Enforcing solution phase nanoscopic aggregation in a palindromic tripeptide†

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C-terminal dimerization of a tripeptide palindrome afforded fibrillation in solution through an assembly probably driven by hydrogen bonding and hydrophobic contributions; such an approach provides an expeditious entry into fabrication of fibrillating peptides from non-fibrillating peptide sequences.

The third hypervariable loop (V3 loop, residues 303–338) of HIV-1 gp120 is essential for viral fusion with the host cell.[1] The V3 loop contains a highly conserved (312) GPGRAF hexapeptide sequence in several HIV-1 isolates, displaying a conformational preference for a double-turn, while truncated tetrapeptide GPGR prefers a type II β-turn.[2–4] Deletion of the GPG palindrome results in the abrogation of HIV-1 infectivity,[5] and consequently, antiviral activity of exogenously administered GPG tripeptide amide has been described for various screens.[6] Our investigations into elaboration of this antiviral activity led us to investigate structural features of GPG and its conjugate in the solid state and in solution owing to our interest in peptide conjugates.[7–9]

We observed the presence of an antiparallel β-sheet orientation for Boc-GPG-OMe (**1**) displaying fully extended conformation ably supported by intermolecular hydrogen bonding interactions in the solid state (Fig. 1) (supporting information)‡. This ordering was not evident when we tried to study its behavior in solution through CD measurements (Fig. 2). Moreover, freshly prepared methanolic solution of GPG tripeptide, fully protected or unprotected, did not afford any aggregation as a consequence of β-sheets orientation present in the solid state, as confirmed by various microscopic techniques (data not shown). This confirmed that the sheer presence of tripeptide β-sheets in the solid state is not readily translatable to and sufficient for solution self-assembly.

We and others have described the use of bifunctional linkers to connect two peptide units for various applications, including stabilization of β-sheet structures.[7–10] We surmised that the propensity of protected GPG to form antiparallel β-sheets could be possibly harnessed to nucleate solution phase self-aggregation in this tripeptide by connecting at least two such units by a diamine linker. It was envisaged that the designed GPG bis-conjugate **3** in its extended conformation might support the formation of layered β-sheets culminating in nanostructured aggregates (Fig. 3). Due to the palindromic nature of the unprotected "monomer" tripeptide, juxtaposition of the bis conjugate **3** can be considered leading to either an antiparallel or a parallel orientation.

† Electronic supplementary information (ESI) available: details of synthesis, crystal structure, molecular simulation studies, CD, TEM, AFM and OM studies. See http://www.rsc.org/suppdata/cc/b5/b500654f/

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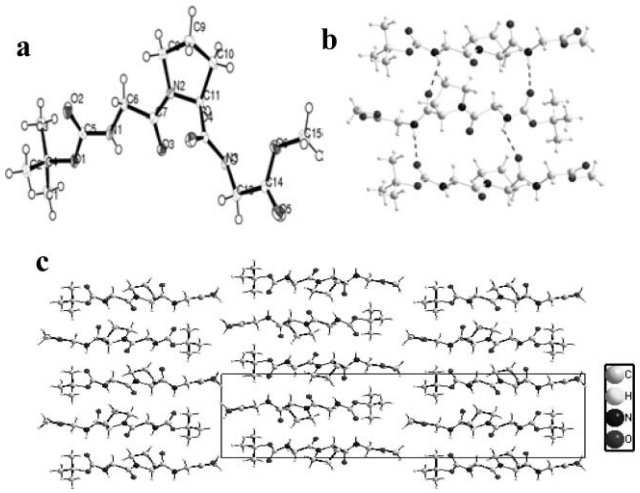

Fig. 1 (a) ORTEP representation of Boc-GPG-OMe with atom numbering scheme. Thermal ellipsoids are shown at the 50% probability level. (b) Backbone hydrogen bonding interactions and antiparallel β-sheets. (c) Packing diagram. Structures (b) and (c) are viewed along the '*a*' axis.

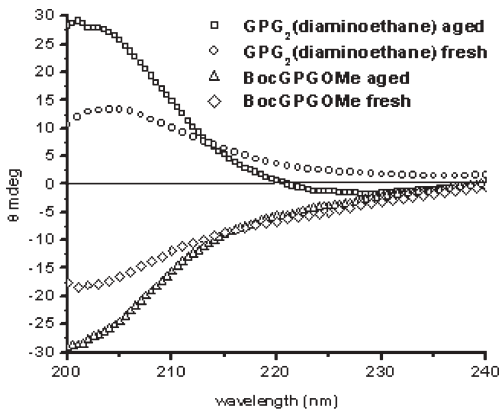

Fig. 2 CD studies of **3** and its fully protected tripeptide monomer (**1**).

3 was synthesized§ by tethering two *N*-protected GPG tripeptides with a flexible 1,2-diaminoethane linker *via* solution-phase chemistry (Fig. 3), characterized and subsequently studied *via* multiple imaging techniques to detect self-aggregation upon aging. We were unable to grow crystals for **3** thereby preventing a comment on its organization in the solid state. However, we have examples where peptide conjugates of similar sequence exhibit fully extended conformation when tethered with diamine linkers, as rendered in Fig. 3.[11]

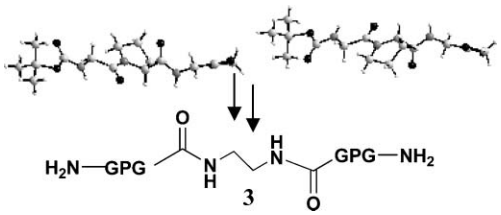

Fig. 3 Design and synthetic outline for **3**.

Solution studies with **3** validated our design strategy and we were able to observe ordered self-aggregated structures in methanolic solution upon aging. The choice of methanol as a solvent for aging studies is dictated by the poor solubility of **3** in water. In contrast to the predominantly random coil nature of both fresh and aged protected tripeptide, the conjugate clearly revealed a more ordered structure in CD studies (Fig. 2). Higher ellipticity values of aged peptide solution confirmed enhanced ordering in the conjugate and prompted us to investigate the nature of aggregates by various microscopic techniques.

Atomic force microscopy was employed to analyze ultrastructural details of the aged aggregates of **3** (15 days incubation in methanol). Extensive fibrillar networks were observed with an average fiber thickness of 27.5 nm and a typical width of 176 nm (Fig. 4a,b). Electron micrographs also confirmed the formation of discrete fibers of $\sim$50 nm cross-sectional diameter (Fig. 4c,d).

Filament formation was further probed by optical microscopy. Congo red dye binding to aged fibrils resulted in staining of **3** and observation of green birefringence when it was subjected to optical microscopic investigations (Fig. 5).

These results prompted us to reinvestigate GPG tripeptide self-assembly in solution using molecular dynamics (MD) simulation. Consequently, we carried out two simulation studies of GPG tripeptides in methanol using the GROMACS forcefield[12] (see supporting information for simulation details and analysis). In the first case, four randomly placed peptides in a box of $\sim$6100 methanol molecules failed to aggregate even after 15 ns of simulation (Fig. 6a,b). All four GPG peptides never came close together during the entire course of this simulation. A second

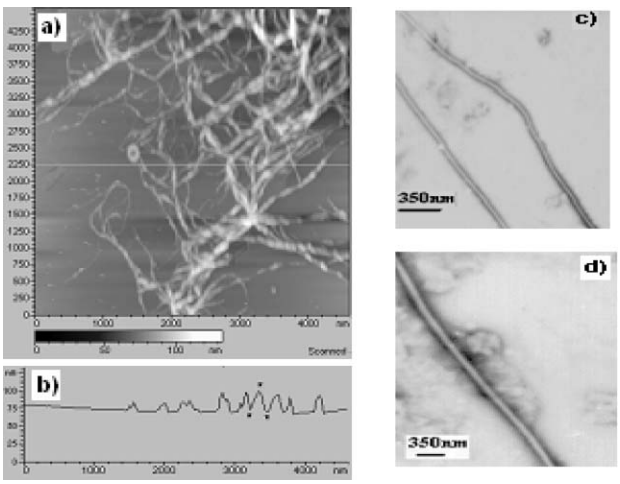

Fig. 4 AFM images: (a) aggregates formation by **3**, and (b) cross-sectional analysis of the image; (c,d) TEM micrographs showing discrete self-assembled peptide filaments of nanometric dimensions.

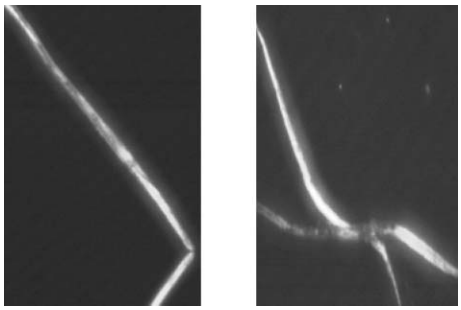

Fig. 5 Optical microscopic images of **3** with Congo red stain.

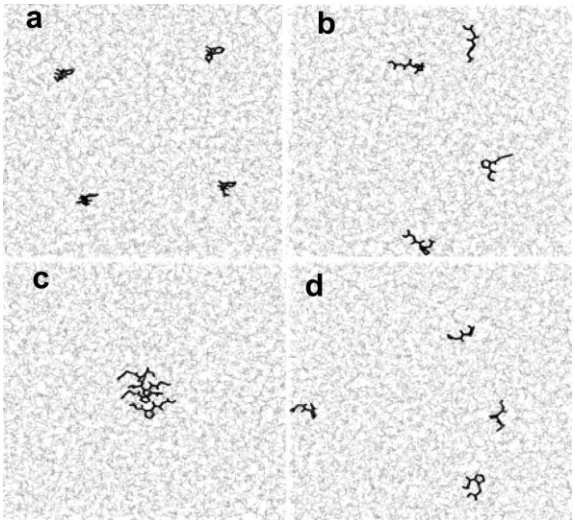

Fig. 6 MD simulations of GPG peptide: (a) four randomly placed tripeptides; (b) snapshot of (a) after 10 ns; (c) four stacked tripeptides; (d) snapshot of (c) after 2.5 ns. Both simulations were extended as described in the text and in the supporting information.

simulation was initiated with an aggregated stack containing four tripeptides akin to Fig. 1c, but they quickly dissociated within 2 ns and subsequently the system essentially behaved like the first simulation (Fig. 6c,d). These results confirm that GPG alone is unable to form fibrils. Therefore, a beneficial role of the bifunctional scaffold in enforcing aggregation is evident and provides a general route to study the determinants of peptide self-assembly in solution.

It is likely that hydrogen bonding in the extended conformation of **3** (Fig. 3) drives intermolecular self-assembly, as the hydrophobic contribution from the *t*-butyl group alone in protected GPG tripeptide did not produce aggregation. However, current results do not permit us to make a definitive statement concerning the dominance of one interaction over another for fibril formation. It will require several precisely designed constructs to understand the molecular mechanism of fibrillation and also to comment on the propensity of the fold-back structure of **3** to generate fibrillar aggregates. We intend to explore the generality and applications of this approach with more rigorous experimental and theoretical models for the generation of short peptide-based nanoscopic molecular scaffolds.[13–18]

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Notes and references

‡ CCDC 249980. See http://www.rsc.org/suppdata/cc/b5/b500654f/ for crystallographic data in .cif or other electronic format.

§ **Synthesis of bis(glycyl-prolyl-glycyl)diaminoethane (3)**: Boc-GPG-OH (1 g, 3.05 mmol, 1 equiv.) was dissolved in dichloromethane–dimethylformamide mixed solvent (1 : 1, 15 mL) and 1-hydroxybenzotriazole (0.41 g, 3.05 mmol, 1 equiv.) was added to it. This mixture was cooled to 0–5 °C. After stirring for 5 min, DCC (0.69 g, 3.34 mmol, 1.1 equiv.) was added and stirring was continued for 30 min and then at room temperature for another 30 min. 1,2-Diaminoethane (0.1 ml, 1.5 mmol, 0.5 equiv.) was added and stirring continued for 8 h at room temperature. Precipitated dicyclohexylurea was filtered and the filtrate washed with dichloromethane. Solvents were evaporated under vacuum. After evaporation, the crude compound was dissolved in dichloromethane (20 mL), washed with 10% $NaHCO_3$ solution (2 × 20 ml), 1 M HCl (2 × 20 ml) and finally with saturated brine solution (2 × 20 ml), followed by drying of the organic layer over anhydrous sodium sulfate. Dichloromethane was evaporated to get the crude compound (0.86 g) which was further purified with a silica gel column (1–8% CH_3OH gradient in $CHCl_3$) to give protected bis conjugate **2**. R_f value is 0.6 (6% methanol in dichloromethane) (0.62 g, 62%). mp: 198–200 °C. $[\alpha]_D^t = -25.45°$ (c = 0.55, in MeOH, t = 25 °C). FAB MS: (M + 1) = 683; ^{1}H NMR: (400 MHz, $CDCl_3$, 25 °C, TMS) δ 1.35(s, Boc 9H); 4.33(m, 1H, Pro α H); 1.93 and 2.07(br, m, 4H, Pro β, γ H); 3.44 and 3.62(m, 2H, Pro δ H); 3.28(appeared as br s, linker 2H); 5.89, 7.36 and 7.69(appeared as br s three –NH). ^{13}C NMR (100 MHz, [D] $CHCl_3$, 25 °C, TMS) δ(ppm) = 24.84, 28.25, 28.97, 38.57, 42.90, 43.06, 46.56, 61.08, 79.60, 156.42, 169.88, 170.04, 172.69; Anal. Calcd. for $C_{30}H_{50}N_8O_{10}$; C, 52.77; H, 7.38; N, 16.41; Found C, 52.67; H, 7.30; N, 16.36%. Deprotection of **2** (0.6 g, 0.73 mmol, 1 equiv.) was achieved by dissolving it in 3 M HCl–dioxane (1 ml) and stirring for 3 h, followed by evaporation of the solvent under reduced pressure. The residue was triturated with diethyl ether, dried and dissolved in water (6 ml). It was passed through an anion exchange column to get pure **3** (0.23 g, 58%). The compound was hygroscopic and prevented an accurate determination of its melting point. $[\alpha]_D^t = -21.62°$ (c = 0.375, in MeOH, t = 25 °C). FAB MS: (M + 1) = 483; ^{1}H NMR: (400 MHz, d^6 DMSO, 25 °C, TMS) δ 4.22(m, 1H, Pro α H); 1.85(m, 4H, Pro β, γ H); 3.61(m, Gly 2H and Pro δ H); 3.42(m, Gly 2H and Pro δ H); 3.31(appeared as br s, linker 2H); 8.32(m, 2H, –NH); 7.73(m, 2H, –NH). ^{13}C (100 MHz, D_2O, 25 °C, TMS) δ(ppm) 25.18, 30.14, 37.614, 43.38, 43.48, 47.58, 61.58, 172.00, 174.55, 176.13. Anal. Calcd. for $C_{20}H_{34}N_8O_6{\cdot}2H_2O$; C, 46.32; H, 7.39; N, 21.61; Found C, 46.11; H, 7.43; N, 21.7%. **Circular dichroism studies**: Methanolic solutions of **1** and **3** (0.5 mM as final concentration) were used as fresh and after 15 days of incubation at 30 °C. Far-UV CD measurements were performed with a JASCO spectropolarimeter (J-810 Model). **Transmission electron microscopy**: 15 days aged solution of **3** (8 μL, 1 mM) was transferred onto Formvar coated TEM grids and dried. Grids were stained with 2% uranyl acetate, dried and examined under a JEOL 2000FXII electron microscope, at an operating voltage of 100 kV. **Optical microscopy**: Congo red solution (2 μL, 150 mM) was added to 15 days aged solution of **3** (98 μL, 1 mM) and the mixture was left for 6 h at room temperature. 50 μL of this solution were transferred on to a glass slide, dried and then viewed under optical microscopy (AX10 Lab, Zeiss) with cross-polarized light (5003). **Atomic force microscopy**: 15 days aged solution of **3** (10 μL, 1 mM) was transferred to a freshly cleaved mica piece, followed by uniform spreading of the sample with the aid of a spin-coater. The mica piece was dried for 30 min followed by AFM imaging (Molecular Imaging, USA).

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