Controlled patterning of aligned self-assembled peptide nanotubes

MEITAL RECHES* AND EHUD GAZIT†

Department of Molecular Microbiology and Biotechnology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel
*Present address: Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, USA
†e-mail: ehudg@post.tau.ac.il

Published online: 5 December 2006; doi:10.1038/nnano.2006.139

Controlling the spatial organization of objects at the nanoscale is a key challenge in enabling their technological application[1–3]. Biomolecular assemblies are attractive nanostructures owing to their biocompatibility, straightforward chemical modifiability, inherent molecular recognition properties and their availability for bottom-up fabrication[4–16]. Aromatic peptide nanotubes are self-assembled nanostructures with unique physical and chemical stability and remarkable mechanical rigidity[14–16]. Their application in the fabrication of metallic nanowires and in the improvement of the sensitivity of electrochemical biosensors have already been demonstrated[14–17]. Here we show the formation of a vertically aligned nanoforest by axial unidirectional growth of a dense array of these peptide tubes. We also achieved horizontal alignment of the tubes through noncovalent coating of the tubes with a ferrofluid and the application of an external magnetic field. Taken together, our results demonstrate the ability to form a two-dimensional dense array of nanotube assemblies with either vertical or horizontal patterns.

The formation of high-density and spatially orient arrays of nanostructures is central to many nanotechnological applications[1–3]. Much effort has been invested in developing fabrication techniques for the growth of organized arrays of carbon and inorganic nanostructures[1–3,18,24]. Well-ordered arrays of nanostructures, such as nanoforests, are formed by physical and chemical vapour deposition techniques, in which the unidirectional growth of the nanotubes and nanowires is controlled by a vapour–liquid–solid mechanism. These procedures are performed under extreme conditions in which the nanoscale building blocks are vaporized and diffused onto a surface, where they condense into molecular films or ordered discrete assemblies. Although this approach is attractive for carbon and inorganic nanoassemblies, it is incompatible with nonvolatile biological and organic building blocks. Peptide building blocks are very attractive organic building blocks for bionanotechnology applications owing to their biocompatibility, chemical flexibility and versatility, biological recognition abilities and straightforward synthesis[6–14].

Aromatic dipeptide nanotubes (ADNTs) constitute a unique class of bio-inspired nanostructures. These hollow tubular nanostructures are formed in aqueous solution by the self-association of a simple aromatic dipeptide, namely diphenylalanine[14]. The peptide tubes are formed as individual entities and have a remarkable micrometre-scale persistence length. These properties, together with their exceptional chemical and thermal stability and extraordinary mechanical strength, make them appealing structural elements for various applications[15,16]. The nanometric dimensions of the ADNTs have already been exploited in the fabrication of a sensitive biosensing device[17], as a casting mould for the fabrication of metallic nanowires[14], and as a scaffold for the organization of platinum nanoparticles[25].

Here we conducted evaporation-initiated unidirectional growth of ADNTs under mild conditions in order to form nanoforests. This controlled patterning of aligned tubes was achieved by spreading monomeric building blocks dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) over siliconized glass, as illustrated in Fig. 1a. The highly volatile fluorinated alcohol is a remarkable solvent that allows the existence of the peptide entities as monodispersed building blocks. Upon the rapid evaporation of the HFP, a thin layer was formed on the substrate. This layer, analysed using a scanning electron microscope (SEM), could be clearly observed as a vertically aligned array of peptide nanotubes (Fig. 1b). Analysis by high-resolution SEM provided us with an insight into the ordered array of tubes and revealed their open-ended structure (Fig. 1c). In addition, the multiwalled nature of the tubes could be clearly observed, as their outer diameter is much wider than their interior cavity (Fig. 1d). We propose that the well-ordered organization of the structures is most likely facilitated by the geometrically restricted stacking of the aromatic moieties in the direction of the growth axis derived by the vapour–liquid–solid system that exists during the rapid evaporation of the HFP solvent (see Supplementary Information, Fig. S1).

To determine the structure of the ADNT array, we performed X-ray diffraction analysis (Fig. 1e). The sixfold symmetry that we observed is very similar to that found for a single crystal of diphenylalanine or powder diffraction under conditions that lead to the formation of nanotubes in solution[26,27]. We therefore assume that the model for the nanotube structure as recently suggested by Gorbitz[27]—in which both hydrogen bonding and aromatic stacking interactions govern the assembly process—is valid for the ADNT nanoforest. However, as these experiments were performed on a crystal or a large collection of nanotubes, we went on to determine the electron diffraction pattern of an individual nanotube (Fig. 1f). The result clearly suggests that the conclusions drawn for a tube collection are also valid at the single tube level (see Supplementary Information data). The level of the ordering of the nanotubes was clearly observed by tilting experiments in which the angle of the sample was changed while

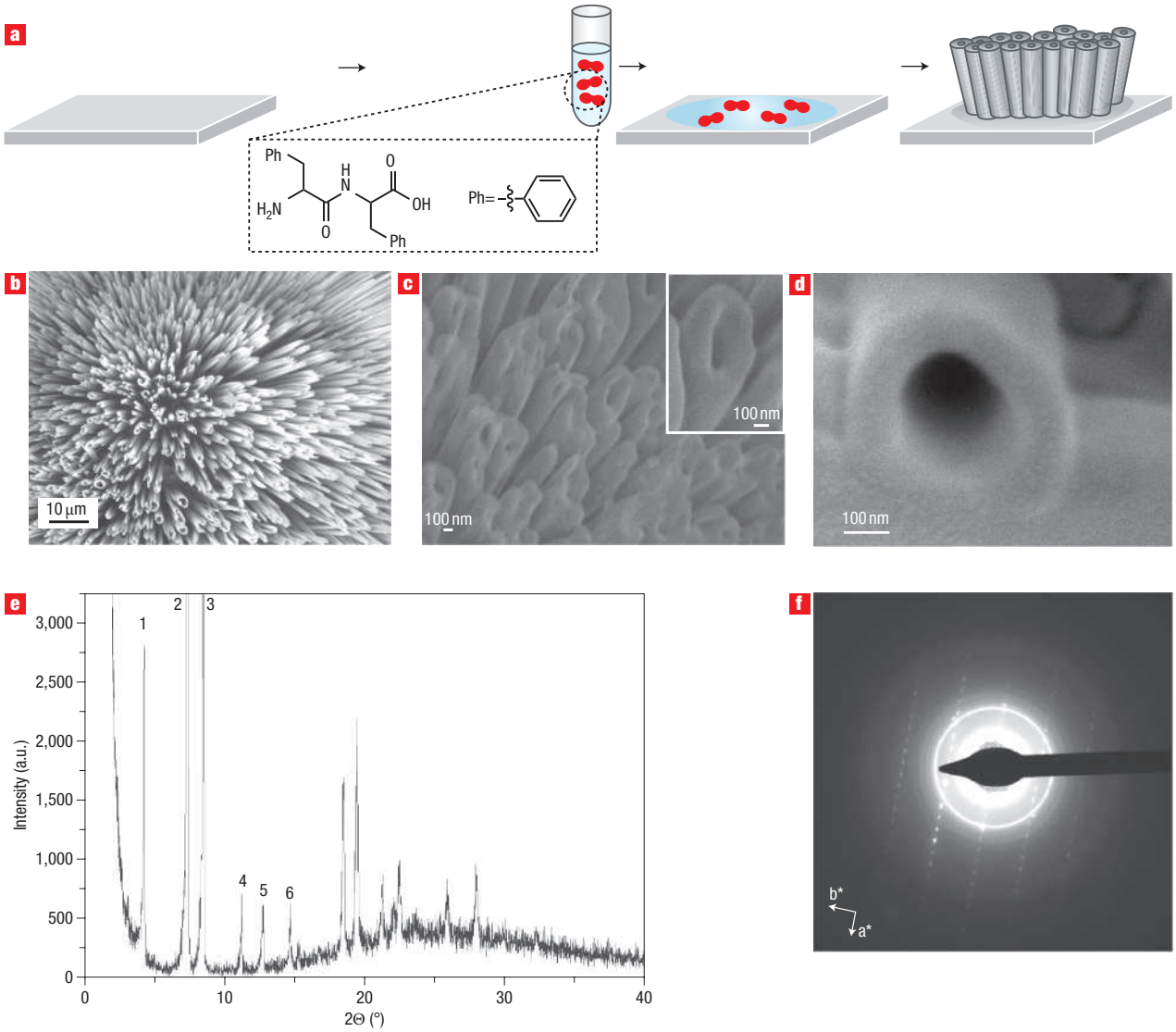


Figure 1 Vertically aligned diphenylalanine-based nanotubes self-assembled into a peptide nanoforest. a, A possible model for the formation of the aligned peptide nanotubes array. Applying the dipeptide monomers dissolved in the organic solvent onto siliconized glass resulted in the formation of a vertically aligned array of peptide nanotubes. **b**, SEM analysis of the vertically aligned peptide nanotubes. **c**, Cold field-emission gun high-resolution scanning electron microscope (CFEG-HRSEM) analysis of the nanotubes array. The inset represents higher magnification of the aligned nanotubes. **d**, High-magnification micrograph (×120,000) of one individual nanotube of the array obtained by CFEG-HRSEM. **e**, X-ray diffractogram of a peptide array on a glass surface. The sixfold symmetry of the structure is shown by the six periodic peaks (labelled 1–6). **f**, Electron-diffraction analysis of a single peptide nanotube. Axis *a* is oriented normal to the long axis of the crystal. a* and b* are the reciprocal lattice vectors in the diffraction pattern.

observations were carried out in a high-resolution scanning electron microscope (HR-SEM) chamber (Fig. 2).

We propose that the rapid evaporation of the HFP solvent results in a supersaturation state that facilitates the formation of numerous nucleation sites on the surface. We suggest that this is followed by unidirectional growth of the nanotubes as more diphenylalanine monomers are stacked on the nucleation sites and sediment away on the surface toward the liquid–air interface. The process of assembly could be observed using light microscopy (see Supplementary Information, movie). Although the peptide solution is completely clear for a few seconds following deposition, a simultaneous and coordinated formation of highly ordered structures occurs as soon as evaporation is allowed. Each nucleation event results in the formation of a peptide nanoforest across an area of 100 μm^2 and the coverage of the whole surface with these ordered structures (Fig. 1b; see Supplementary Information, movie). It should be noted that other mechanisms of assembly could not be ruled out. The most significant is solution-initiated assembly, which could be followed by organization of the preformed tubes into a nanoforest.

Although the peptide building blocks are highly soluble in HFP, their alignment onto the surface could be observed only in peptide concentrations ranging from 160 mg ml^{-1} to 60 mg ml^{-1} (see Supplementary Information, Fig. S2). At lower concentrations, nanotube formation could be detected, but with no specific orientation (see Supplementary Information, Fig. S2a, b). This lower limit most likely reflects the inability of the peptide monomer to nucleate with supersaturation-driven growth at the

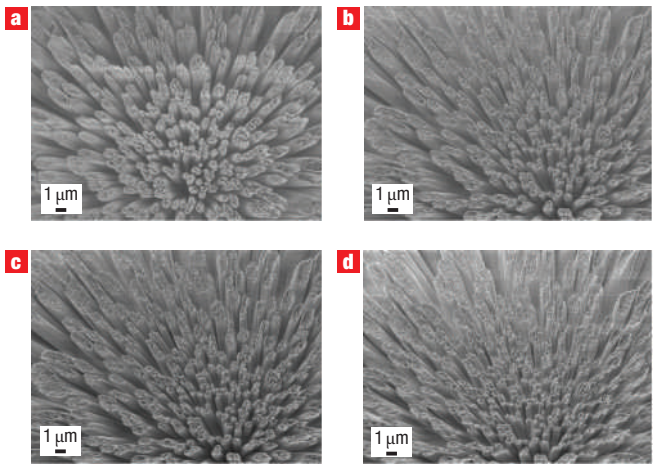


Figure 2 Cold field-emission gun high-resolution scanning electron microscope (CFEG-HRSEM) analysis with various tilting angles of the diphenylalanine-based peptide nanotubes array assembled on a siliconized glass. a–d, Micrographs taken for the same area in the sample while the angle of the sample was continuously changed. Tilting angles of 10° (**a**), 20° (**b**), 30° (**c**) and 40° (**d**) were viewed.

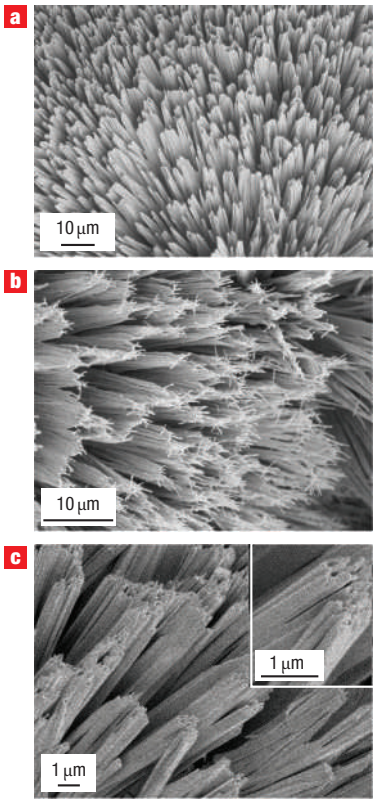
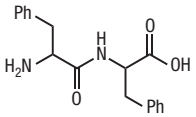
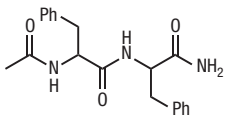
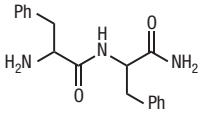
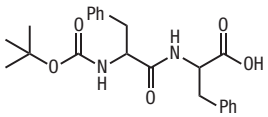
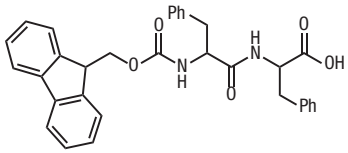
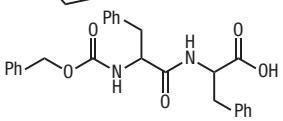


Figure 3 A positively charged diphenylalanine peptide analogue can self-assemble on siliconized glass in the same manner as the diphenylalanine peptide. a, SEM micrograph of the very small diameter assemblies aligned on siliconized glass. **b**, Higher SEM magnification of the assembled tubular structures. **c**, CFEG-HRSEM analysis of the self-assembled tubular structures; the inset represents higher magnification.

Table 1 The chemical structure of the diphenylalanine peptide analogues used in this study. It should be noted that at neutral pH, these compounds exist in a range of charge states. The parent diphenylalanine (NH_2–Phe–Phe–COOH) is zwitterionic and bears a positively charged ammonium group and a negatively charged carboxylate group. The *N*-acetylated (Ac=acetyl) compound (Ac–Phe–Phe–NH_2) is neutral and carries no charge. The amide derivative of diphenylalanine (NH_2–Phe–Phe–NH_2) carries a single positive charge, whereas the final three entries each carry a single negative charge, but differ in the nature of the group attached to the *N*-terminus of the dipeptide, namely *t*-butoxycarbonyl (Boc), fluorenylmethoxycarbonyl (Fmoc) or benzyloxycarbonyl (Cbz). The phenyl (Ph) group is defined in Fig. 1a.

Peptide Name	Molecular Structure
NH_2–Phe–Phe–COOH	
Ac–Phe–Phe–NH_2	
NH_2–Phe–Phe–NH_2	
Boc–Phe–Phe–COOH	
Fmoc–Phe–Phe–COOH	
Cbz–Phe–Phe–COOH	

surface. The higher limit probably results from the uncontrolled growth of the monomers along multiple axes (see Supplementary Information, Fig. S2f). The assembly of the peptide monomers into highly ordered nanostructures when evaporation is allowed, as reflected by the diffraction experiment, may be the result of a preferred crystallization process. The concentration of the building blocks upon evaporation results in the transition of the solution into the labile phase. In the case of aggregation into amorphous clusters, no phase transition and phase separation are observed. These findings further support a nucleation-driven mechanism of formation[28].

To resolve the molecular basis for this vertical self-organization, we monitored the self-assembly of various diphenylalanine analogues (Table 1). These analogues were previously shown to be able to form nano-assemblies by self-association in aqueous solution[29], yet they differ from the diphenylalanine parent compound in their electrostatic charge state. It should be noted that the native peptide is zwitterionic under neutral conditions with a positively charged amine group and a negatively charged carboxyl group. SEM analysis of these analogues revealed that charge effects this self-assembly

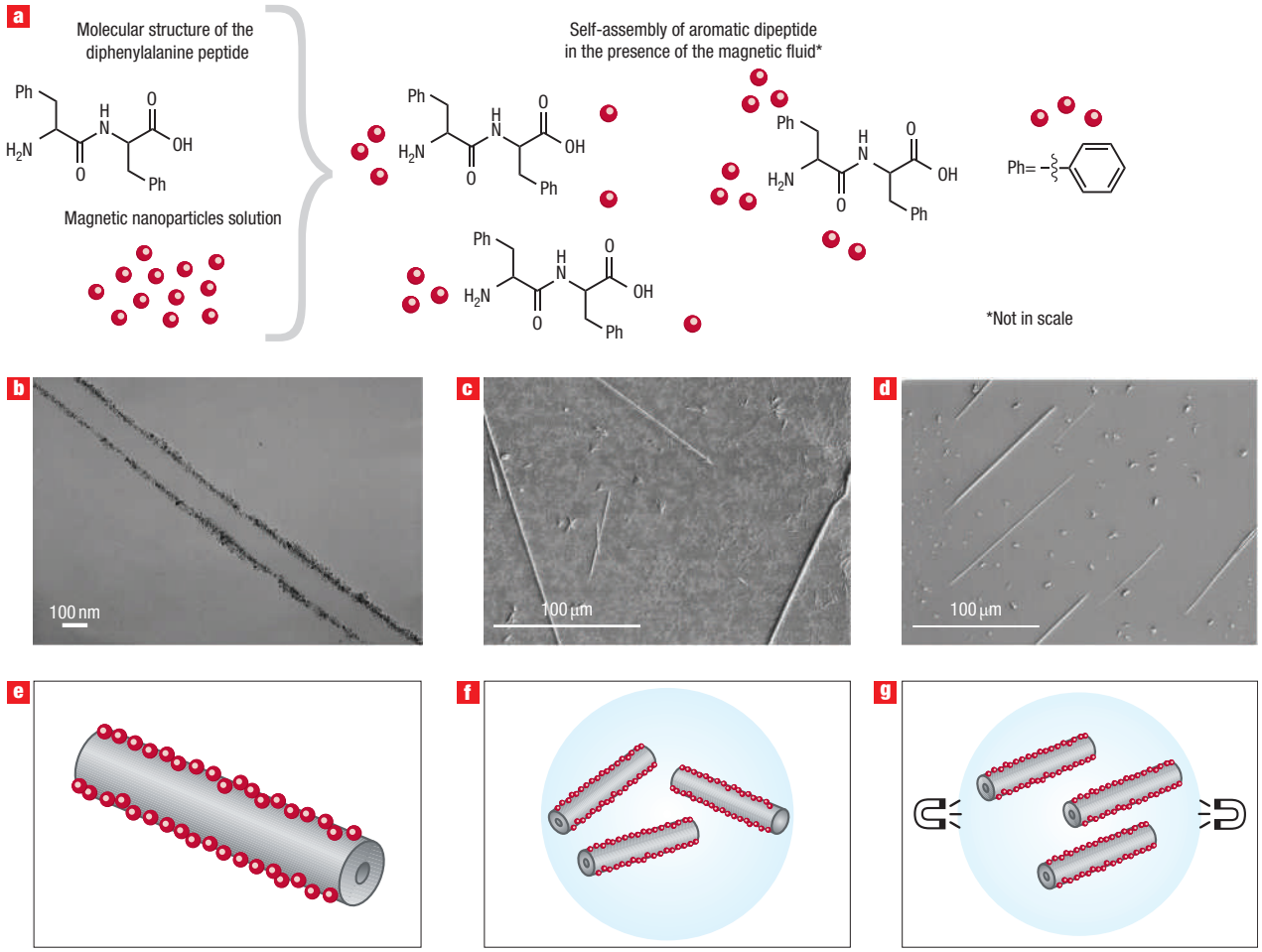


Figure 4 The self-assembly of the diphenylalanine-based peptide nanotubes in the presence of a ferrofluid and their exposure to an external magnetic field resulted in control over their horizontal alignment. a, Schematic representation of the dipeptide monomers self-assembled in the presence of a ferrofluid solution containing magnetite nanoparticles of about 5 nm in diameter. **b**, TEM image of a self-assembled peptide tube coated with magnetic particles. **c**, Low-magnification SEM micrograph of the self-assembled magnetic tubes. **d**, Horizontal arrangement of the self-assembled magnetic tubes after their exposure to a magnetic field, observed by low-magnification SEM. **e**, Schematic representation of the self-assembled magnetic tubes. **f**,**g**, Schematic representations of the magnetic tubes randomly oriented before exposure to the magnetic field (**f**) and horizontally aligned on exposure to the magnetic field (**g**).

process—a non-charged analogue, Ac–Phe–Phe–NH_2, formed non-oriented tubular structures on the surface (Fig. S3), and a positively charged peptide, NH_2–Phe–Phe–NH_2, formed well-aligned structures (Fig. 3).

A *t*-butyl carbamate–Phe–Phe–COOH (Boc–Phe–Phe–COOH) peptide was used to explore the role of negative charge in this process. This peptide did not form an aligned tubular structure. This was also the case with other negatively charged peptide analogues that were modified with larger moieties. Moreover, a cyclic analogue, which has no net charge, assembled into well-ordered tubular structures with a random orientation on the surface (see Supplementary Information, Fig. S3). We suggest that this random assembly on the surface results from the lack of repulsive forces needed to facilitate the tubes' orientation. In the case of the negatively charged peptides, although tubular structures have previously been formed in solution[29], in this case, interactions with the surface seem to prohibit assembly.

In order to further explore the role of charge in this process, the assembly of the diphenylalanine peptide on a surface in its deprotonated state was studied by the addition of a base, *N*,*N*-diisopropylethylamine (DIAE), to the dissolved peptide monomers. In the presence of 0.5% DIAE, aligned tubular structures were formed; however, when the DIAE concentration was increased, the order and directionality of the tubes was affected and a random orientation was observed (see Supplementary Information, Fig. S4). Moreover, when applied to a positively charged surface, the diphenylalanine peptide did not self-assemble into an aligned array. These experimental results further suggest that extremely ordered but discrete peptide arrays are being stabilized by repulsive electrostatic interactions between the self-assembled nanostructures.

For many nanotechnological applications, a horizontal alignment of the nano-objects is desired. For that purpose another line of research was directed towards the horizontal alignment of the tubes using a ferrofluid and an external magnetic field. The use of a magnetic field to align carbon nanotubes and inorganic nanoparticles has been widely studied[30]. However, in the case of carbon nanotubes, introducing magnetic properties represents a major challenge.

Here we used a simple technique for making the ADNT ferromagnetic by assembling the diphenylalanine monomers in the presence of magnetite nanoparticles (Fig. 4a). While the ADNTs assembled into tubular structures, the magnetic nanoparticles formed a non-covalent coating layer of magnetic nanoparticles (Fig. 4b). As this coating also occurs around the walls of the non-charged nanotubes, we believe that the magnetite particles adhere to the walls by hydrophobic interactions. The presence of the magnetic particles did not affect the high yield of tube formation. In addition, the high efficiency of this process could be observed by TEM analysis, which revealed that all tubular structures were coated with magnetic particles. These magnetic-coated ADNTs spread randomly when applied onto a surface (Fig. 4c). SEM analysis revealed that upon exposure to an external magnetic field, all tubes responded to the field. This simple and rapid process resulted in the spatial organization of the tubes onto a surface and their alignment according to the direction of the magnetic field (Fig. 4d). This form of orientation may be very useful for various nanofluidics and nanoelectromechanical systems (NEMS).

In summary, we have demonstrated the axial unidirectional growth of ADNTs into a dense ordered array. This is the first reported case of the formation of a vertically aligned nanoforest from a self-assembled peptide nanostructure. We also established the spatial horizontal organization of the tubes using a magnetic field. The controlled alignment of these highly ordered self-assembled peptide nanotubes, which have unique chemical and mechanical stability, along with the ability to decorate them with functional groups, may enable their integration into multi-array sensitive sensors, field-emission settings, nano(electro)mechanic and nanofluidic devices.

METHODS

MATERIALS

The diphenylalanine and diphenylalanine peptide analogues were purchased from Bachem. Fresh solutions of the diphenylalanine peptide and analogues were prepared by dissolving the lyophilized form of the peptides in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma). To avoid any pre-aggregation, fresh stock solutions were prepared for each experiment.

VERTICAL ALIGNMENT OF THE DIPHENYLALANINE PEPTIDE NANOTUBES

After dissolving the peptide in HFP to a final concentration of 100 mg ml^{-1}, 30 μl of the solution was placed on 22-mm-diameter siliconized glass (Hampton Research).

HORIZONTAL ALIGNMENT OF THE DIPHENYLALANINE PEPTIDE NANOTUBES

A fresh stock solution of the diphenylalanine peptide was dissolved in HFP to a concentration of 100 mg ml^{-1}. The stock solution was then diluted to a final concentration of 2 mg ml^{-1} in 20% ferrofluid diluted in water (EMG 508, Ferrotec Corporation). This commercially available water-based ferrofluid carries 1.07% magnetite (Fe_3O_4) particles with a characteristic diameter of 10 nm in the presence of a stabilized anionic surfactant. After dilution in water, the final concentration of the magnetite nanoparticles was 0.214%. After overnight incubation, which is required for nanotube self-assembly, a 10-μl drop of the solution was deposited on a cover slip slide. In the next step, the slide was placed between two permanent magnets that produce a constant field of 0.5 T taken from a Hall Measurement System (Bridge Technology). The sample was exposed to the external magnetic force until the solution was dried; this was followed by SEM analysis of the sample as described above. For TEM analysis a 10-μl aliquot of the peptide solution was placed on a 200-mesh copper grid, covered by carbon-stabilized Formvar film. After 1 min, excess fluid was removed and the grid and samples were viewed using a JEOL 1200EX TEM operating at 80 kV.

SCANNING ELECTRON MICROSCOPY

Samples were coated with gold and analysed using a JSM JEOL 6300 SEM operating at 5 kV. For analysis by cold field-emission gun (CFEG) high-resolution scanning electron microscopy (HRSEM), the samples were coated with Cr and viewed using a JSM-6700 field-emission SEM equipped with a CFEG operating at 1 kV.

ELECTRON DIFFRACTION

Electron diffraction experiments were performed on an FEI Tecnai F20 microscope FEI at 200 kV with a field-emission gun. Low-dose methods were used; searching was performed at extremely low doses and low magnification. Electron diffraction patterns were recorded directly to the camera: a TVIPS F415 camera with 4,000 × 4,000 pixels, with a 500-ms exposure time.

X-RAY DIFFRACTION

The peptide array was formed on siliconized glass as described above. The deposited peptide array was subjected to X-ray diffraction (XRD) analysis. XRD measurements were made using a Ttrax theta-theta diffractometer (Rigaku) with copper anode, parallel beam optics and generator power of 12 kW.

Received 1 September 2006; accepted 31 October 2006; published 5 December 2006.

References

1. Zhong, Z. H., Wang, D. L., Cui, Y., Bockrath, M. W. & Lieber, C. M. Nanowire crossbar arrays as address decoders for integrated nanosystems. *Science* **302,** 1377–1379 (2003).
2. Modi, A., Koratkar, N., Lass, E., Wei, B. Q. & Ajayan, P. M. Miniaturized gas ionization sensors using carbon nanotubes. *Nature* **424,** 171–174 (2003).
3. Patolsky, F. *et al.* Electrical detection of single viruses. *Proc. Natl Acad. Sci. USA* **101,** 14017–14022 (2004).
4. Whitesides, G. M., Mathias, J. P. & Seto, C. T. Molecular self-assembly and nanochemistry: A chemical strategy for the synthesis of nanostructures. *Science* **254,** 1312–1319 (1991).
5. Sarikaya, M., Tamerler, C., Jen, A. K., Schulten, K. & Baneyx, F. Molecular biomimetics: Nanotechnology through biology. *Nature Mater.* **2,** 577–585 (2003).
6. Ghadiri, M. R., Granja, J. R., Milligan, R. A., McRee, D. E. & Hazanovich, N. Self-assembling organic nanotubes based on a cyclic peptide architecture *Nature* **366,** 324–327 (1993).
7. Banerjee, I. A., Yu, L. & Matsui, H. Cu nanocrystal growth on peptide nanotubes by biomineralization: size control of Cu nanocrystals by tuning peptide conformation. *Proc. Natl Acad. Sci. USA* **100,** 14678–14682 (2003).
8. Hartgerink, J. D., Beniash, E. & Stupp. S. I. Self-assembly and mineralization of peptide-amphiphile nanofibers. *Science* **294,** 1684–1688 (2001).
9. Aggeli, A. *et al.* Responsive gels formed by the spontaneous self-assembly of peptides into polymeric beta-sheet tapes. *Nature* **386,** 259–262 (1997).
10. Mao, C. *et al.* Viral assembly of oriented quantum dot nanowires. *Science* **303,** 213–217 (2004).
11. Vauthey, S., Santoso, S., Gong, H. Y., Watson, N. & Zhang, S. Molecular self-assembly of surfactant-like peptides to form nanotubes and nanovesicles. *Proc. Natl Acad. Sci. USA* **99,** 5355–5360 (2002).
12. Zhao, X. & Zhang, S. Fabrication of molecular materials using peptide construction motifs. *Trends Biotechnol.* **22,** 470–476 (2004).
13. Hamada, D., Yanagihara, I. & Tsumoto, K. Engineering amyloidogenicity towards the development of nanofibrillar materials. *Trends Biotechnol.* **22,** 93–97 (2004).
14. Reches, M. & Gazit, E. Casting metal nanowires within discrete self-assembled peptide nanotubes. *Science* **300,** 625–627 (2003).
15. Adler-Abramovich, L. *et al.* Thermal and chemical stability of diphenylalanine peptide nanotubes: implications for nanotechnological applications. *Langmuir* **22,** 1313–1320 (2006).
16. Kol, N. *et al.* Self-assembled peptide nanotubes are uniquely rigid bioinspired supramolecular structures. *Nano Lett.* **5,** 1343–1346 (2005).
17. Yemini, M., Reches, M., Rishpon, J. & Gazit, E. Novel electrochemical biosensing platform using self-assembled peptide nanotubes. *Nano Lett.* **5,** 183–186 (2005).
18. Li, W. Z. *et al.* Large-scale synthesis of aligned carbon nanotubes. *Science* **274,** 1701–1703 (1996).
19. Terrones, M. *et al.* Controlled production of aligned-nanotube bundles. *Nature* **388,** 52–55 (1997).
20. Ren, Z. F. *et al.* Synthesis of large arrays of well-aligned carbon nanotubes on glass. *Science* **282,** 1105–1107 (1998).
21. Melosh, N. A. *et al.* Ultrahigh-density nanowire lattices and circuits. *Science* **300,** 112–115 (2003).
22. Wang, Z. L & Song, J. Piezoelectric nanogenerators based on zinc oxide nanowire arrays. *Science* **312,** 242–246 (2006).
23. Huang, Y., Duan, X. F., Wei, Q. Q. & Lieber, C. M. Directed assembly of one-dimensional nanostructures into functional networks. *Science* **291,** 630–633 (2001).
24. Thurn-Albrecht, T. *et al.* Ultrahigh-density nanowire arrays grown in self-assembled diblock copolymer templates. *Science* **290,** 2126–2129 (2000).
25. Song, Y. J. *et al.* Synthesis of peptide-nanotube platinum-nanoparticle composites. *Chem. Commun.* 1044–1045 (2004).
26. Gorbitz, C. H. Nanotube formation by hydrophobic dipeptides. *Chem. Eur. J.* **7,** 5153–5159 (2001).
27. Gorbitz, C. H. The structure of nanotubes formed by diphenylalanine, the core recognition motif of Alzheimer's beta-amyloid polypeptide. *Chem. Commun.* 2332–2334 (2006).
28. Asherie, N. Protein crystallization and phase diagrams. *Methods* **34,** 266–272.
29. Reches, M. & Gazit, E. Self-assembly of peptide nanotubes and amyloid-like structures by charged-termini-capped diphenylalanine peptide analogues. *Israel J. Chem.* **45,** 363–371 (2005).
30. Banerjee, I. A. *et al.* Magnetic nanotube fabrication by using bacterial magnetic nanocrystals. *Adv. Mater.* **17,** 1128–1131 (2005).

Acknowledgements

The authors acknowledge support from the Israel Science Foundation (the F.I.R.S.T program). We thank members of the Gazit Laboratory for helpful discussions. M.R. gratefully acknowledges the support of the Clore Foundation Scholars Programme and the Dan David Scholarship Award. The authors would like to thank E. Wachtel for the XRD analysis, S. Wolf for the electron diffraction analysis, and M. Pauzner for graphical assistance.
Correspondence and requests for materials should be addressed to E.G.
Supplementary information accompanies this paper on www.nature.com/naturenanotechnology.

Author contributions

M.R and E.G designed the experimental setup, analysed the data and co-wrote this manuscript. M.R performed the experiments.

Competing financial interests

The authors declare that they have no competing financial interests.