, Rees CW, Trost BM, Lehn J, Von Ragné Schleyer P, Zahradnik R (eds). Springer Verlag: Berlin, 1984, 202–227.
26 Johansson A, Åkerblom E, Ersmark K, Lindeberg G, Hallberg A. An improved procedure for N- to C-directed (inverse) solid-phase peptide synthesis. *J. Comb. Chem.* 2000; **2**: 496–507. DOI:10.1021/cc000022h
27 Mihala N, Bódi J, Gömöry A, Süli-Vargha H. An alternative solid phase peptide fragment condensation protocol with improved efficiency. *J. Pep Sci* 2001; **7**: 565–568. DOI:10.1002/psc.352
28 Thieriet N, Alsina J, Guibé F, Albericio F. In *Peptides for the New Millennium*, Fields GB, Tam JP, Barany G (eds). Kluwer Academic Publisher: Dordrecht, 2000, 78–79.
29 Thieriet N, Guibé F, Albericio F. Solid-phase peptide synthesis in the reverse (N → C) direction. *Org. Lett.* 2000; **2**: 1815–1817, DOI:10.1021/ol0058341
30 Wang P, Landon M, Layfield R, Mayer RJ, Ramage R. In *Innovation and Perspectives in Solid Phase Synthesis and Combinatorial Libraries*, Epton R (ed.). Mayflower Scientific Limited: Birmingham, 1999, 415–416.
31 Huang YD, Yang SZ, Feng YM, Niu CI. An insulin analogue with γ-amino butyric acid substitution for A13Leu-A14Tyr. *J. Pept. Res.* 1999; **54**: 18–22. DOI:10.1034/j.1399-3011.1999.00068.x
32 Davis M-TB, Preston JF. A simple modified carbodiimide method for conjugation of small-molecular-weight compounds to immunoglobulin G with minimal crosslinking. *Anal. Biochem.* 1981; **116**: 402–407. DOI:10.1016/0003-2697(81)90380-8
33 Han S-Y, Kim Y-A. Recent development of peptide coupling reagents in organic synthesis. *Tetrahedron* 2004; **60**: 2447–2467. DOI:10.1016/j.tet.2004.01.020
34 Hϕeg-Jensen T, Jakobsen MH, Olsen CD, Holm A. Formation of peptide thioamides by use of Fmoc amino monothioacids and PyBOP. *Tetrahedron Lett.* 1991; **32**: 7617–7620. DOI:10.1016/0040-4039(91)80549-L

35 Kemp DS. Racemisation in peptide synthesis. In *The Peptides: Analysis, Synthesis, Biology*. Gross E, Meienhofer J (eds). Academic Press: New York, 1979, 1–10.
36 Atherton E, Cameron LR, Sheppard RC. Peptide synthesis: Part 10. Use of pentafluorophenyl esters of fluorenylmethoxycarbonylamino acids in solid-phase peptide synthesis. *Tetrahedron* 1988; **44**: 843–857.
37 Kaiser E, Colescott RL, Bossinger CD, Cook PI. Colour test for the detection of free terminal amino groups in solid phase synthesis of peptides. *Anal. Biochem*. 1970; **34**: 595–598. DOI:10.1016/0003-2697(70)90146-6
38 Gisin BF. The monitoring of reactions in solid-phase peptide synthesis with picric acid. *Anal. Chim. Acta* 1972; **58**: 248–249.
39 Escamilla GH, Newkome GR. Bolaamphiphiles: from golf balls to fibers. *Angew. Chem. Int. Ed Engl.* 1994; **33**: 1937–1940.
40 Balaram RP. A model for the interaction of trifluoroethanol with peptides and proteins. *Int. J. Pept. Prot. Res.* 1996; **48**: 328–336.
41 Kogiso M, Okada Y, Hanada T, Yase K, Shimizu T. Self-assembled peptide fibers from valylvaline bola-amphiphiles by a parallel β-sheet network. *Biochim. Biophys. Acta* 2000; **1475**: 346–352. DOI:10.1016/S0304-4165(00)00088-X
42 Snyder RG, Hsu SL, Krimm S. Vibrational spectra in the CH stretching region and the structure of the polymethylene chain. *Spectrochim. Acta, Part A* 1978; **34**: 395–406. DOI:10.1016/0584-8539(78)80167-6
43 Snyder RG, Strauss HL, Elliger CA. CH stretching modes and the structure of n-alkyl chains. 1. Long, disordered chains. *J. Phys. Chem.* 1982; **86**: 5145–5150. DOI:10.1021/j100223a018
44 Masuda M, Vill V, Shimizu T. Conformational and thermal phase behaviour of oligomethylene chains constrained by carbohydrate hydrogen-bond networks. *J. Am. Chem. Soc.* 2000; **13**: 12327–12333. DOI:10.1021/ja001884p
45 Kubelka J, Keiderling TA. Differentiation of β-sheet-forming structures: Ab initio-based simulations of IR absorption and vibrational CD for model peptide and protein β-sheets. *J. Am. ChemSoc* 2001; **123**: 48, 12048–12058 DOI:10.1021/ja0116627
46 Krimm S, Bandekar J. Normal mode spectrum of the parallel-chain β-sheet. *Biopolymers* 1988; **27**: 909–921.
47 Myshakina NS, Ahmed Z, Asher SA. Dependence of amide vibrations on hydrogen bonding. *J. Phys. Chem. B* 2008; **112**: 11873–11877. DOI:10.1021/jp8057355
48 Krimm S, Bandekar J. Vibrational spectroscopy and conformation of peptides polypeptides and proteins. *Adv. Protein Chem.* 1986; **38**: 181–365. DOI:10.1016/S0065-3233(08)60528-8
49 Lehn J-M, *Supramolecular Chemistry*. VCH, Verlagsgesellschaft: Weinheim, 1995, 139.
50 Philp D, Stoddart JF. Self-assembly in natural and unnatural systems. *Angew. Chem. Intl. Ed. Engl.* 1996; **35**: 1154. DOI:10.1002/anie.199611541
51 Sadatmousavi P, Soltani M, Nazarian R, Jafari M, Chen P. Self-assembling peptides: potential role in tumor targeting. *Curr. Pharm. Biotechno.* 2011; **12**: 1089–1100. DOI:10.2174/138920111796117409
52 Zhao X. Design of self-assembling surfactant-like peptides and their applications. *Curr. Opin. Colloid In.* 2009; **14**: 340–348. DOI:10.1016/j.cocis.2009.07.002
53 Reches M, Gazit E. Molecular self-assembly of peptide nanostructures: mechanism association and potential uses. *Curr. Nanosci.* 2006; **2**: 105–111. DOI:10.2174/157341306776875802
54 Zhang S, Marini DM, Hwang W, Santoso S. Design of nanostructured biological materials through self-assembly of peptides and proteins. *Curr. Opin. Chem. Biol.* 2002; **6**: 865–871. DOI:10.1016/S1367-5931(02)00391-5
55 Santoso SS, Vauthey S, Zhang S. Structures, function and application of amphiphilic peptides. *Curr. Opin. Colloid In.* 2002; **7**: 262–266. DOI:10.1016/S1359-0294(02)00072-9
56 Fuhrhop J-H, Wang T. Bolaamphiphiles. *Chem. Rev.* 2004; **102**: 2901–2937. DOI:10.1021/cr030602b
57 Lin Y, Mao C. Bio-inspired supramolecular self-assembly towards soft nanomaterials. *Front. Mater. Sci.* 2011; **5**: 247–265. DOI:10.1007/s11706-011-0141-5
58 Hirst AR, Escuder B, Miravet JF, Smith DK. High-tech applications of self-assembling supramolecular nanostructured gel-phase materials: from regenerative medicine to electronic devices. *Angew. Chem. Intl. Ed. Engl.* 2008; **47**: 8002–8018. DOI:10.1002/anie.200800022
59 Chen P. Self-assembly of ionic-complementary peptides: a physico-chemical viewpoint. *Colloid. Surface A* 2005; **261**: 3–24. DOI:10.1016/j.colsurfa.2004.12.048
60 Zhang S, Marini DM, Hwang W, Santoso S. Design of nanostructured biological materials through self-assembly of peptides and proteins. *Curr. Opin. Chem. Biol.* 2002; **6**: 865–871. DOI:10.1016/S1367-5931(02)00391-5
61 Katz E, Willner I. Integrated nanoparticle-biomolecule hybrid systems: synthesis, properties, and applications. *Angew. Chem. Intl. Ed. Engl* 2004; **43**: 6042–6108. DOI:10.1002/anie.200400651

