Synthesis and Physicochemical Properties of Lipophilic Polyamide Dendrimers

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Purpose. To synthesise symmetrical dendritic macromolecules with external lipid surfaces, to investigate their behaviour at the air-water interface and their ability to form supramolecular aggregates, and to gain an understanding of their potential as drug carriers.

Methods. Dendrimeric compounds were synthesised with molecular weights ranging from 737 (1st generation dendrimer) to 25,246 (6th generation dendrimer) with carbon numbers ranging from 40 to 1404. The surface behaviour of these compounds was determined using spread films at the air/water interface on a Langmuir trough, and transmission electron microscopy was used to study the supramolecular aggregates formed by the more hydrophobic members of the series.

Results. Dendrimers up to a maximum of 6 generations were synthesised. Surface saturation did not allow the completion of the synthesis of the 7th generation. The limiting surface areas at the air/water interface ranged from 0.4 nm^2 to 16.1 nm^2 values in good agreement with the areas derived from computer generated molecular models (0.5 nm^2 to 14 nm^2).

Conclusions. The synthesised dendrimers exhibited a linear relationship between area per molecule and the molecular weight of the compounds. A dendrimer with 16 lipoamino acid branches formed tubular supramolecular aggregates with a helical structure and dimensions in the long axis of 140–200 nm.

KEY WORDS: dendrimers; lipidic polyamides; drug carriers; surface area.

INTRODUCTION

Increasing interest has been shown in the molecular architecture of dendritic macromolecules (1–4). The three dimensional globular structures of dendrimers are characterised by a large number of branches emanating from a single core molecule or focal point, with a branch at each monomer unit. The synthetic strategies available for the preparation of dendritic macromolecules allow not only the terminal functional groups, but also the internal building blocks and the functionality at the focal point, to be varied. Dendrimers can be envisaged as unimolecular encapsulants, or so-called "molecular boxes". They have potential uses as drug delivery vehicles, molecular ball bearings, flow regulators in fluids, and highly mono-dispersed "dwarf latexes" for coatings (5). Peptide dendrimers with branched structures have been found to be useful in developing vaccines (6), diagnostic products and artificial enzymes. Lipid modified peptide dendrimeric adjuvants are used to increase the immunogenicity of synthetic peptides (7,8). Different peptide dendrimer systems are also under investigation for drug, peptide and gene delivery in our laboratories (9). Roy and coworkers (10) used the solid-phase approach to prepare inhibitors of influenza A virus haemagglutinins.

Two fundamentally different approaches have been developed for the stepwise synthesis of dendritic macromolecules (11). In the *divergent approach,* (12,13) growth is initiated at a polyfunctional core molecule and occurs by a stepwise series of coupling/activation steps involving a monomer capable of at least one branch per repeat unit. The *convergent growth approach* (14,15) is the opposite of the divergent approach: growth begins at what will become the chain ends of the final molecule and, by the use of protected monomer units and stepwise series of coupling/activation steps, larger and larger dendritic fragments are produced. The final reaction is attachment to a polyfunctional core molecule.

We have adopted the divergent approach for the synthesis of lipophilic peptide dendrimers using solid support (16). Lipoamino acids combine structural features of lipids with those of aminoacids (17) and are highly lipophilic due to their long alkyl side chains, while possessing polar and conformational behaviour characteristic of amino acids and peptides.

We describe in this paper the synthesis of dendritic macromolecules with lipidic surfaces. Their surface behaviour at the air-water interface has been determined using a Langmuir trough, and we have investigated the ability of some of the compounds in the series to form supramolecular aggregates. The surface behaviour of the dendrimers afforded some insight into their cross-sectional areas, and assisted in the understanding of how the molecules form the supramolecular assemblies. These characteristics were studied as a prelude to the further exploration of these lipophilic dendrimers in drug delivery.

MATERIALS AND METHODS

4-Methyl benzhydrylamine (MBHA) resin, protected aminoacids from Novabiochem, UK, 2-(1H benzotriazole-1-yl)-1,3,3-tetramethyluronium hexafluoro phosphate (HBTU) from Phase Separations Ltd, UK, trifluoroacetic acid (TFA) from Halocarbon Products Corporation, USA, hydrogen fluoride gas (HF) from BOC, UK, diisopropyl ethyl amine (DIEA) from Fluka, Switzerland and dimethylformaamide (DMF) from Rathburn, UK were all used as received. The protected lipidic aminoacids were synthesised and purified in our laboratory as described elsewhere (17).

Synthesis

The peptide dendrimers with lipophilic surfaces were synthesised employing a solid phase procedure (18) on a MBHA resin (1g, substitution 0.46 mmol/g resin). The synthesis of the first and each subsequent level of the peptide construct was achieved using a three fold excess of HBTU activated Boc-aminoacids in DMF. In all couplings the efficiency was greater than 99% as indicated by a quantitative ninhydrin test. Deprotection of the *N*-termini was performed in 100% TFA. The resin peptide was carefully flow washed before and after the deprotection. After the completion of synthesis the terminal

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Table I. Structural and Mass Spectral Data for the Lipidic Dendrimers

Compound	Molecular formula	Yield (%)	Molecular weight	MS m/e (%)
1	$C_{40}H_{76}O_6N_6$	85	736.59	759(20)(M+Na), 652(18),568(100),386(24)
2	$C_{84}H_{154}O_{12}N_{12}$	78	1523.18	1549(25)(M+Na), 1360(63), 1191(100), 794(53)
3	$C_{172}H_{322}O_{24}N_{24}$	74	3114.52	3138(31)(M+Na), 2942(100), 2773(62), 2378(64), 2210(66), 1594(60)
4	$C_{348}H_{650}O_{48}N_{48}$	74	6270.98	6294(77)(M+Na), 5873(100), 3240(62), 3062(75), 1560(45), 1058(66)
5	$C_{700}H_{1306}O_{96}N_{96}$	48	12596.03	12600(59), 11783(75), 9142(53), 7473(51), 6288(100)
6	$C_{1404}H_{2618}O_{192}N_{192}$	38	25246.09	25269(33)(M+Na), 24432(36), 23138(42), 21861(45), 17098(46), 11531(83), 8214(93)
7	$C_{284}H_{522}O_{48}N_{48}$	72	5373.98	5396(M+Na)(28), 5374(67), 5039(100), 4574(87), 2720(25)
8	$C_{412}H_{778}O_{48}N_{48}$	60	7167.99	7191(M+Na)(70), 6993(74), 5693(50), 3206(52),2018(83)

amino groups were acetylated with acetic anhydride in the presence of diisopropylethylamine. Finally, the resin was washed with dichloromethane and dried in air. The peptide was removed from the resin support with a high HF method (2 g resin peptide, 20 ml HF, 1.5 h at −5°C) to yield the crude peptide which was dissolved in 95% acetic acid solution and lyophilised.

Purification of Dendrimers

Analytical HPLC separation of the synthesised dendrimers was carried out on a 25 cm Vydac C_{18} 5 RAC column with 5 μm pore size and 4.6 mm internal diameter. Following standard degassing techniques, particulate matter was removed from HPLC grade acetonitrile and water using membrane filters.

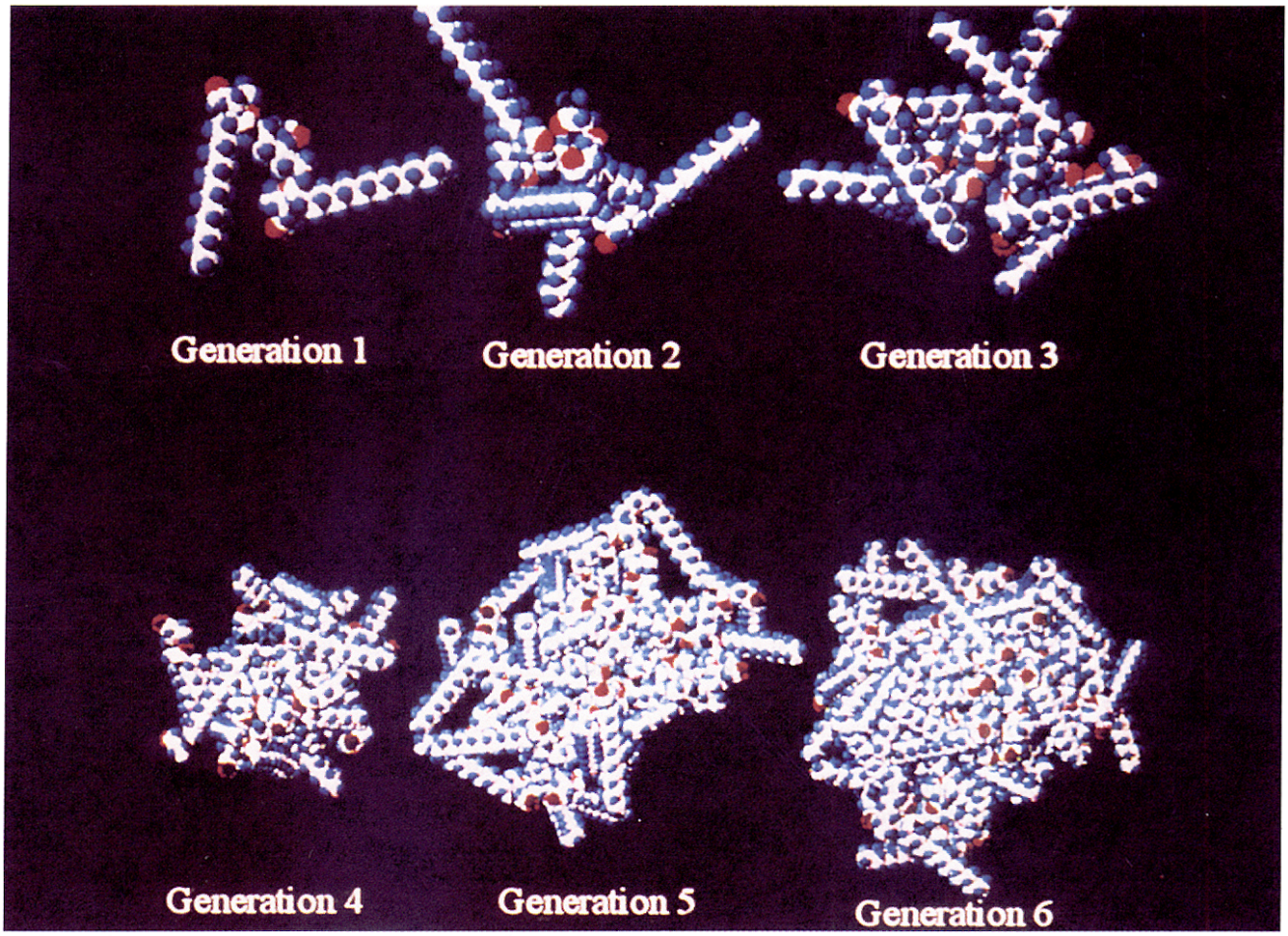

Fig. 1. Molecular models of the compound 1–6.

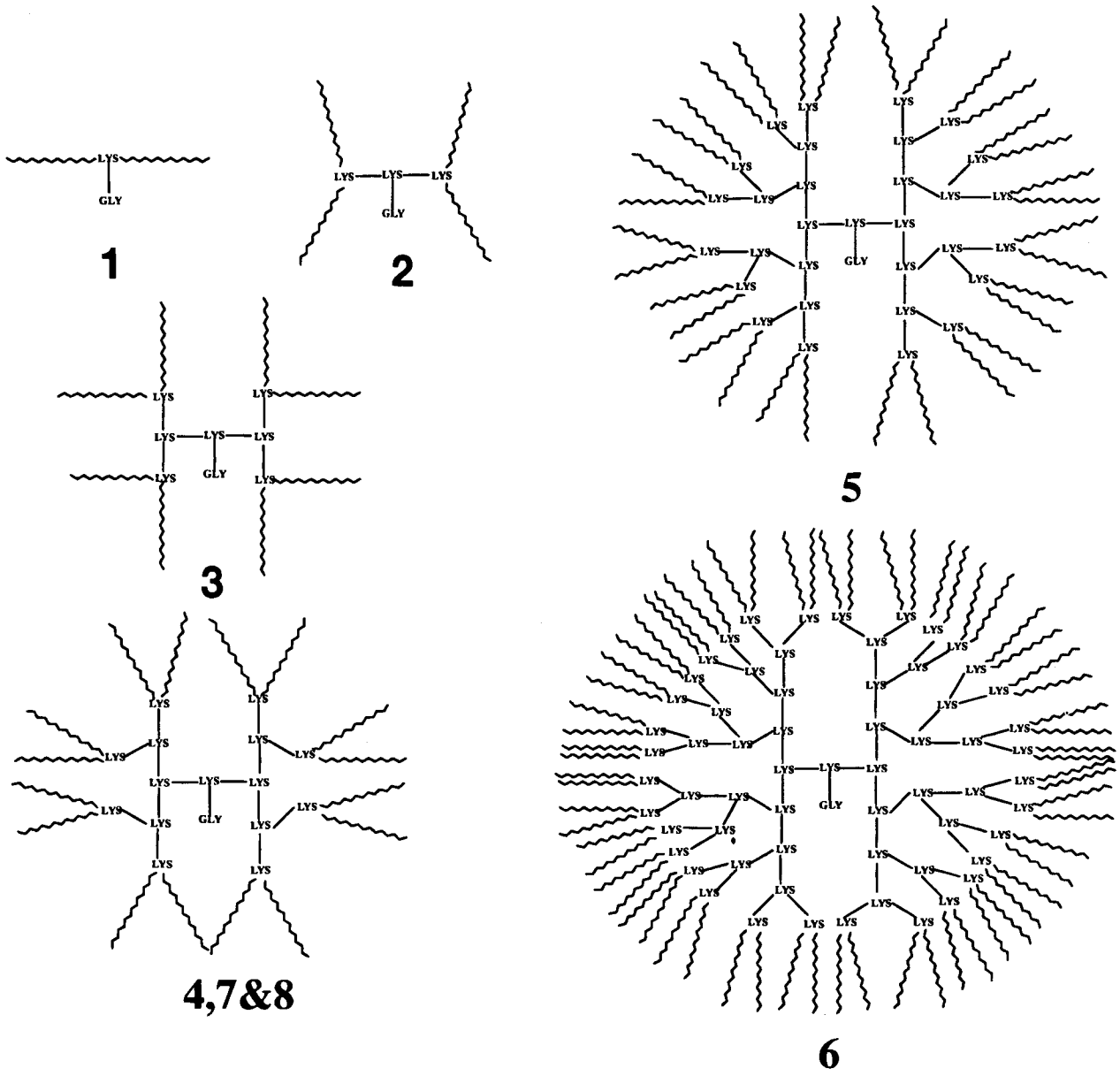

Fig. 2. Schematic representation of the compounds synthesised.

Analytical separation was achieved with a solvent gradient beginning with 0% acetonitrile, increasing to 60% acetonitrile at 20 min, maintaining this concentration for 20 min and decreasing steadily to 0% acetonitrile for 10 min at a constant flow of 1.2 ml min^{-1}. For preparative separation a TSK-GEL preparative C_{18} column with 10 μm pore size and 2.5 cm internal diameter was used. Separation was achieved with a solvent gradient beginning with 0% acetonitrile, increasing constantly to 18% acetonitrile at 60 min then 60% acetonitrile at 80 min, staying at this concentration for a further 30 min and decreasing steadily to 0% acetonitrile for 30 min at a constant flow of 8 ml min^{-1}. The gradient was effected by two microprocessor-controlled Gilson 302 single piston pumps. Compounds were detected with a Waters 486 tunable absorbance detector at 214 nm or a Holochrome UV-VIS detector 220 nm and collected as diastereomeric mixtures. Mass spectra were run on VG Analytical Tofspec instrument, using matrix assisted laser desorption (MALD) ionisation at a wavelength of 337 nm generated by a nitrogen laser. The characteristics of the synthesised lipophilic peptide dendrimers are summarised in Table I.

Surface Pressure Studies

Monolayers of the dendrimers were spread from solutions in chloroform (50 μl) on purified water, swept to remove adsorbed impurities, allowing 10 min for solvent evaporation before commencing compression at a rate of 100 cm^2/min. Surface pressure (II) versus molecular area (A) isotherms were recorded at 25°C using an automated Langmuir film balance

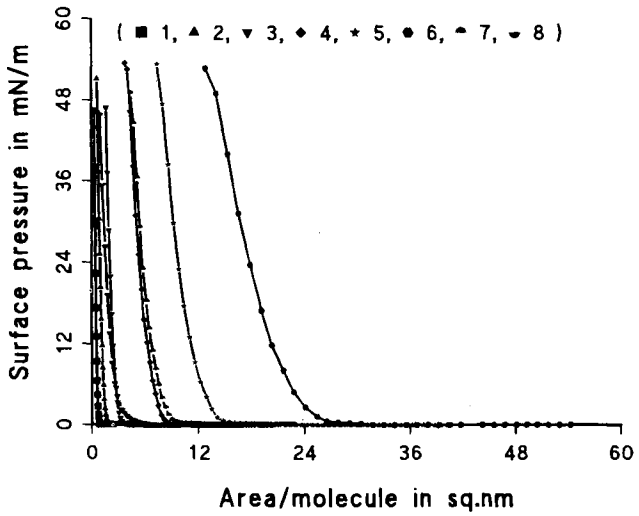

Fig. 3. Surface pressure-isotherms of lipidic peptide dendrimers Conditions: Dendrimers spread on water as solutions in chloroform (50 μl) allowing 10 min for solvent evaporation before commencing compression at a rate of 100 cm^2/min at 25°C.

(Nima Technology, Coventry, UK) equipped with two barriers for symmetric compression, a pressor sensor and a filter paper Wilhelmy plate capable of an accuracy of measurement of 0.1 mN/m. Limiting areas/molecule were obtained from the II-A isotherms by extrapolating II surface pressure plots to zero. The surface pressure measurements were replicated and the results were reproducible, with the experimental error not exceeding ±2%.

Formation of Supramolecular Aggregates

To study the potential of these dendritic molecules to aggregate into higher order structures, solutions (1 mg ml^{-1}) of the compounds in chloroform were prepared. A thin film of the compound was formed in a round bottomed flask by vacuum drying under an atmosphere of nitrogen. A small amount of water was added to hydrate the films and the suspension stirred at 37°C for 24 h. The samples were then examined by transmission electron microscopy (Philips 201).

RESULTS AND DISCUSSION

The MS data for compounds 1–8 and the molecular models of compounds 1–6 are given in Table I and Fig. 1 respectively. The molecules range from C_{40} to C_{1404} and their molecular weights from 737 (1st generation) to 25,246 (6th generation). A schematic representation of the synthesised compounds are shown in Fig. 2.

The II-A isotherms obtained for monolayers of the compounds are shown in Fig. 3. The characteristics of the dendrimers, derived from these experiments, are given in Table II. A typical rise in surface pressure for the compounds was recorded during compression at a molecular area between 0.802 nm^2 and 30.38 nm^2. On further compression the surface pressure increased without evidence of transition. The low compressibility (dII/dA) of the films was a sign that the complex dendrimer molecules were packed tightly and that the cohesive forces between them were considerable. All the compounds gave characteristic isotherms with a high collapse pressure, between 46.4 mN m^{-1} and 53.42 mN m^{-1}.

The area per molecule of the compounds ranged between 0.41 nm^2 and 16.1 nm^2. The molecular area increased, as anticipated, with the increasing generations of the dendrimer family. A reasonably linear correlation between the logarithmic area/molecule in nm^2 and the logarthmic molecular weight of dendrimer generations was observed.

The projected area of the compounds were calculated by arranging the hydrophilic moieties in a spherical form and measuring the average diameter using the Sybyl 6.2 program (Tripos UK Ltd, UK). The correlation coefficient of area per molecule observed by surface pressure measurements and projected area was found to be 0.96.

Compounds 7 and 8 were synthesised to determine the effect on the area per molecule of the length of the alkyl chain attached to the dendrimers, Compound 7, which contained shorter chains of 8 carbon atoms had a low apparent area per molecule of 1.2 nm^2 possibly due to its ability to dissolve in the subphase during compression. Compound 8, which contained 16 carbon chains, was poorly soluble and its films stable at the air/water interface producing a limiting area per molecule of 5.2 nm^2 (Fig. 3).

Saville *et al.* (19) found that polyether dendrimers could form surface multilayers. Their compounds had surfactant like properties up to the third generation, the fourth generation marking the end of such behaviour. Our lipophilic peptide dendrimers were insufficiently soluble in water to show conventional surfactant properties but the behaviour of the compounds at the air/water interface was clearly dependent on the length of the lipophilic chain attached to the core molecule.

Bo *et al.* (20) synthesised a new kind of amphiphilic polyether dendrimer bearing eight alkyl chains and found that these dendrimers could also form stable monolayers at air-water interface, but with lower collapse pressures.

Formation of Supramolecular Aggregates

The hydrophobic effect is widely seen as a vital driving force in self-organisation processes in nature. Because dendrimers are amphiphilic, in water there is the possibility for them to self assemble into micelles or bilayers. The structure and stability of the aggregate is dependent on both internal conditions such as the critical packing parameter, i.e. the relative size of the hydrophilic to hydrophobic portions, external conditions such as concentration and temperature (21).

The low generation dendrimers (compounds 1–3) did not form supramolecular aggregates visible by TEM. Only the 4th generation dendrimer (4) was found to form supramolecular aggregates spontaneously. Probably due to their overwhelming lipophilic nature and geometry, dendrimers of a higher generation were not found to form vesicles or other aggregates. When filmed, the highly branched dendrimers (compounds 5 and 6) could not be hydrated, so did not form supramolecular aggregates. The structures formed from (4) as seen in Fig. 4. had a length of about 140 nm and a thickness of about 40 nm. Apart from the helical pattern adopted by individual structures, some appear as bundles of three to four aggregates (Fig. 5).

Fig. 6 speculates on the manner in which dendrimer (4) forms cylindrical tubule in aqueous systems. All lipophilic chains of the dendrimer units (A) may associate to form tubules

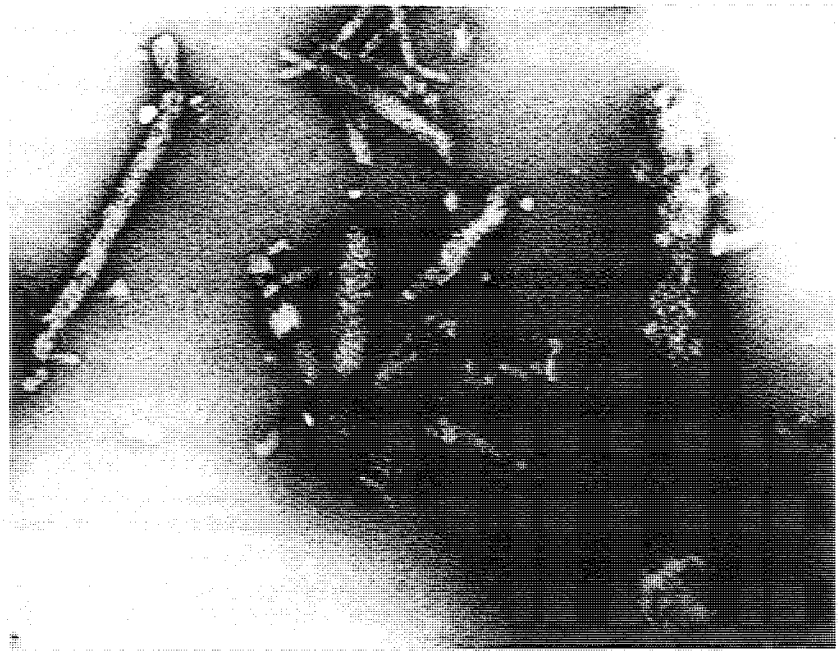

Fig. 4. Supramolecular structures formed with compound 4 (1 mm = 9.5 nm). Conditions: A thin film of the compound was formed in a round bottomed flask by vacuum drying under an atmosphere of nitrogen. A small amount of water was added to hydrate the films and the suppression stirred at 37°C for 24 hrs.

(B) with a cross section as shown (C). It is possible that due to hydrogen bonding interactions these tubules may associate to form bundles (D).

Newkome *et al.* (22) have not only confirmed the stacked aggregation of an arborol alkyne gel but also revealed a unique helical morphology presumably promoted by the increased core rigidity and electron repulsion of the juxtaposed alkynes. The large diameters of the twisted rods (~60 nm) demonstrated in the electron micrographs probably resulted from the packing of individual rods into grooves of adjacent helical rods, or aggregates, thereby producing "supercoil" or "molecular rope" nanostuctures. In the case of our lipidic peptide dendrimers the "twisted" morphology seen in Fig. 7 might have similar origins.

Recently Hudson *et al.* (23) outlined the mechanisms by which flat tapered and conical monodendrons of polyether dendrimers generate cylindrical and spherical supramolecular dendrimers and subsequent self-organisation. This opens new strategies for design at the molecular level.

The fate of the dendrimers series *in vivo* is being studied after administration by a variety of routes. The diversity of monomer and aggregate structures possible in these series implies that there are numerous opportunities for the fabrication of delivery systems with predetermined dimensions and properties.

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Table II. Characteristics of the Lipidic Peptide Dendrimers

Compound	Generation number	Number of branches and lipidic ends	Length of the alkyl chain attached	Number of lysine residues	Maximum surface pressure achieved ($mN\ m^{-1}$)	Area per molecule (nm^2)[a]	Projected area by the simulated computer model (nm^2)
1	1	2	12	1	46.4	0.41	0.53
2	2	4	12	3	51.1	0.76	1.01
3	3	8	12	7	46.7	1.85	1.92
4	4	16	12	15	53.4	4.71	4.63
5	5	32	12	31	53.3	8.82	6.48
6	6	64	12	63	52.6	16.1	13.97
7	4	16	8	15	46.0	1.21	4.63
8	4	16	16	15	48.2	5.17	4.63

[a] Measured at $\Pi = 36\ mNm^{-1}$.

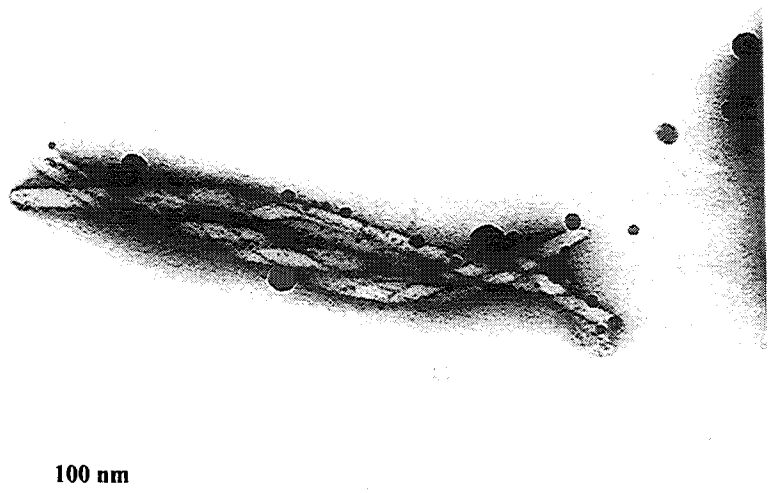

Fig. 5. A bundle of vesicles coiling among themselves of the compound 4 (1 mm = 14 nm). Conditions: A thin film of the compound was formed in a round bottomed flask by vacuum drying under an atmosphere of nitrogen. A small amount of water was added to hydrate the films and the suspension stirred at 37°C for 24 hrs.

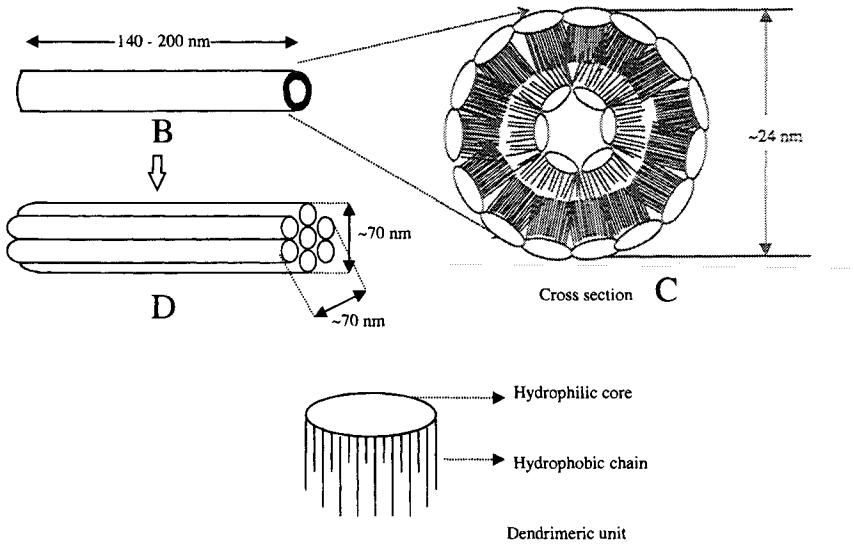

Fig. 6. Possible modes of association of lipophilic dendrimer in aqueous dispersions. A: a typical tubule with the dimensions of 140–200 nm and ~24 nm thickness measured from Fig. 6. with cross section. B: Shown enlarged, with the possible association to bundles.

REFERENCES

1. D. A. Tomalia, A. M. Naylor, and W. A. Goddard. Starburst dendrimers: molecular level control of size, shape, surface chemistry, topology and flexibility from atoms to macroscopic matter. *Angew. Chem. Int. Ed. Engl.* **29**:138–175 (1990).
2. G. R. Newkome, A. Nayak, R. Behera., C. N. Moorefield, and G. R. Baker. Chemistry of micelle series. 22. Cascade polymers-synthesis and characterization of 4-directional spherical dendritic macromolecules based on adamantane. *J. Org. Chem.* **57**:358–362 (1992)
3. R. G. Denkalwalter, J. Kolc, and W.J. Lukasavage. Macromolecular highly branched homogeneous compound based on lysine units. *U. S. Patent.* 4 289 872, Sept 15,1981.
4. J. Issberner, R. Moors, and F. Vogtle. Dendrimers: from generations and functional groups to functions. *Angew. Chem,. Int. Ed. Engl.* **33**:2413–2420 (1994).
5. P. R. Dvornic and D. A. Tomalia. Molecules that grow like trees, *Science Spectra.* **5**:36–41 (1996).
6. J. Shao and J.P. Tam. Unprotected peptides as building blocks for the synthesis of peptide dendrimers with oxime, hydrazone and thiazolidine linkages. *J. Amer. Chem. Soc.* **117**: 3893–3899 (1995).
7. I. Toth. A novel approach to Drug Delivery. Lipidic Amino acid conjugates. *Journal of Drug Targeting* **2**:217–239 (1994).
8. I. Toth, M. Danton, N. Flinn, and W. A. Gibbons. A combined adjuvant and carrier system for enhancing synthetic peptides immunogenicity utilizing lipidic amino acids. *Tetrahedron Lett.* **34**:3925–3928 (1993).
9. I. Toth, T. Sakthivel, A. Wilderspin, and A. T. Florence. a) Dendrimers, Eu. Pat. Appl. No. 97304060.3 (1997). b) Cationic dendrimers for gene delivery (paper in preparation).
10. R. Roy, D. Zanini, S. J. Meunier, and A. Romanowska. Solid-phase synthesis of dendritic sialoside inhibitors of influenza A virus hemagglutinin. *J. Chem. Soc. Chem. Commun.* 1869 (1993).
11. C. J. Hawker, K. L. Wooley, and J. M. J. Frechet. Unimolecular micelles and globular amphiphiles: dendritic macromolecules as novel recyclable solubilization agents.*J. Chem. Soc. Perkin. Trans. I.* 1287–1297 (1993).
12. A. Morikawa, M. Kakimoto, and Y. Imai. Synthesis and characterization of new polysiloxane starburst polymers. *Macromolecules,* **24**:3469–3474 (1991).
13. J. Zhang, J. S. Moore, Z. Wu, and R. A. Aguirre. Nanoarchitectures. I. Controlled synthesis of phenylacetylene sequences. *J. Amer. Chem. Soc.* **114**:2273 (1992).
14. T. M. Miller, T. X. Neenan, R. Zayas, and H. E. Blair. Synthesis and characterization of a series of monodisperse, 1, 3, 5, phenylene-based hydrocarbon dendrimers including $C_{276}H_{186}$ and their fluorinated analogs. *J. Amer. Chem. Soc.* **114**:1018–1025 (1992).
15. A. Morikawa, M. Kakimoto, and Y. Imai. Convergent synthesis of siloxane starburst dendrons and dendrimers via hydrosilylation. *Macromolecules.* **25**:3247–3253 (1992).
16. K. E. Uhrich, S. Boegeman, J. M. J. Fréchet, and S. R. Turner. The solid-phase synthesis of dendritic polyamides. *Polymer Bull.* **25**:551–558 (1991).
17. W. A. Gibbons, R. A. Hughes, M. Charalambous, M. Christodoulou, A. Szeto, A. E. Aulabaugh, P. Mascagni, and I. Toth. Synthesis, resolution and structural of lipidic amino acids and their homo- and hetero-Oligomers. *Liebigs. Ann. Chem.* 1177–1183 (1990).
18. R. B. Merrifield. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Amer. Chem. Soc.* **85**:2149–2154 (1963).
19. E. P. M. Saville, P. A. Reynolds, J. W. White, C. J. Hawker, J. M. J. Fréchet, K. L. Wooley, J. Penfold, and P. Webster. Jr. Neutron reflectivity and structure of polyether dendrimers as Langmuir films. *J. Phys. Chem.* **99**:8283–8289 (1995).
20. Z. Bo, X. Zhang, X. Yi M. Yang, J. Shen, Y. Rehn, and S. Xi. The synthesis of dendrimers bearing alkyl chains and their behaviour at air-water interface *Polymer Bulletin* **38**:257–264 (1997).
21. F. Zeng and S. C. Zimmerman. Dendrimers in Supramolecular Chemistry: From Molecular Recognition to Self-Assembly. *Chem. Rev.* **97**:1681–1712 (1997).
22. G. R. Newkome, C. N. Moorefield, G. R. Baker, R. K. Behera, G. H. Escamillia, and M. J .Saunders. Supramolecular self-assemblies of two directional cascade molecules: Automorphogenesis. *Angew. Chem. Int. Ed. Engl.* **31**:917–919 (1992).
23. S. D. Hudson, H. T. Jung, V. Percec, W. D. Cho, G. Johansson, G. Ungar, and V. S. K. Balagurusamy. Direct Visualization of Individual Cylindrical and Spherical Supramolecular Dendrimers. *Science* **278**:449–452 (1997).