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Facilitation of peptide fibre formation by arginine-phosphate/ carboxylate interactions

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Abstract. This study describes peptide fibre formation in a hexapeptide, derived from the V3 loop of HIV-1, mediated by the interactions between arginine residues and phosphate/carboxylate anions. This charge neutralization approach was further confirmed when the deletion of arginine residue from the hexapeptide sequence resulted in fibre formation, which was studied by a combination of microscopic techniques.

Keywords. Peptide; self-assembly; arginine; microscopy.

1. Introduction

The process of peptide self-assembly, leading to fibre formation, critically depends on a subtle interplay of a variety of factors such as hydrogen bonding, hydrophobicity, π–π interactions, pH and electrostatic effects.[1] The latter property, in particular, observed in amphiphilic peptide constructs and related systems has been ingeniously exploited to result in interesting design paradigms useful for the construction of fibrous peptide-based materials[2a–f] and for surface deposition of peptides.[2g,h] Charge neutralization between acidic and basic amino acid side chains is the most common motif in such cases.

Chiti *et al* have recently reviewed that electrostatic charges are key factors in protein aggregation as a high net charge, either globally or locally, may hinder self-association.[3] Various biological entities viz. proteins, certain RNA molecules and DNA molecules fold into unique three-dimensional structures essential for their biological activity. In certain cases, the presence of counter ions facilitates neutralization of electrostatic forces which may interfere with optimal folding process.[4] Numerous examples of fibre formation through charge neutralization method are described in literature.[5] For instance, one of the major factors responsible for the self-assembly of microtubule-associated tau protein is abnormal hyperphosphorylation. This phosphorylation event is responsible for the neutralization of inhibitory basic charges, thus resulting in the self-assembly of tau protein into filamentous tangles.[6]

The third hypervariable region 3 (V3 loop) of HIV-1 gp120 is a critical determinant of viral infectivity.[7] It contains a highly conserved hexapeptide sequence (319) GPGRAF possessing a double β-turn character in solution. We have previously studied peptide fibre formation in GPG palindrome, derived from this sequence, via a *bis*-conjugation approach where formation of stable fibres was attributed to increased number of hydrogen bonding interactions in folded or extended conformation.[8] In continuation, the present study deals with GPGRAF hexapeptide where we demonstrate a combined beneficial effects of charge mitigation and extended hydrogen bonding interactions, as factors responsible for the growth of peptide fibres in a time-dependent fashion.

This hexapeptide sequence contains an arginine residue which is the most basic amongst all naturally occurring amino acids.[9] Side chain guanidino group can participate both in electrostatic interactions and directed hydrogen bonds, through its planar, fork-like geometry.[9] The carboxylate anion-guanidinium cation interaction is crucial in natural and non-natural systems as it allows stable, complementary ion-pair interaction due to the formation of two hydrogen bonds. Charged groups, such as carboxylates and guanidiniums, are frequently encountered on the surface of proteins and help solubilise these large molecules in an aqueous environment. Additionally, these functionalities also play a critical role in tuning selectivity of molecular interactions, recognition events and biological function.[10] For example, arginine-

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containing alamethicin peptide displayed self-assembly behaviour and the helix bundle channels thus formed exhibited remarkable anion-selectivity.[11]

The present work investigates bioinspired arginine-phosphate/carboxylate interactions to enforce peptide fibre formation via self-assembly process. Such charge mitigation approach also offers an intriguing possibility of biological anions inducing or assisting protein/peptide aggregation *in vivo*.

2. Materials and methods

All reactions, except saponification reactions, were performed under nitrogen atmosphere. Solvents like dichloromethane, *N,N′*-dimethylformamide, methanol were dried following the standard procedures prior to use. 1,2-diaminoethane was purchased from Lancaster and distilled before use. Triethylamine was purchased from S.D. Fine Chemicals Ltd. Mumbai, India, and distilled before use. *N,N′*-dicyclohexylcarbodiimide, was purchased from Spectrochem Pvt. Ltd., Mumbai, India used without further purification. 1-hydroxybenzotriazole which was purchased from Spectrochem Pvt. Ltd. Mumbai, India, was recrystallized from 50% aqueous ethanol prior to use. ^{1}H and ^{13}C NMR were recorded on JEOL-JNM LAMBDA 400 model operating at 400, 100 MHz respectively. ESI High Resolution Mass spectra were recorded at Indian Institute of Technology Kanpur, India on a Waters, Q-ToF Premier micromass HAB 213 mass spectrometer using capillary voltage 2·6–3·2 kV. Sample concentration were 1 mM (1 mg of sample was dissolved in 1 mL methanol) and 10 μL of the sample prepared was injected. Melting points of the compounds determined are uncorrected (Navyug India Limited). The precursor penta- and hexapeptides were synthesized via routine solution-phase methods and will be reported elsewhere.

2.1 *Synthesis of peptides*

2.1a *Synthesis of H_2N–Gly–Pro–Gly–Arg–Ala–Phe–COOH (1):* Boc–GPGR(Tos)AF–OH (0·1 g, 0·11 mmol) was completely dissolved in dichloromethane (2 mL), then added few drops of trifluoromethane sulphonic acid and stirred for 3 h. After this time, the solvent was evaporated under reduced pressure. The gummy compound triturated with diethyl ether and the round bottomed flask was kept in an ice bath. After sometime ether was decanted and this process repeated till the gummy compound becomes solid. This solid compound was dissolved in 50% aqueous methanol (2 mL) and passed through an anion exchange column to get pure **1** (0·05 g, 80%). The compound was hygroscopic and prevented the (accurate) determination of its melting point. $[\alpha]^{D}_{t}$ = –45° (c = 0·73 in 50% aqueous methanol, t = 25°C). HRMS: found (M + 1) = 604·3204; $C_{27}H_{42}N_9O_7$ requires 604·3207 [ESI +ve mode]. ^{1}H NMR: (400 MHz, D_2O, 25°C) δ (ppm) 1·15 (*d*, Ala, –CH_3); 1·6, 1·8 and 2·1 (*m*, for guanidine 6H and Pro β, γ 4H); 2·9 and 3·1 (*m*, Phe 2 βH); 3·3, 3·75 and 3·93 (*m*, Gly 4H and Pro 2 δ H); 4·10, 4·3 and 4·63 (*m*, 4α H of Pro, Arg, Ala and Phe); 7·01, 7·03, 7·14 and 7·17 (*m*, for Phe protons). ^{13}C NMR: (100 MHz, D_2O, 25°C) δ (ppm) 17·43, 25·14, 30·18, 38·24, 41·34, 43·41, 47·49, 50·41, 53·80, 56·86, 61·38, 125·93, 126·64, 126·88, 127·52, 129·25, 130·22, 138·11, 157·42, 172·14, 173·91, 174·19, 176·32, 178·14.

2.1b *Synthesis of H_2N–Gly–Pro–Gly–Ala–Phe–COOH (2):* Boc–GPGAF–OH (0·1 g, 0·18 mmol) was dissolved in 50% TFA–DCM (2 mL) and then stirred for 2·5 h, and then solvent was evaporated under reduced pressure. The obtained gummy compound was triturated with diethyl ether and the round bottomed flask was kept in an ice bath. After sometime the ether was decanted and this process repeated till the gummy compound becomes solid. This solid compound was dissolved in 50% aqueous methanol (2 mL) and passed through an anion exchange column to get pure **2** (0·06 g, 80%). The compound was hygroscopic and prevented the (accurate) determination of its melting point. $[\alpha]^{D}_{t}$ = –50° (c = 0·6 in 50% aqueous methanol, t = 25°C). HRMS: found (M + 1) = 448·2192; $C_{21}H_{30}N_5O_6$ requires 448·2196 [ESI +ve mode]. ^{1}H NMR: (400 MHz, D_2O, 25°C) δ (ppm) 1·14 (*d*, Ala, –CH_3); 1.88 and 2·14 (*m*, Pro β, γ 4H); 2·93 (*m*, Phe 2β H); 3.36 (*m*, Pro δ H); 3·67, 3·71 and 3·96 (*m*, 4 Gly H); 4·25, 4·35 and 4·7 (*m*, 3α H of Pro, Ala and Phe); 6·98 (*d*), 7·00, 7·05, 7·20 (*m*, corresponding to Phe aromatic protons)· ^{13}C NMR (100 MHz, D_2O, 25°C) δ (ppm) 17·53, 25·72, 29·17, 30·22, 38·38, 43·01, 47·54, 50·20, 56·87, 61·42, 126·64, 127·46, 129·91, 130·15, 130·51, 157·76, 171·85, 174·05, 175·79, 178·23.

2.1c *Synthesis of Bis(Gly–Pro–Gly–Arg–Ala–Phe) diaminoethane (3):* The protected bisconjugate of the hexapeptide (0·04 g, 0·02 mmol) was dissolved in dry dichloromethane (2 mL), stirred for 10 min and after completely forming a clear solution, 0·3 mL of tri-

fluoromethane sulphonic acid was added and stirred for 3 h. After this time, the solvent was evaporated under reduced pressure. The syrupy residue was triturated with diethyl ether, and the round bottomed flask was kept in an ice bath. After sometime ether was decanted and this process repeated till the gummy compound becomes solid. The solid was dissolved in 50% aqueous methanol (3 mL) and passed through an anion exchange column to get pure **3** (0·02 g, 81%). The compound was hygroscopic and prevented the (accurate) determination of its melting point. $[\alpha]^{D}_{t}$ = 44·4° (c = 0.45 in methanol, t = 25°C). HRMS: found (M + 2) = 1233.6913; while $C_{56}H_{88}N_{20}O_{12}$ requires 1233·6972 [ESI +ve mode]. ^{1}H NMR: (400 MHz, D_2O, 25°C) δ (ppm) 1·07 (*d*, Ala $-CH_3$); 1·36, 1·53, 1·70 (*m*, for guanidine 6H and Pro β, γ 4H); 2·78 to 3·4 (*m*, Phe 2βH, 2H of DAE, Pro δ 2H, gly 4H); 3·99 and 4·18 (*m*, 4α H of Pro, Arg, Ala and Phe); 6·99 and 7·07 (*m*, aromatic protons of Phe). ^{13}C NMR: (100 MHz, D_2O, 25°C) δ (ppm) 17·14, 23·06, 24·98, 25·10, 28·88, 30·12, 32·30, 37·95, 43·40, 50·37, 53·97, 55·95, 61·43, 62·57, 127·90, 129·45, 125·95, 137·16, 157·44, 166·57, 173·46, 175·08, 176·06, 176·45, 180·21, 180·63.

2.1d *Synthesis of Bis (Gly–Pro–Gly–Ala–Phe) diaminoethane (**4**):* Deprotection of protected bis pentapeptide (0·3 g, 0·27 mmol) was achieved by dissolving in 4 mL of 50% TFA–DCM mixture, stirred for 3 h and then evaporated the solvent under reduced pressure. The gummy compound triturated with diethyl ether and the round bottomed flask was kept in an ice bath. After sometime ether was decanted and this process repeated till the gummy compound becomes solid. This solid compound was dissolved in 3 mL 50% aqueous methanol and passed through an anion exchange column to get pure 4 (0·23 g, 58%). The compound was hygroscopic and prevented the (accurate) determination of its melting point. $[\alpha]^{D}_{t}$ = 33·3° (c = 0.3 in water, t = 25°C). HRMS: (M + Na) = 941·4611 while $C_{44}H_{62}O_{10}N_{12}Na$ requires 941·4610 [ESI +ve mode]. ^{1}H NMR: (400 MHz, D_2O, 25°C) δ (ppm) 1·12 (*d*, Ala $-CH_3$); 1·75–1·85 and 2·06–2·13 (*m*, Pro β, γ 4H); 2·82–3·11 (*m*, Phe 2β H and DAE 2H); 3·3, 3·4 and 3·7 (*m*, for Gly 4H and Pro δ H) 4·09, 4·26 and 4·38 (*m*, 3α H of Pro, Phe, Ala); 7·08–7·22 (*m*, 5H of Phe)· ^{13}C NMR (100 MHz, D_2O, 25°C) δ (ppm) = 26·39, 27·46, 39·69, 43·07, 43·41, 44·51, 55·56, 109·55, 112·68, 119·02, 120·17, 122·78, 125·20, 127·59, 136·87, 171·21, 171·55, 172·14, 172·85, 174·76.

2.1e *FPLC Studies:* The peptide conjugates 3 and 4 are found to be pure when run through a μRPC C2/C18 ST4.6/100 column with an applied gradient of 50% methanol-water system with 1 mL/min flow rate. The FPLC used here is an analytical FPLC (Akta Basic, Amersham Pharmacia). The concentration of the conjugates for an analytical run is 1 mg/ml and the purity of peptides and their conjugates was >95%.

2.1f *Scanning Electron Microscopy:* 15 μL of 4 (5 mM, 20 days aged diluted to 1 mM as final concentration), 3 in phosphate buffer (5 mM pH = 7·0, 15 d aged diluted to 1 mM), 3-ADP and 3-ATP complexes (5 mM, 1 : 1 ratio, diluted to 1 mM as the final concentration, 20 d aged) were dried on a clean brass stub and gold coated for 1 h and then used for SEM imaging. SEM imaging was accomplished with FEI Quanta 200 instrument operated with a voltage of 20 kV. The samples of aged peptides 1 and 2 were also prepared following the same procedure as mentioned above.

2.1g *Atomic force microscopy:* 15 μL of 20 days aged solution of **4** (5 mM but diluted to 1 mM during sample preparation), 15 μL of ~20 days aged solutions of **3**-succinic acid (5 mM, 1 : 1 ratio, diluted to 1 mM as the final concentration) **3**-glutaric acid (5 mM, 1 : 1 ratio, diluted to 1 mM as the final concentration), **3** in phosphate buffer alone (5 mM pH = 7·0, 15 d aged diluted to 1 mM) were transferred on to freshly cleaved mica pieces (3 × 5 μL). These mica pieces were dried for a period of 10 min under a table lamp (100 W) followed by imaging with atomic force microscope (Molecular Imaging, USA), operated under Acoustic AC mode (AAC) with the aid of cantilever (NSC36, Mikro Masch). The force constant was 0·6 N/m, while the resonant frequency used was 150 kHz. The images were taken in air at room temperature, with a scan speed of 1·5–2·2 lines/s. Data acquisition was performed by Pico Scan 5 software and the analysis was done with the aid of visual SPM.

2.1h *Optical microscopy:* Congo red solution (2 μL, 50 μM saturated solution in 80% ethanol/water) was added to aged solutions of **4** (23 μL, 5 mM), **3** in phosphate buffer (23 μL, 5 mM), **3**-succinic acid, **3**-glutaric acid (23 μL, 5 mM) and **3**-ATP (23 μL, 5 mM) and the mixture sonicated for 8 s and left for 6 h at 37°C. These solutions were then transferred on to clean glass slides, dried and

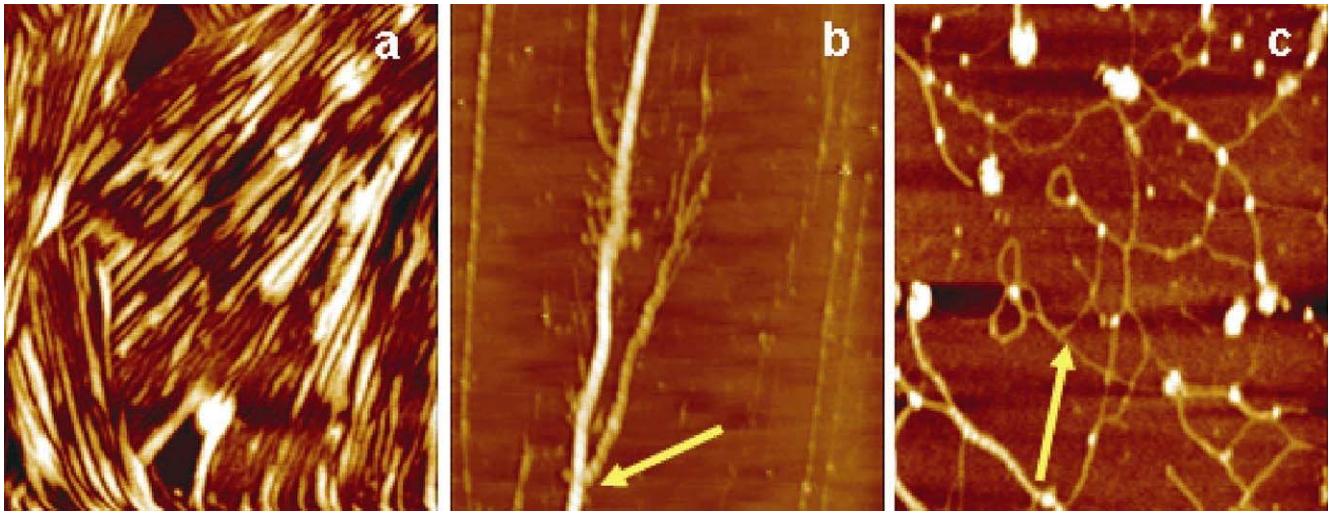


Figure 1. AFM micrographs of aged samples and cross-sections of (**a**) **2** (~30 nm); (**b**) **1**-ATP (~90 nm) and (**c**) **1**-glutaric acid (~15 nm). (Arrowheads indicate the branching points).

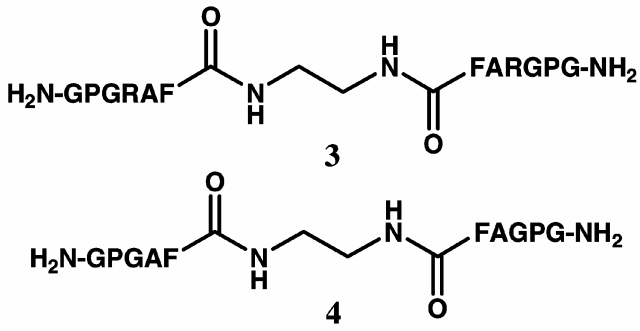


Figure 2. Molecular structures of peptide conjugates, **3** and **4**.

then viewed under optical microscope (Labomed, Digi 3, 10X) with cross-polarized light, interfaced with a PC. Images were obtained by using a CCD camera attachment and processed with the help of Digipro V 2.0 software.

3. Results and discussion

Initial experiments involved the study of aggregative behaviour of the full hexapeptide GPGRAF (**1**) and a control GPGAF (**2**) pentapeptide sequence with arginine deletion to ascertain its role, if any, in the formation of stable peptide fibres upon aging. Interestingly, ~ 45 days incubation of **1** and **2** (5 mM in each case) in pure water at 37°C revealed fibre formation in **2**, (formation of protofilaments observed after 10 d; data not shown) lacking the arginine residue (figure 1a), while **1** alone did not reveal distinct fibre formation perhaps due to the electrostatic repulsion between the two guanidino side chains (data not shown). However, **1** alone could only afford poorly structured oligomeric aggregates when its aqueous solution was aged for 45 days (data not shown). Thus, we decided to counter this structural (charge) lacuna with the help of biologically relevant anions such as phosphates and carboxylates. The premise of charge neutralization was validated when **1** self-assembled in the presence of ATP and glutaric acid (2·5 mM as final concentration), revealing emergence of peptide fibres at the end of 45 days (figure 1b, c).

We have been experimenting with the beneficial effects of linked bis- and tris-peptide conjugates where self-assembly is accentuated due to increased number of non-covalent stabilizing interactions, such as hydrogen bond and π–π interactions.[8,12] In a related example, we were able to enforce aggregation in a truncated V3 loop GPG tripeptide when conjugated to 1,2-diaminoethane (DAE).[8] Thus, it was of interest to determine whether this linker, in combination with charge neutralization, would further control emergence of peptide fibres through self-assembly. Two *bis*-conjugates, $(GPGRAF)_2$ DAE (**3**) and $(GPGAF)_2$ DAE (**4**) (figure 2) were synthesized using solution phase protocols.

Preliminary aging experiments with **3** and **4** were concurrent to our observations with **1** and **2**. Arginine-containing hexapeptide conjugate **3** (5 mM) failed to reveal fibres upon incubation in water (figure 3a), while the arginine-deleted bis construct **4**

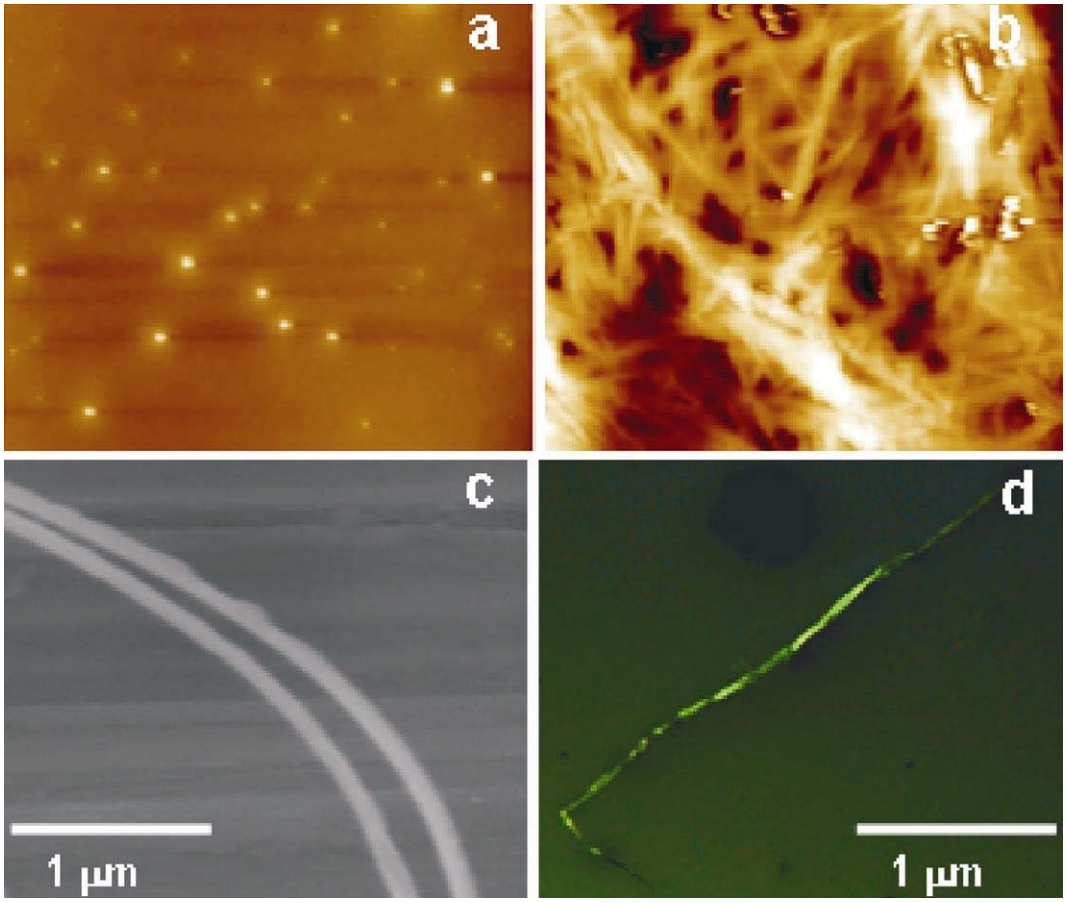


Figure 3. Aging studies with bis conjugates. (**a**) AFM micrograph of aged **3** displaying punctuated structures suggesting lack of aggregation; (**b**–**d**) AFM (~20 nm) and SEM micrographs and optical micrograph of Congo red-stained image of individual peptide fibre from aged solution of **4**.

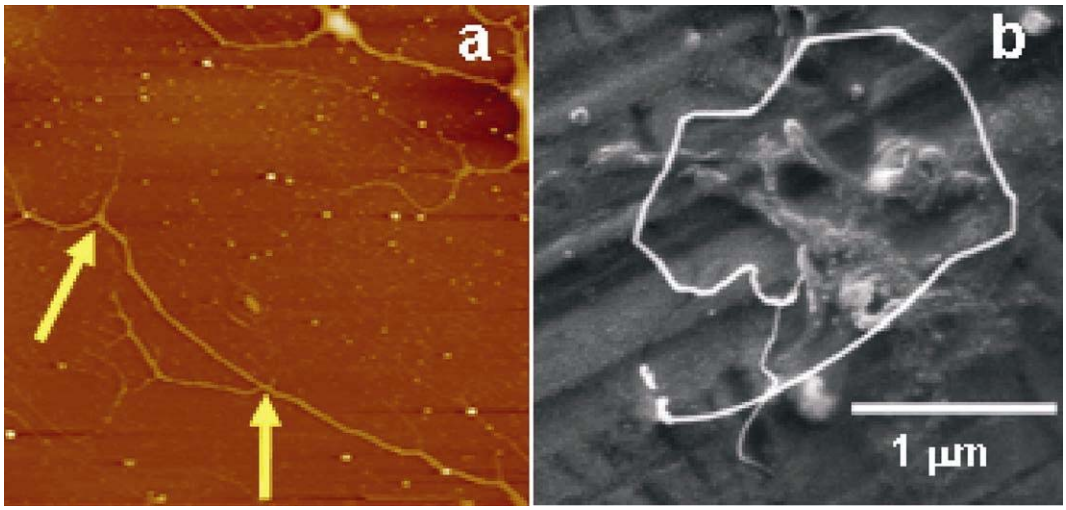


Figure 4. (**a**) AFM image of aged **3** (fibre cross-section: ~90 nm) in the presence of phosphate buffer (arrowheads indicate branching points); (**b**) SEM image of mature **3** fibres in phosphate buffer.

(5 mM), afforded dense fibrillation within ~20 days of incubation in water at 37°C (figure 3b–d). This observation suggested that bis-scaffolds reduced the aggregation time by half possibly

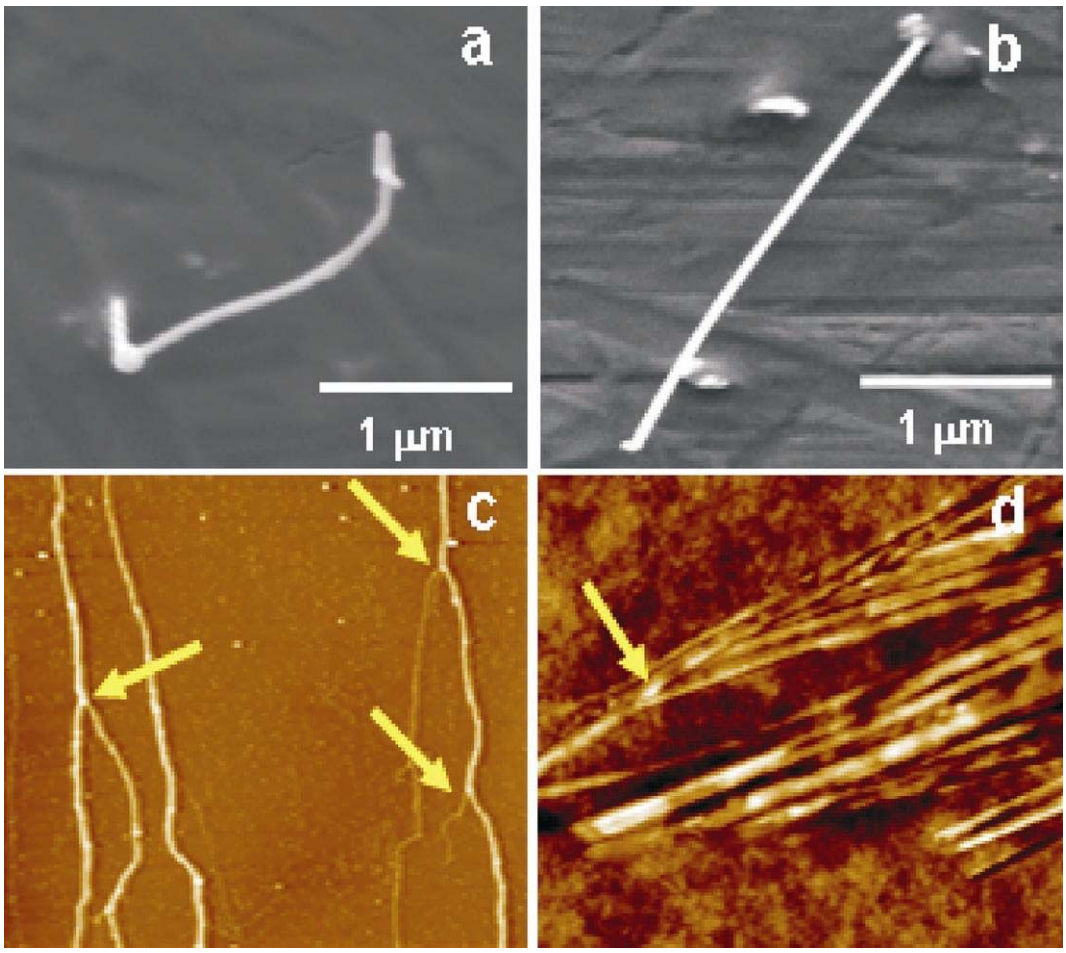


Figure 5. SEM images peptide fibre from incubation of **3** with (**a**) ADP; and (**b**) ATP; AFM images of peptide fibres from incubation of **3** with (**c**) succinic acid (~140 nm) and (**d**) glutaric acid (~20 nm). Arrowheads indicate branching points.

by enhancing the number of stabilizing interactions.

Importantly, we were able to force aggregation in **3** (5 mM) in the presence of simple phosphate buffer (5 mM, pH = 7·0), where self-assembled structures were observed within 15 days of incubation (figure 4). Branching of fibrils in phosphate buffer was observed in AFM (figure 4a). Natural protein fibres, such as those formed by actin, collagen and fibrin also form branches. Formation of branches in self-assembling filaments has been reported by Woolfson and coworkers.[13]

Encouraged by these results, we probed nucleotides for their ability to induce aggregation. SEM micrographs revealed facile self-assembly of **3**-ADP and **3**-ATP complexes (5 mM as final concentration of **3** and corresponding nucleotides), culminating into fibre formation (figure 5a, b). Such an interaction between phosphate groups and an arginine moiety was recently reported for the self-assembly of peptide amphiphiles.[2e] The interaction between guanidinium and phosphate groups is generally attributed to a combined effect of electrostatics and hydrogen bonding.[14] Frankel and co-workers, while studying HIV-1 Tat protein and TAR RNA interaction, have also described the role of an arginine moiety for RNA binding.[15] Another literature example has described interaction of adenine nucleotides with positively charged residues, such as arginine and lysine, in the case of a pore-forming protein.[16,17]

The guanidinium group can also bind to other anionic species such as carboxylates.[18–20] On the lines of arginine-phosphate interaction, incubation of 3 in the presence of diacids such as succinic and glutaric acid (5 mM as final concentration of 3 and corresponding diacids) resulted in peptide fibre formation (figure 5c, d). Interestingly, diacid induced fibres also displayed branched features similar to the struc-

tures observed in the phosphate buffer (figure 5c, d). We also resorted to optical microscopy to capture the occurrence of Congo-red stained fibres which once again confirmed the presence of peptide fibres due to electrostatic effects manifested by arginine-anion interactions (figure 6).

Induction of peptide assembly solely on the basis of charge neutralization can be understood by a simple model which also suggests reasons through which fibril growth and branching may occur (scheme 1). Lateral overlaps are expected to elongate peptide fibre growth in a given direction, while spatial predisposition of phosphates and carboxylates and tethering of two growing chains may eventually result in the emergence of the branchpoints. It is expected that a suitable choice of anions may result in interesting ultrastructural morphologies of self-assembled peptides.

Interestingly, neutralization of arginine residues via salt bridges is reported for HIV-1 interaction with antibodies and arginine interaction with sulphated proteoglycans, for the infection of macrophages and T-cell lymphocytes.[21]

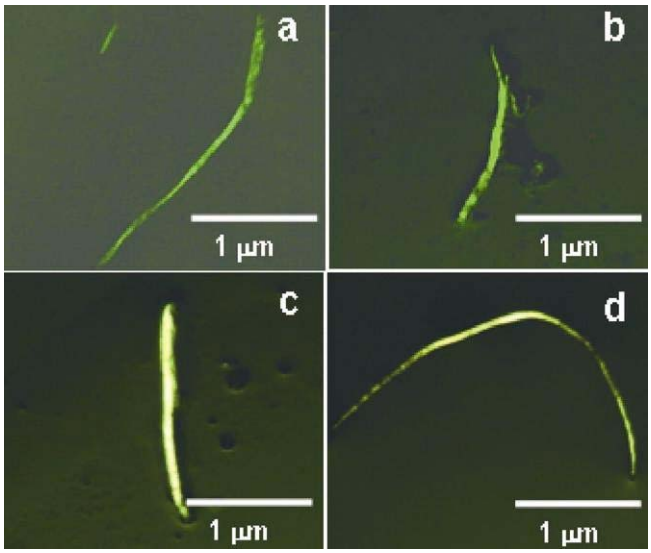
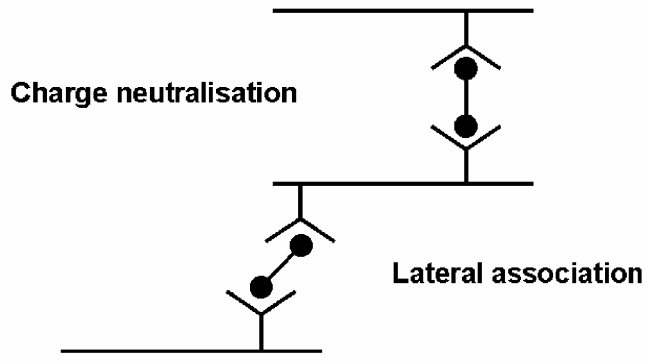


Figure 6. The phenomenon of green-birefringence of **3** with (**a**) succinic acid (**b**) glutaric acid, (**c**) phosphate buffer alone and (**d**) ATP.



Scheme 1. Proposed model of electrostatic neutralization of arginine moieties on peptide chains by anions (red dumbbells) and lateral association to give self-assembled fibres of persistent length.

4. Conclusions

This study entails a bioinspired approach in enforcing peptide aggregation via neutralization of positive charges on the arginine residues and applies electrostatics based design paradigm for the construction of peptide fibres and filaments, with a distinct dependence on flexible linkers for an accelerated self-assembly process. The study of the properties of peptide nanofibrillar assemblies and the mechanisms of formation might be a source of inspiration for the development of ordered, rationally designed nanostructures with potentially interesting applications.[22]

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