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Biochimica et Biophysica Acta 1371 (1998) 168–184

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Formation of high axial ratio microstructures from peptides modified with glutamic acid dialkyl amides

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Received 29 September 1997; accepted 7 October 1997

Abstract

A growing number of amphiphiles are known to form high axial ratio microstructures (HARMs) such as the hollow cylindrical microstructures called lipid tubules. As a prelude to exploring the potential of HARMs formed from lipopeptides in controlled release drug delivery, several microstructure formation conditions were investigated. We report the preparation of several glutamic acid dialkyl amides with varying alkyl chain lengths bearing a variety of peptides (1–4 amino acids) [peptide-Glu-$(NHC_nH_{2n+1})_2$, $n = 12, 14, 16$]. These surfactants have been rapidly and efficiently converted into HARMs in aqueous buffer at physiological pH and ionic strength, or in buffer containing MeOH or EtOH. Helical ribbons and tubular HARMs were produced that were stable for as long as 6 months below the phase transition temperatures of the compounds. To estimate the stability of HARMs in vivo, HARMs formed from $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ were incubated with DOPC liposomes or fetal calf serum at 40°C. HARM size and shape did not change significantly, suggesting that such lipopeptide particles can retain their morphology long enough in vivo to be useful as drug delivery vehicles.

Keywords: Lipopeptide; Self-organizing lipid microstructure; Chiral lipid; Tubule; Drug delivery; Bilayer

Abbreviations: AcOH: acetic acid; ACN: acetonitrile; BuOH: butanol; Boc: *t*-butoxycarbonyl; $(Boc)_2O$: di-*tert*-butyl dicarbonate; *Z*: carbobenzoxy; COSY: correlation spectroscopy; DCC: dicyclohexylcarbodiimide; DCU: dicyclohexylurea; DEPC: diethyl phosphorocyanidate; DIEA: *N,N*-diisopropylethylamine; DSC: differential scanning calorimetry; EDC: 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride; DOPC: dioleoylphosphatidylcholine; EtOH: ethanol; FCS: fetal calf serum; HBS: 20 mM HEPES, 120 mM NaCl, 1 mM EDTA, 0.2% NaN_3 pH 7.4; HOBT: hydroxybenzotriazole; OSu: *N*-hydroxysuccinimidyl; MeOH: methanol; $DC_{8,9}PC$: 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine; Sar: sarcosine; TEA: triethylamine; TFA: trifluoroacetic acid

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0005-2736/98/$19.00
PII S0005-2736(97)00267-8

1. Introduction

Liposomes are the most common form of self-assembled microstructure that two-chain amphiphiles adopt when dispersed in aqueous media. In some cases, however, the molecular packing of amphiphiles produces bilayers that self-assemble into particles with strikingly different geometries. Among the most unusual of these lipid particles are high axial ratio microstructures (HARMs) formed from ribbons with differing degrees of helicity or rolled-up bilayers. For example, the polymerizable phospholipid, 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}PC$), forms cylindrical structures called tubules. In this case, the structure appears to be a helical ribbon, often with fused edges [1–5].

In addition to $DC_{8,9}PC$, several other amphiphiles have been found to form HARMs. Among them are derivatives of glutamic acid containing alkyl amines amide linked to its α- and γ-carboxyl groups and bearing poly-glutamate or poly-aspartate [6,7] or short peptides [8] attached to its α-amino group. HARMs were formed from the *N*-acyl and the *N*-α-hydroxy fatty acyl fractions of galactocerebrosides [9]. Synthetic and semi-synthetic sphingolipids can also be made to efficiently convert to HARMs [10]. Some lipid mixtures containing phosphatidylserine convert from liposomes to another HARM, the cochleate cylinder, in the presence of Ca^{2+} [11–13]. Tubules and helices have also been found in aged suspensions of saturated-chain phosphatidylcholines and as transient intermediates in the crystallization of cholesterol from mixed micellar suspensions [14,15].

A few attempts have been made to explore the pharmaceutical potential of HARMs. It has been suggested that the hollow lumen of $DC_{8,9}PC$ tubules could be used for controlled release of biological response modifiers such as growth factors and cytokines to facilitate regeneration of wounded soft tissue [16,17]. Encapsulation of transforming growth factor-β into $DC_{8,9}PC$ tubules (with or without subsequent embedding of the tubules in agarose hydrogel) provides first-order release of the growth factor over 10 h at 37°C with preservation of biological activity [16,17]. Johnson et al. [18] have investigated aerosol delivery of $DC_{8,9}PC$ tubules using a Collison nebulizer, demonstrating that upon nebulization the particles undergo only minor fragmentation. Significant success has been achieved in development of vaccines based on cochleate cylinders formed from phosphatidylserine-containing lipid mixtures precipitated by Ca^{2+}. It has been shown that complexes of such cochleate cylinders with peptides and/or proteins effectively stimulate antibody production and cell-mediated immune responses in the absence of additional adjuvants, particularly when administered orally [12,13]. Apparently peptides and proteins immobilized in this way are protected from proteolysis in the stomach.

Based on their potential as pharmacologically active molecules, we are studying the ability of lipopeptides to form HARMs [10]. Many peptides affect biological functions at very low concentrations [19]. Because of this, peptides can be very effective drugs, but practical pharmacological application of peptides has been hampered by their very short half-life in vivo, which is mostly due to filtration by the kidneys and/or proteolysis [19]. Because of their fast clearance, and premature degradation, it is very difficult to maintain peptides at concentrations within their therapeutic windows. The appropriate hydrophobic modification of biologically active peptides could cause them to self-assemble to form HARMs, which will serve as a drug depot. The lipopeptides might be biologically active if their hydrophobic moieties do not interfere with binding of the peptides to their sites of action. Alternatively, the lipopeptides might function as prodrugs that become active after their hydrophobic moieties are cleaved.

The large dimensions of the HARMs make them ideally suited for retention of drug at intramuscular, subcutaneous, intraperitoneal, topical, and intralesional sites. The tight molecular packing on the surface of the structure might provide protection of the biologically active peptides against premature proteolysis. The high curvature at the HARM edges suggests much looser molecular packing in these regions. If such bilayer edges are in contact with bulk solvent, these regions are the most likely sites of dissolution and enzymatic attack. We have shown that the tubule morphology can lead to a nearly constant rate of enzymatic hydrolysis of the surfactant [20].

As a first step in the exploration of the pharmaceutical potential of HARMs formed from lipopeptides, we tested several approaches to quick and simple formation of HARMs designed to be thermally stable

under physiological conditions. We have used several peptides bearing glutamic acid dialkyl amides [$Glu(NHC_nH_{2n+1})_2$] as model compounds. Although the short peptide moieties of the amphiphiles studied have no known biological activity, these compounds should have physical properties close to those of biologically active lipopeptides. We have chosen glutamic acid dialkyl amides as a hydrophobic anchor because of its known property to self-assemble into HARMs when coupled to a number of short peptides [6–8]. We have synthesized some of these previously reported lipopeptides as well as similar surfactants with novel peptide head groups and varying lengths of hydrocarbon chain. Since HARMs form only at temperatures below the phase transition temperature (T_m), we have determined T_m values of the lipopeptides using differential scanning calorimetry (DSC). Shimizu and Hato [8] have shown that sonicated dispersions of some peptides lipidated with glutamic acid dialkyl amides are capable of forming HARMs spontaneously in pure water through ''... gradual change from a very small particle to another specific morphology over a period of hours or days...''. One of our primary goals was to obtain similar particles in a much shorter time. To this end, we applied methods that form HARMs from one of the most studied tubule forming compounds, the phospholipid $DC_{8,9}PC$. These methods include thermal cycling of lipopeptide dispersions in aqueous media [1,21,22] or in MeOH (EtOH)/aqueous media mixtures [23].

To insure biological compatibility of the resulting suspensions, we used an aqueous medium at physiological pH and ionic strength in all experiments. To determine if HARMs have the potential for pharmacologically useful lifetimes for continuous release in vivo, HARMs formed from one of the compounds were challenged by exposure to solutions containing potential acceptors for lipid molecules. The challenges included incubation in the presence of dioleoylphosphatidylcholine (DOPC) liposomes, which were introduced as model cell membranes, and in the presence of fetal calf serum, modeling extracellular fluids.

2. Materials and methods

All chemicals and solvents from commercial sources were of reagent grade. All reactions were carried out under argon. Amino acids used were of the L-configuration. Dioleoylphosphatidylcholine was obtained from Avanti Polar Lipids (Birmingham, AL). Crude peptides, Boc-Gly-Lys-(ε-*Z*)-Sar-Pro and Boc-Lys-(ε-*Z*)-Ala-Sar-Pro, were custom made by Anaspec (San Jose, CA) and were purified by silica gel chromatography. TLC was run on silica gel 60 F254 plates (EM Science), and spots were visualized with Cl_2/*o*-tolidine and/or ninhydrin, and/or I_2 vapor. Flash chromatography was performed using silica gel 60 (230–400 mesh, EM Science). The structures of intermediate compounds were confirmed by ^1H NMR (Brucker 200, 300, or 500 MHz). Mass spectroscopy (ES–MS) was performed using a Kratos Profile HV-4 instrument with an electrospray ionization source, and samples were mixed with MeOH:water (1:1) containing 1% acetic acid.

2.1. Synthesis

Several lipopeptides were synthesized, and their chemical structures are shown in Fig. 1. Compounds were synthesized according to the scheme in Fig. 2 using either DEPC, EDC, or DCC as the coupling reagent.

2.1.1. $HCl \cdot (Pro)_3$–$Glu(NHC_{14}H_{29})_2$

A solution of Boc–Pro–Pro (29.31 mg, 93.82 mmol) in CH_2Cl_2 (0.4 ml) was mixed with $HCl \cdot$ Pro–$Glu(NHC_{14}H_{29})_2$ (60.0 mg, 89.4 mmol) dissolved in DMF:$CHCl_3$ (1.0 ml:1.6 ml), and the mixture was cooled to 0°C with stirring. DEPC (16.0 μg, 98.3 μmol) in DMF (0.3 ml) and TEA (79.4 μl, 196.6 μmol) in DMF (0.3 ml) were added. The mixture was stirred at room temperature for 24 h before diluting with $CHCl_3$ (50 ml) and was washed successively with saturated NH_4Cl (3 × 10 ml), H_2O

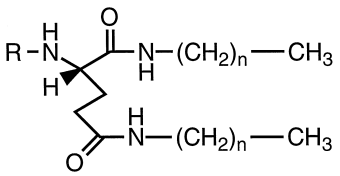

Fig. 1. Schematic representation of lipopeptide compounds synthesized. R = Pro, $(Pro)_3$, Gly-Lys-Sar-Pro, and Ac-Lys-Ala-Sar-Pro. The value of *n* was 11, except in the case of the $(Pro)_3$ R headgroup, in which case *n* was 11, 13, or 15.

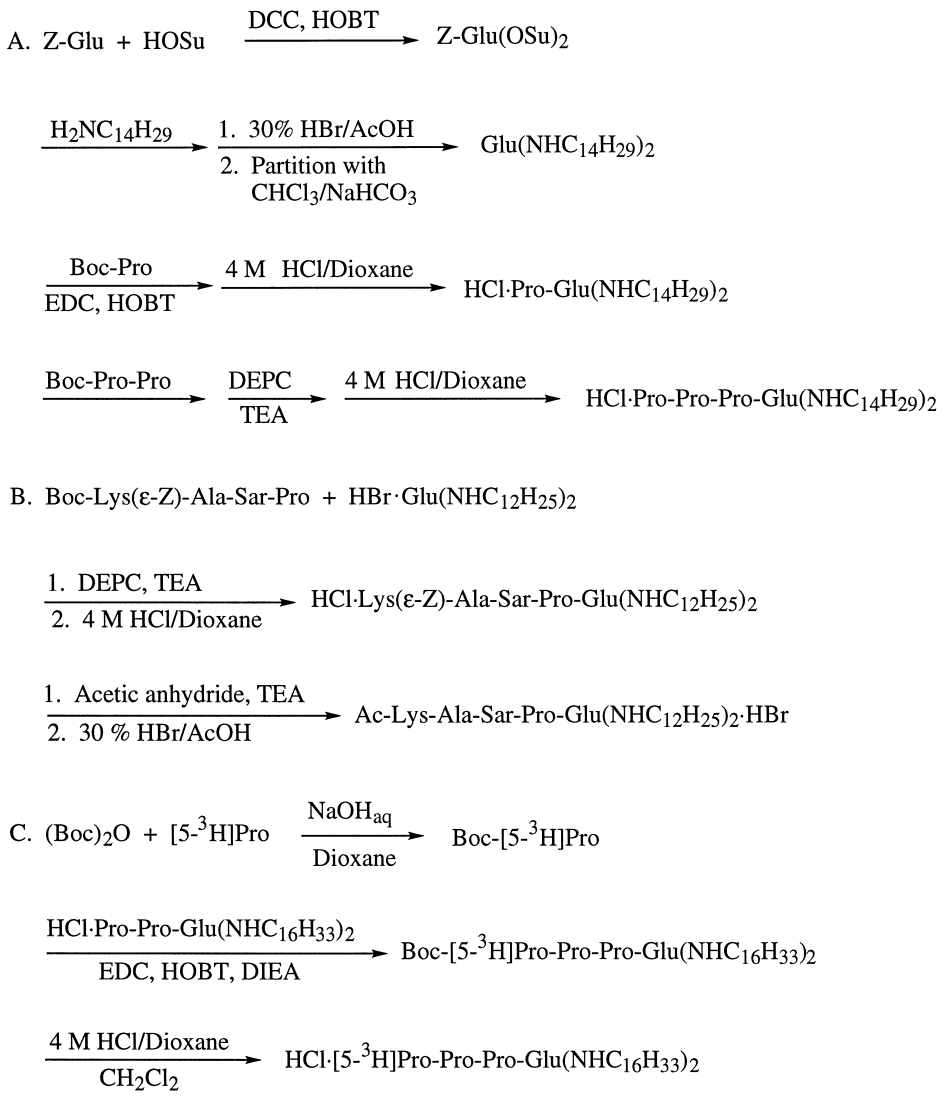

Fig. 2. The three synthetic schemes employed. $HCl \cdot (Pro)_3–Glu(NHC_{12}H_{25})_2$ and $HCl \cdot (Pro)_3–Glu(NHC_{16}H_{33})_2$ were prepared using scheme A.

(1 × 10 ml), saturated $NaHCO_3$ (3 × 10 ml), H_2O (1 × 10 ml), brine (1 × 10 ml), and dried over Na_2SO_4. After filtration, the solvent was evaporated in vacuo, and the residue was purified by flash chromatography on silica gel with $CHCl_3$:MeOH (97.5:2.5) to give the product in 83% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.49. The product (60.0 mg, 89.4 μmol) was deprotected in CH_2Cl_2 (1.5 ml) with 4 M HCl/dioxane (2 ml) at room temperature for 2 h, concentrated, dried in vacuo, and lyophilized from ACN/H_2O (1:3) to give $HCl \cdot (Pro)_3–Glu(NHC_{14}H_{29})_2$ in 100% yield. TLC (4:1:1 butanol:acetic acid:H_2O): R_f 0.43. ES–MS: $[M + H]^+$ 830.0, calcd. 830.3, $[M + 2H]^{+2}$ 415.5, calcd. 415.5.

2.1.2. $HCl \cdot Pro–Glu(NHC_{14}H_{29})_2$

To a solution of Boc–Pro (44.0 mg, 204.5 μmol) in $CHCl_3$ (2 ml), $Glu(NHC_{14}H_{29})_2$ (100.0 mg, 185.9 μmol) and HOBT (39.2 mg, 204.5 μmol) were added, and the mixture was cooled to 0°C with stirring. After the addition of EDC (39.2 mg, 204.5 μmol) and DIEA (35.6 μl, 204.5 μmol), the stirring was continued for 2 h at 0°C, and then at room

temperature overnight. The mixture was diluted with $CHCl_3$ (40 ml) and washed successively with saturated NH_4Cl (2 × 20 ml), H_2O (1 × 20 ml), saturated $NaHCO_3$ (2 × 20 ml), H_2O (1 × 20 ml), and brine (1 × 20 ml). Subsequent to drying over Na_2SO_4, the solvent was evaporated in vacuo, and the residue was purified by flash chromatography with $CHCl_3$:MeOH (97:3) to give 91% yield. TLC (97:3 $CHCl_3$:MeOH): R_f 0.15. Deprotection of the product (104 mg, 141.5 μmol) in CH_2Cl_2 (2.5 ml) was achieved with 4 M HCl/dioxane (2.5 ml) at room temperature for 1.5 h. The mixture was concentrated and dried in vacuo to give HCl · Pro–Glu($NHC_{14}H_{29}$)$_2$ in 100% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.25.

2.1.3. *Glu($NHC_{14}H_{29}$)$_2$*

Tetradecylamine (0.92 g, 4.31 mmol) was combined with a solution of *Z*-Glu(OSu)$_2$ (1.0 g, 2.1 mmol) in $CHCl_3$ (25 ml), and the reaction mixture was stirred at room temperature for 2 days [8]. The mixture was diluted with $CHCl_3$ (100 ml) and washed successively with saturated $NaHCO_3$ (2 × 30 ml), H_2O (1 × 30 ml), 5% HCl (2 × 30 ml), H_2O (1 × 30 ml), and brine (1 × 20 ml). Subsequent to drying over Na_2SO_4, the solvent was evaporated, the residue solidified from MeOH, and further purified by flash chromatography with $CHCl_3$:MeOH (97:3) to give the product in 56% yield. The product was deprotected as described by Shimizu and Hato [8] for the preparation of HBr · Glu($NHC_{12}H_{25}$)$_2$. The deprotected product was then filtered, washed with a small amount of AcOH, suspended in ethyl ether, filtered, and dried in vacuo. Desalting of the product (0.75 g), HBr · Glu($NHC_{14}H_{29}$)$_2$, was achieved by partitioning with $CHCl_3$ (80 ml) and saturated $NaHCO_3$ (3 × 20 ml). The organic layer was washed with H_2O (1 × 20 ml), dried over Na_2SO_4, and the solvent was evaporated to dryness to give Glu($NHC_{14}H_{29}$)$_2$ in 84% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.30. ^{1}H NMR ($CDCl_3$/TMS): δ 7.33 [–$(CH_2)_{11}$–N*H*–CO–], 6.11 (H_2N–), 3.40 (α-CH–), 3.22 (NH–CH_2–), 2.32 (γ-CH_2–), 1.95 (β-CH_2–), 1.50 [–$(CH_2)_{10}$–], 0.88 (–CH_2–CH_3).

2.1.4. *Z-Glu(OSu)$_2$*

Z-Glu (5.0 g, 17.8 mmol) in dry THF (100 ml) was combined with OSu (4.64 g, 40.3 mmol) and the mixture was cooled to 0°C with stirring. DCC (7.69 g, 37.3 mmol) was added, and the stirring was continued for several hours at 0°C, and then at room temperature overnight. DCU was removed by filtration, the solvent evaporated, and the residue was taken up in ethyl acetate and filtered again. The filtrate was washed with $NaHCO_3$, H_2O, brine, and dried over Na_2SO_4. Upon evaporation of the solvent, the residue was solidified from ethyl ether to give *Z*-Glu(OSu)$_2$ in 87% yield. ^{1}H NMR (300 MHz, $CDCl_3$/TMS): δ 7.35 (C_6H_5–), 5.64 (NH), 5.12 (–*C*H_2–C_6H_5), 4.88 (α-CH), 2.80 (γ-CH_2 and –N–CO–CH_2–CH_2–CO–), 2.45 (β-CH), 2.33 (β-CH).

2.1.5. *HCl · (Pro)$_3$–Glu($NHC_{12}H_{25}$)$_2$*

The synthesis of this compound has been reported by Shimizu and Hato [24]. However, we have prepared this compound via different routes of synthesis and purification. A solution of Boc–Pro–Pro (36.3 mg, 116.1 μmol) in DMF (300 μl) was mixed with HCl · Pro–Glu($NHC_{12}H_{25}$)$_2$ (68.0 mg, 110.5 μmol) in DMF (2 ml) at 0°C. DEPC (19.8 μl, 121.6 μmol) in DMF (300 μl) and TEA (33.9 μl, 243.1 μmol) in DMF (300 μl) were added, and stirring continued for 1 h at 0°C, and then at room temperature overnight. The mixture was washed and purified as described for HCl · (Pro)$_3$–Glu($NHC_{14}H_{29}$)$_2$ to give the product in 71% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.49. The protected peptide lipid (68 mg, 77.9 μmol) in CH_2Cl_2 (1.5 ml) was stirred with 4 M HCl/dioxane (2 ml) at room temperature for 1.5 h, concentrated, and dried in vacuo to give HCl · (Pro)$_3$–Glu($NHC_{12}H_{25}$)$_2$ in 100% yield. M.p. 58–60°C. TLC (9:1 $CHCl_3$:MeOH): R_f 0.30. ES–MS: $[M + H]^+$ 773.6, calcd. 774.2, $[M + H]^{+2}$ 387.5, calcd. 387.6.

2.1.6. *HCl · Pro–Glu($NHC_{12}H_{25}$)$_2$*

Boc–Pro (46.89 mg, 0.22 mmol) and HOBT (29.4 mg, 0.22 mmol) were combined with Glu($NHC_{12}H_{25}$)$_2$ (100 mg, 0.21 mmol) in 3 ml $CHCl_3$/CH_2Cl_2 (2:1), and the mixture was cooled to 0°C. EDC (43.8 mg, 0.23 mmol) was added, and the mixture stirred for 1 h at 0°C, and then at room temperature overnight. The solution was washed and purified as described for Boc–Pro–Glu($NHC_{14}H_{29}$)$_2$ to give the product in 80% yield. The protected peptide lipid (111 mg, 0.16 mmol) in CH_2Cl_2 (1 ml) was stirred with 4 M HCl/dioxane (1 ml) at room temperature for 1 h, concentrated, and purified by

flash chromatography with $CHCl_3$:MeOH (9:1) to give HCl · Pro–Glu($NHC_{12}H_{25}$)$_2$ in 82% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.20. ES–MS: $[M + H]^+$: 579.6, calcd. 579.9.

2.1.7. Glu($NHC_{12}H_{25}$)$_2$

This compound was prepared as described for Glu($NHC_{14}H_{29}$)$_2$ to give 69% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.30. ^{1}H NMR ($CDCl_3$/TMS): δ 7.33 [–(CH_2)$_{11}$–N*H*–CO–], 6.12 (H_2N–), 3.42 (α-CH–), 3.23 (NH–C*H*$_2$–), 2.32 (γ-CH_2–), 1.94 (β-CH_2–), 1.50 [–(CH_2)$_{10}$–], 0.89 (–CH_2–CH_3).

2.1.8. HCl · (Pro)$_3$–Glu($NHC_{16}H_{33}$)$_2$

Boc–(Pro)$_3$–Glu($NHC_{16}H_{33}$)$_2$ was prepared as described for Boc–(Pro)$_3$–Glu($NHC_{14}H_{29}$)$_2$ to give 84% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.49. The removal of Boc was achieved as described for HCl · (Pro)$_3$–Glu($NHC_{14}H_{29}$)$_2$ to give HCl · (Pro)$_3$–Glu($NHC_{16}H_{33}$)$_2$ in 99.6% yield. TLC (4:1:1 BuOH:AcOH:H_2O): R_f 0.43. ES–MS: $[M + H]^+$ 885.7, calcd. 885.9, $[M + 2H]^{+2}$ 443.5, calcd. 443.5.

2.1.9. HCl · Pro–Glu($NHC_{16}H_{33}$)$_2$

This compound was prepared as described for HCl · Pro–Glu($NHC_{14}H_{29}$)$_2$ to give 85% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.21.

2.1.10. Glu($NHC_{16}H_{33}$)$_2$

This compound was prepared as described for Glu($NHC_{14}H_{29)2}$ to give 93% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.30. ^{1}H NMR ($CDCl_3$/TMS): δ 7.37 [–(CH_2)$_{11}$–N*H*–CO–], 6.15 (H_2N–), 3.45 (α-CH–), 3.22 (NH–C*H*$_2$–), 2.34 (γ-CH_2–), 1.95 (β-CH_2–), 1.50 [–(CH_2)$_{10}$–], 0.88 (–CH_2–CH_3).

2.1.11. Ac-Lys-Ala-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ · HBr

Acetic anhydride (7.53 μl, 79.78 μmol) and TEA (11.12 μl, 79.78 μmol) were added to the ice-H_2O cooled solution of HCl · Lys-(ε-*Z*)-Ala-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ (65.4 mg, 66.49 μmol) in DMF (2 ml) and stirred for 1.25 h, and then at room temperature overnight. Upon dilution with $CHCl_3$ (20 ml), the mixture was washed as described for HCl · (Pro)$_3$–Glu($NHC_{14}H_{29}$)$_2$ and purified by flash chromatography with $CHCl_3$:MeOH (95:5) to give the product in 97% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.25. ^{1}H NMR (DMSO-d_6/TMS): δ 8.20–7.20 (NH), 7.35 (CH_2–*C*$_6$*H*$_5$–), 5.01 (*C*H_2–C_6H_5), 4.80–3.49 (α-CH), 3.12–2.93, 2.79 (NH–*C*H_2–CH_3, *N*–*C*H_3), 1.84 (CH_3CO–), 1.50–1.20 [–(CH_2)$_{10}$–], 1.20, 1.10 (β-CH_3), 0.86 (–(CH_2)$_{11}$–*C*H_3–). The product (66.0 mg, 64.4 μmol) in $CHCl_3$ (2 ml) was deprotected by treatment with 30% HBr/AcOH (5 ml) at room temperature for 2 h. After the solvent evaporation, the residue was dried in vacuo and lyophilized from MeOH:ACN:H_2O (1:1:2) to give the product in 64.2% yield. TLC (4:1:1 BuOH:AcOH:H_2O): R_f 0.33. M.p.: 130–134°C. ^{1}H NMR (DMSO-d_6/TMS): δ 8.4–7.5 (NH), 4.80–3.75 (α-CH), 3.04–2.97, 2.84–2.72 (–NH–*C*H_2–), 1.84 (CH_3–CO–), 1.36–1.24 [–(*C*H_2)$_{10}$–CH_3], 1.20, 1.10 (β-CH_3), 0.85 [–NH–(CH_2)$_{11}$–*C*H_3)]. ES–MS: $[M + H]^+$ 892.1 calcd. 892.30, $[M + 2H]^{+2}$ 446.1, calcd. 446.65.

2.1.12. HCl · Lys-(ε-Z)-Ala-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$

Boc-Lys-(ε-*Z*)-Ala-Sar-Pro (100.0 mg, 161.3 μmol) was coupled to HBr · Glu($NHC_{12}H_{25}$)$_2$ (82.5 mg, 146.6 μmol) as described for HCl · (Pro)$_3$–Glu($NHC_{14}H_{29}$)$_2$. The product was purified by flash chromatography with $CHCl_3$:MeOH (96:4) to give the product in 58% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.44. ^{1}H NMR (DMSO-d_6/TMS): δ 8.21–6.80 (NH), 7.34 (CH_2–*C*$_6$*H*$_5$), 5.00 (*C*H_2–C_6H_5), 4.80–3.45 (α-CH), 3.10–2.90, 2.79 (NH–*C*H_2-, *N*–*C*H_3), 1.47–1.13 [–(CH_2)$_{10}$–], 1.36 [(CH_3)$_3$C–], 1.19, 1.10 (β-CH_3), 0.85 [–(CH_2)$_{11}$–*C*H_3]. The peptide lipid was deprotected with 4 M HCl/dioxane as described for HCl · (Pro)$_3$–Glu($NHC_{14}H_{29}$)$_2$ and purified by flash chromatography with $CHCl_3$:MeOH (9:1) to give HCl · Lys-(ε-*Z*)-Ala-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ in 81% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.26. ^{1}H NMR (DMSO-d_6/TMS): δ 8.34–7.14 (NH), 7.34 (CH_2–*C*$_6$*H*$_5$), 5.00 (*C*H_2–C_6H_5), 4.80–3.42 (β-CH), 3.12–2.90, 2.78 (NH–*C*H_2–, *N*–*C*H_3), 1.45–1.20 [–(CH_2)$_{10}$–], 1.19, 1.10 (β-CH_3), 0.85 [–(CH_2)$_2$–*C*H_3).

2.1.13. 2HBr · Gly-Lys-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$

Boc-Gly-Lys-(ε-*Z*)-Sar-Pro (70 mg, 115.38 μmol) was coupled to Glu($NHC_{12}H_{25}$)$_2$ (52.9 mg, 109.88 μmol) as described for HCl · (Pro)$_3$–Glu($NHC_{14}H_{29}$)$_2$ to give the product in 96% yield. TLC (9:1 $CHCl_3$:MeOH): R_f 0.36. The product (45 mg, 42.1 μmol) was dissolved in CH_2Cl_2 (1 ml) and deprotected by treatment with 30% HBr/AcOH (9

ml) at room temperature for 2 h. Evaporation of the solvent and lyophilization gave 2HBr · Gly-Lys-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ in 80% yield. TLC (4:1:1 BuOH:AcOH:H_2O): R_f 0.31. ES–MS: $[M+H]^+$ 836.0, calcd. 836.7.

2.1.14. HCl · [5-^{3}H](Pro)$_3$–Glu($NHC_{16}H_{33}$)$_2$

To a solution of HCl · Pro–Pro–Glu($NHC_{16}H_{33}$)$_2$ (48.8 mg, 59.14 μmol) in $CHCl_3$ (3 ml), Boc-[5-^{3}H]Pro (14.0 mg, 65.06 μmol) and HOBT (8.8 mg, 65.06 μmol) were added. Upon cooling the mixture to 0°C, EDC (12.47 mg, 65.06 μmol) and DIEA (21.63 μl, 124.20 μmol) were added, and the mixture was stirred at 0°C for 45 min before allowing to warm up to room temperature overnight. The reaction solution was diluted with $CHCl_3$ (30 ml) and washed with saturated NH_4Cl, H_2O, saturated $NaHCO_3$, H_2O, brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was further purified by flash chromatography with $CHCl_3$:MeOH (97.5:2.5) to give the product in 93% yield. The protected peptide lipid (54.2 mg, 54.9 μmol) was deprotected using 4 M HCl/dioxane (2.5 ml) at room temperature for 2 h, concentrated, and dried in vacuo to give HCl · [5-^{3}H](Pro)$_3$–Glu($NHC_{16}H_{33}$)$_2$ in 100% yield. The product co-migrated with the unlabeled HCl · (Pro)$_3$–Glu($NHC_{16}H_{33}$)$_2$ on TLC plate and is estimated to be 97.5% radiochemically pure by TLC and fluorography. TLC (4:1:1 BuOH:AcOH:H_2O): R_f 0.43.

2.1.15. Boc-[5-^{3}H]Pro

Synthesis of this compound is a modification of the procedure of Tarbell et al. [25]. [5-^{3}H]Pro (1 mCi, specific activity = 15 Ci/mmol) in H_2O (500 μl) was diluted with unlabeled Pro (11.5 mg). Dioxane (1 ml) was added to the mixture followed by 1 N NaOH (100 μl). Upon cooling the stirred mixture to 0°C, $(Boc)_2O$ (24.0 mg, 109.9 μmol) was added, and the mixture was allowed to warm up to room temperature overnight. The solution was diluted with H_2O (40 ml) and washed with hexane (3 × 10 ml). CH_2Cl_2 (10 ml) was added to the aqueous layer and cooled to 0°C. The pH was adjusted to 1–2 with 1 N HCl, and the product was extracted with CH_2Cl_2 (5 × 15 ml). The combined CH_2Cl_2 extracts were washed with H_2O (2 × 15 ml), dried over Na_2SO_4, concentrated under reduced pressure, and dried in vacuo to give Boc-[5-^{3}H]Pro (specific activity = 10 Ci/mole) in 65% yield. The product co-migrated with the unlabeled compound on TLC plate. TLC (9:1 $CHCl_3$:MeOH): R_f 0.25.

2.1.16. HCl · Pro–Pro–Glu($NHC_{16}H_{33}$)$_2$

Boc–Pro–Pro (57.86 mg, 185.2 μmol) was coupled to Glu($NHC_{16}H_{33}$)$_2$ (100.0 mg, 168.3 μmol) as described for HCl · [5-^{3}H](Pro)$_3$–Glu($NHC_{16}H_{33}$)$_2$. The product was purified by flash chromatography with $CHCl_3$:MeOH (97.5:2.5) to give Boc-Pro-Pro-Glu($NHC_{16}H_{33}$)$_2$ in 100% yield. TLC (95:5 $CHCl_3$:MeOH): R_f 0.30. The deprotection of Boc was achieved also as described for HCl · [5-^{3}H](Pro)$_3$–Glu($NHC_{16}H_{33}$)$_2$ to give HCl · Pro–Pro–Glu($NHC_{16}H_{33}$)$_2$ in 79% yield. TLC (4:1:1 BuOH:AcOH:H_2O): R_f 0.42.

2.2. Differential scanning calorimetry

To prepare samples for DSC, 70 μl of 20 mM HEPES, 120 mM NaCl, 1 mM EDTA, 0.2% NaN_3, pH 7.4 (HBS) was added to 1.0–2.0 mg of lipopeptide powders. The pH of the resulting suspensions was adjusted to 7.4 using 0.1 M NaOH. These suspensions were weighed and sealed in 70 μl silver calorimetry pans in aliquots containing 50 mg of HBS. A pan of the same type with 50 mg of HBS only was used as a reference. In the case of Ac-Lys-Ala-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ and (Pro)$_3$–Glu($NHC_{16}H_{33}$)$_2$, additional DSC measurements were made in the presence of 46% EtOH and 30% MeOH respectively. DSC was performed using a Seiko DSC-100 high sensitivity calorimeter (Torrance, CA). Samples were heated from 3°C to 95°C at 1°C/min. DSC of each sample was run twice. Reported T_m values were recorded during the second run. The first run was used to insure proper hydration of the peptide lipids.

To determine the integrity of the compounds after thermal scanning, the DSC pans were opened and their contents dissolved in MeOH to make solutions with final concentrations of the lipopeptides of 0.5 mg/ml. The solutions were analyzed by TLC in BuOH:AcOH:H_2O (4:1:1). TLC revealed no decomposition of the studied compounds after DSC.

2.3. Preparation of HARMs

HARMs were formed either by thermal cycling of dispersions of the compounds in HBS, or by thermal cycling of HBS dispersions in the presence of MeOH or EtOH, or by precipitation upon dilution of concentrated MeOH solutions of the compounds with HBS. When alcohols were used for HARM preparation, the particles were subsequently transferred to pure HBS using centrifugal-driven filtration. To do this, 200 μl aliquots of particle suspensions were centrifuged in Ultrafree®-CL Centrifugal Filters with a 30,000 Da nominal molecular weight limit (Millipore, Bedford, MA) for 15 min at $3000 \times g$ at room temperature, washed with 1 ml of fresh HBS under the same conditions, and reconstituted in the original volume of HBS.

2.3.1. Heating and cooling of aqueous dispersions

Samples of 0.5 mg of either $(Pro)_3$–$Glu(NHC_{14}H_{29})_2$ or $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ in 1 ml of HBS each were sonicated for 15 s at room temperature using a high power bath sonicator (Laboratory Supplies, Hicksville, NY). Dispersions obtained were rapidly heated to 65°C, incubated for 15 min, then cooled to room temperature at 0.2°C/in in an RTE-110P programmable water bath (Neslab Instruments, Newington, NH).

2.3.2. Heating and cooling of HBS dispersions in the presence of MeOH

Samples of either 0.1 mg of $(Pro)_3$–$Glu(NHC_{14}H_{29})_2$ or $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ dissolved in 20 μl of MeOH each were added to 200 μl of HBS while vortexing. Concentrations of MeOH in the samples were adjusted to the values shown in Fig. 4. Sealed samples were heated to 65°C, incubated for 15 min and then cooled to room temperature at 0.2°C/min. In the case of Boc-protected intermediates listed in Table 4, 0.3 mg/ml dispersions of the compounds in 30% MeOH in HBS, obtained as described below, were used. Upon heating to 65°C, all dispersions formed semi-transparent solutions.

2.3.3. Precipitation upon dilution of concentrated MeOH solution

Samples of 0.2 mg of Gly-Lys-Sar-Pro-$Glu(NHC_{12}H_{25})_2$ or $(Pro)_3$–$Glu(NHC_{12}H_{25})_2$ dissolved in 40 μl of MeOH were rapidly added to 400 μl of HBS while vortexing; the mixtures were then incubated for 2 h at room temperature. Aliquots of 0.5 ml of the intermediates (Table 4) dissolved in MeOH in concentrations of 1 mg/ml were added to 1.2 ml of HBS while vortexing, and incubated overnight at room temperature.

2.3.4. Heating and cooling of HBS dispersions in the presence of EtOH

Samples of 0.2 mg of Ac-Lys-Ala-Sar-Pro-$Glu(NHC_{12}H_{25})_2$, Gly-Lys-Sar-Pro-$Glu(NHC_{12}H_{25})_2$, or $(Pro)_3$–$Glu(NHC_{12}H_{25})_2$ were dissolved in 50 μl of EtOH. The minimum fractions of HBS that induced precipitation of the lipopeptides were determined by addition of HBS in 10 μl portions while vortexing, with 5 min incubations after each addition. The critical concentrations of EtOH for each compound are shown in Table 1. Dispersions obtained by HBS precipitation were heated to 50°C, incubated for 15 min, then cooled rapidly to room temperature (50°C to 20°C within 4 min).

2.4. Morphology

The morphology of the precipitates was studied using a Zeiss ICM-405 inverted phase contrast microscope (Carl Zeiss, Thornwood, NY) equipped with a Dage 66 SIT video camera (Dage-MTI, Michigan City, IN). Images obtained were digitized using a Data Translation QuickCapture frame grabber board in a Macintosh II. In addition, the precipitates were studied by transmission electron microscopy (TEM). Samples for TEM were prepared by applying 20 μl aliquots of suspensions to Formvar-coated 150 mesh copper TEM sample grids. No stain was used. The samples were dried in air for at least 24 h before observation with a Philips EM 410 transmission elec-

Table 1
Final concentrations of EtOH at which precipitation occurs upon dilution of 4 mg/ml solutions of the lipopeptides with HBS

Compound	Concentration of EtOH (volume %)
Ac-Lys-Ala-Sar-Pro-$Glu(NHC_{12}H_{25})_2$	46
Gly-Lys-Sar-Pro-$Glu(NHC_{12}H_{25})_2$	42
$(Pro)_3$–$Glu(NHC_{12}H_{25})_2$	36

tron microscope. The outer dimensions of HARMs were measured using NIH Image 1.58 software.

2.5. Light scattering of $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ HARMs as a function of temperature

Light scattering at 300 nm of $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ HARM suspension was measured using an LS-5B fluorescence spectrometer (Perkin-Elmer) equipped with a magnetic stirrer and a thermostated cuvette holder. An aliquot of 0.4 mM $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ HARMs in 30% MeOH in HBS obtained by heating and cooling (see above) was heated from 25° to 75°C in 2–5°C steps in a sealed cuvette. The temperature of the suspension was measured using a thermocouple inserted through a septum in the cuvette cap. The sample temperature was allowed to stabilize for 5–10 min after each step. The data obtained were fit to a sigmoidal (Boltzmann) function.

The same experiment was done with $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ HARMs transferred to pure HBS using an Ultrafree®-CL Centrifugal Filter. In order to estimate the concentration of $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ after MeOH removal, the lipidated peptide was doped with a trace of [5-^{3}H]-$(Pro)_3$–$Glu(NHC_{16}H_{33})_2$. The yield of MeOH free $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$, estimated by scintillation counting, was found to be $62 \pm 0.43\%$ ($n = 3$). This value was used to adjust the concentration of $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ to 0.4 mM.

2.6. Stability of $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ HARMs

A suspension of $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ HARMs formed by heating and cooling of the lipopeptide in HBS/MeOH (see above) was added to either fetal calf serum or sonicated DOPC liposomes in HBS at pH 7.4 at a DOPC concentration of 5 mg/ml at a ratio of 1/10 (v/v). The resulting suspensions were incubated for 45 h at 40°C.

3. Results and discussion

Two lipopeptides, $(Pro)_3$–$Glu(NHC_{12}H_{25})_2$ and Gly-Lys-Sar-Pro-$Glu(NHC_{12}H_{25})_2$, reported by Shimizu and Hato [8] were chosen as 'control lipopeptides' for generating HARMs. The lengths of the alkyl chains were increased by 2 and 4 carbons with the same head group to study the dependence of their hydrocarbon chain melting temperature, T_m, on chain length, as well as to find peptide lipids with convenient melting temperatures for future in vivo studies. Ac-Lys-Ala-Sar-Pro-$Glu(NHC_{12}H_{25})_2$, a variant with a potential tryptic cleavage site in which the α-amino terminus was blocked, was also prepared. The lipopeptide, however, proved to be a very poor substrate for trypsin (data not shown).

Both Sar and Pro provide challenges as structural elements for surfactant headgroups. ^{1}H NMR of 2HBr · Gly-Lys-Sar-Pro-$Glu(NHC_{12}H_{25})_2$ and Ac-Lys-Ala-Sar-Pro-$Glu(NHC_{12}H_{25})_2$ · HBr (as well as the peptides without hydrocarbon chains) exhibited multiple resonance peaks that were further investigated by COSY. The cross peaks indicated that the lipopeptides (and the corresponding peptide) exist in multiple, stable conformational isomers on the NMR time scale. The existence of *cis/trans* isomers of Sar and Pro containing cyclic peptides has been previously documented [26]. The existence of multiple structures due to *cis/trans* isomerization of N-substituted peptide bonds was evident even in such short linear sequences. The exact nature of these isomers and their influence on head group-head group interactions in HARMs such as tubules have not yet been investigated.

3.1. DSC

The value of T_m must be known to develop protocols for forming, storing, and observing HARMs. To determine T_m, DSC of the lipopeptides dispersed in HBS was performed. Each compound gave a single endothermic peak on heating in the range of 3°C to 95°C. The phase transitions are summarized in Table 2. The T_m value for $(Pro)_3$–$Glu(NHC_{12}H_{25})_2$ is close to that previously reported for this compound in pure water at pH 5–6 (31°C, Shimizu and Hato [8]), whereas the value for Gly-Lys-Sar-Pro-$Glu(NHC_{12}H_{25})_2$ is slightly different from the reported value (39°C, Shimizu and Hato [8]). This suggests that the presence of the extra NH_2 group (due to the Lys) makes this class of compounds more sensitive to the environment, e.g., pH and ionic strength, compared to those with only prolines. All

Table 2
Phase transition temperatures as determined by DSC from the endotherm maxima in heating scans

Compound	Solvent	T_m (°C)
$(Pro)_3$–Glu$(NHC_{12}H_{25})_2$	HBS	34.6
$(Pro)_3$–Glu$(NHC_{14}H_{29})_2$	HBS	51.2
$(Pro)_3$–Glu$(NHC_{16}H_{33})_2$	HBS	61.7
$(Pro)_3$–Glu$(NHC_{16}H_{33})_2$	30% MeOH in HBS	59.7
Gly-Lys-Sar-Pro-Glu$(NHC_{12}H_{25})_2$	HBS	50.1
Ac-Lys-Ala-Sar-Pro-Glu$(NHC_{12}H_{25})_2$	HBS	67.6
Ac-Lys-Ala-Sar-Pro-Glu$(NHC_{12}H_{25})_2$	46% EtOH in HBS	29.5

lipopeptides tested except $(Pro)_3$–Glu$(NHC_{12}H_{25})_2$ have T_m values higher than 37°C. $(Pro)_3$ derivatives with longer hydrocarbon chains have the higher T_m values as anticipated. Since all HARMs observed to date consist of lipids below their T_m values, HARMs formed from all but one of the compounds studied here should be thermally stable at physiological temperatures. Note that the DSC experiments were not performed on pre-formed HARMs, so caution should be used in predicting HARM melting temperatures from these data.

3.2. HARM formation

The first approach tested for accelerating lipopeptide HARM formation was heating and cooling of aqueous dispersions of the surfactant through T_m, which has been demonstrated to be effective for formation of $DC_{8,9}PC$ tubules [1,21,22]. In the present study, HBS dispersions of $(Pro)_3$-containing amphiphiles with different hydrocarbon chain lengths

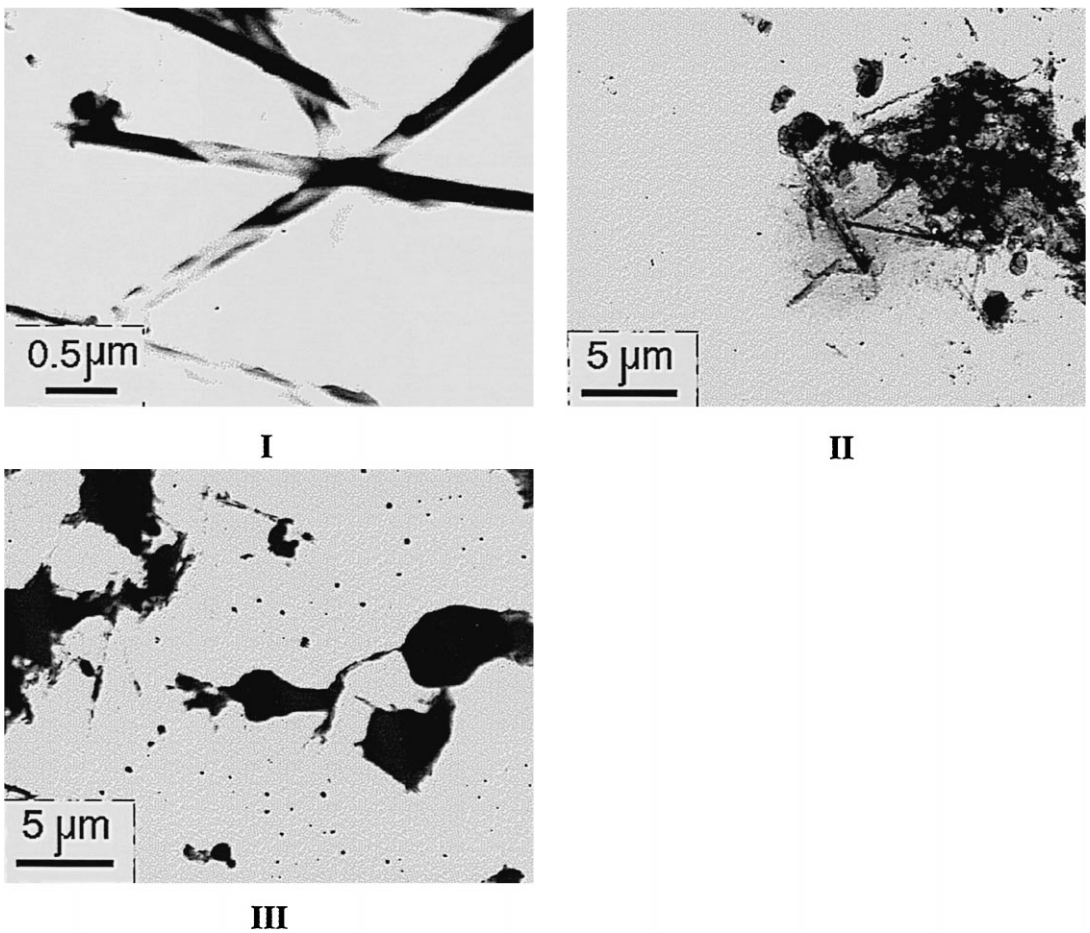

Fig. 3. TEM images of particles formed with $(Pro)_3$–Glu$(NHC_{12}H_{25})_2$ (I), $(Pro)_3$–Glu$(NHC_{14}H_{29})_2$ (II), and $(Pro)_3$–Glu$(NHC_{16}H_{33})_2$ (III) after heating and slow cooling of these lipopeptides in HBS.

were heated to 65°C in HBS and cooled to room temperature at 0.2°C/min. TEM images of the resulting particles are shown in Fig. 3. In this and in all cases below, TEM results were consistent with observations made by optical microscopy. Heating and slow cooling through T_m transforms $(Pro)_3$–$Glu(NHC_{12}H_{25})_2$ into helical ribbons with nearly 100% efficiency. The width of these ribbons is similar to the helical pitch, but fusion of adjacent ribbon edges is observed in rare cases. These HARMs have an average diameter of 0.14 ± 0.05 μm and lengths of 7.0 ± 2.5 μm (Table 3).

The morphology of $(Pro)_3$–$Glu(NHC_{12}H_{25})_2$ HARMs is close to that observed by Shimizu and Hato [8] in sonicated aqueous dispersions of the same material in early stages of incubation. The major difference between the results of Shimizu and Hato and ours is that the HARMs formed by slow cooling in this study did not change their morphology significantly over time. The helical structures formed just after cooling were present after storage for 6 months at room temperature (data not shown). In the study of Shimizu and Hato [8], HARMs continued to convert from helical ribbons to tubules and changed length over time. Slow cooling through T_m, unlike incubation at room temperature, causes self-assembly of $(Pro)_3$–$Glu(NHC_{12}H_{25})_2$ molecules directly into HARMs with stable morphology. The fact that the resulting HARMs are mostly helical ribbons, rather than tubules, may be related to the presence of HBS electrolytes in the formation mixture. Effects of ionic strength on HARM formation have been predicted, although not often observed [27–29].

Slow cooling in HBS resulted in HARM formation by $(Pro)_3$–$Glu(NHC_{14}H_{29})_2$ and $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ as well. However, the conversion efficiencies in these cases were much lower; significant fractions of the material formed amorphous aggregates (Fig. 3). This may be due to more rapid precipitation of these longer chain, less soluble lipopeptides. In an attempt to increase the conversion efficiencies, MeOH was included in the formation medium. $(Pro)_3$–$Glu(NHC_{14}H_{29})_2$ and $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ were dispersed in MeOH/HBS mixtures with varying percentages of MeOH. The influence of MeOH on HARM formation has been studied previously with $DC_{8,9}PC$. It has been shown that cooling of $DC_{8,9}PC$ dispersions in the presence of alcohols can result in more efficient tubule formation than that found with pure aqueous dispersions [23]. The data obtained on $(Pro)_3$–$Glu(NHC_{14}H_{29})_2$ and $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ are consistent with this finding. As shown in Fig. 4, slow cooling of lipopeptides in the presence of 30% or 50% MeOH increases the efficiency of HARM formation to almost 100%. It should be noted that only the particles formed in 30% MeOH are relatively straight and uniform in size. Slow cooling in 50% MeOH induced formation

Table 3
Morphologies and dimensions of HARMs formed by various methods from the peptides modified with glutamic acid dialkyl amide

Compound	Formation method	Morphology	Length[a] (μm)	Diameter[a] (μm)
$(Pro)_3$–$Glu(NHC_{12}H_{25})_2$	Slow cooling of HBS dispersion	Helical ribbons/tubules	7.0 ± 2.5 ($n = 10$)[b]	0.14 ± 0.05 ($n = 7$)
	Dilution of MeOH solution	Helical ribbons/tubules	6.6 ± 2.3 ($n = 7$)	0.15 ± 0.04 ($n = 9$)
$(Pro)_3$–$Glu(NHC_{14}H_{29})_2$	Slow cooling in 30% MeOH in HBS	Helical ribbons/tubules	7.8 ± 2.9 ($n = 7$)	0.22 ± 0.02 ($n = 5$)
$(Pro)_3$–$Glu(NHC_{16}H_{33})_2$	Slow cooling in 30% MeOH in HBS	Helical ribbons/tubules	7.9 ± 3.2 ($n = 7$)	0.28 ± 0.03 ($n = 5$)
Gly-Lys-Sar-Pro-$Glu(NHC_{12}H_{25})_2$	Dilution of MeOH solution	Helical ribbons	3.1 ± 0.3 ($n = 7$)	0.12 ± 0.02 ($n = 5$)
	Cooling in 42% EtOH in HBS	Rod-like aggregates	> 70	0.65 ± 0.18 ($n = 9$)
Ac-Lys-Ala-Sar-Pro-$Glu(NHC_{12}H_{25})_2$	Cooling in 46% EtOH in HBS	Rod-like aggregates	> 70	0.91 ± 0.39 ($n = 8$)

[a]Average value $\pm$ S.D.
[b]Number of measurements.

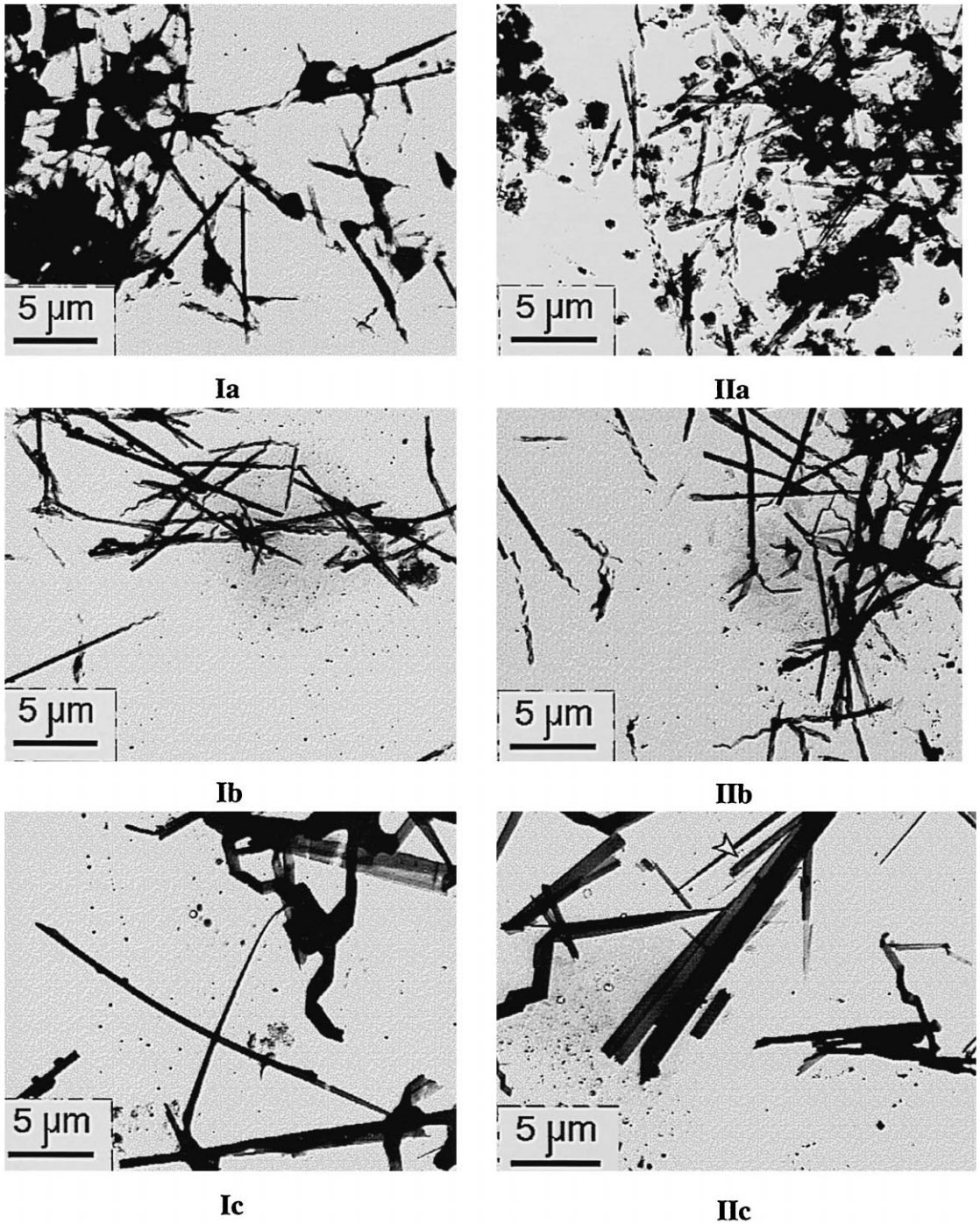

Fig. 4. TEM images of particles formed with $(Pro)_3$–$Glu(NHC_{14}H_{29})_2$ (I) and $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ (II) after heating and cooling of the lipopeptides in HBS/MeOH mixture with MeOH concentrations of 20% (a), 30% (b), and 50% (c). Note: some tubules wrapped with helical ribbons were observed in (IIc) as highlighted by arrow.

of ribbons with broad width variation; some ribbons were tapered, and others were irregularly wound into imperfect helices. Tubules were also observed in rare cases. Formation of tubules in less than 30% MeOH resulted in only partial conversion to HARMs. Particles obtained from $(Pro)_3$–$Glu(NHC_{14}H_{29})_2$ and

Table 4
Morphologies of HARMs formed from Boc-protected peptides modified with glutamic acid dialkyl amides

Compound	Formation method	Morphology
Boc-Gly-Lys-(ε-*Z*)-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$	Dilution of MeOH solution	Amorphous aggregates
	Slow cooling in 30% MeOH in HBS	HARMs
Boc-Lys-(ε-*Z*)-Ala-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$	Dilution of MeOH solution	Amorphous aggregates
	Slow cooling in 30% MeOH in HBS	HARMs/amorphous aggregates
Boc–Pro–Glu($NHC_{14}H_{29}$)$_2$	Dilution of MeOH solution	Amorphous aggregates
	Slow cooling in 30% MeOH in HBS	HARMs
Boc–Pro–Glu($NHC_{16}H_{33}$)$_2$	Dilution of MeOH solution	Amorphous aggregates
	Slow cooling in 30% MeOH in HBS	HARMs
Boc–(Pro)$_2$–Glu($NHC_{16}H_{33}$)$_2$	Dilution of MeOH solution	HARMs/amorphous aggregates
	Slow cooling in 30% MeOH in HBS	Amorphous aggregates
Boc–(Pro)$_3$–Glu($NHC_{14}H_{29}$)$_2$	Dilution of MeOH solution	HARMs/amorphous aggregates
	Slow cooling in 30% MeOH in HBS	HARMs/amorphous aggregates
Boc–(Pro)$_3$–Glu($NHC_{16}H_{33}$)$_2$	Dilution of MeOH solution	HARMs
	Slow cooling in 30% MeOH in HBS	HARMs/amorphous aggregates

(Pro)$_3$–Glu($NHC_{16}H_{33}$)$_2$ by heating and cooling in 30% MeOH had diameters of 0.22 ± 0.02 μm and lengths of 7.8 ± 2.9 μm, and diameters of 0.28 ± 0.03 μm and lengths of 7.9 ± 3.2 μm, respectively (Table 3). These HARMs have morphologies close to those formed by (Pro)$_3$–Glu($NHC_{12}H_{25}$)$_2$ by heating and slow cooling in pure HBS. Transferring these HARMs to pure HBS and consequent storage for up to 4 months at room temperature did not induce observable changes in their morphology (data not shown). The intermediate, protected Boc–(Pro)$_3$–Glu($NHC_{14}H_{29}$)$_2$ and Boc–(Pro)$_3$–Glu($NHC_{16}H_{33}$)$_2$ formed HARMs in 30% MeOH in HBS upon cooling at 0.2°C/min as well (Table 4, images not shown). The morphology of particles was different in this case. HARMs were not uniform in size, and amorphous aggregates were often observed. These results give additional examples of the modification of peptides with glutamic acid dialkylamides producing HARMs. This also shows that a minor modification in the peptide head group may affect the morphology of HARMs obtained under a given set of formation conditions.

Heating and cooling of $DC_{8,9}PC$ aqueous dispersions in the presence of EtOH induces tubule formation as well [23]. This approach for formation of HARMs was applied to some of the lipopeptides. A single concentration of EtOH was used for each compound—the concentration at which the lipopeptide precipitates from solution in absolute EtOH upon gradual addition of HBS at room temperature. Heating to 50°C and fast subsequent cooling (50°C to 24°C within about 4 in) of the resulting suspensions produced HARMs from both Ac-Lys-Ala-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ and Gly-Lys-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ (Fig. 5). The compound formed a mixture of very long (> 70 μm) and sometimes bent rod-like aggregates with rather broad variations in diameter (Table 3). Cooling at a slower rate, in this case, did not significantly alter the morphology of the particles (data not shown). The rest of the compounds processed this way, Pro–Glu($NHC_{12}H_{25}$)$_2$ and (Pro)$_3$–Glu($NHC_{12}H_{25}$)$_2$, did not form HARMs (not shown). Although the highest temperature used in this technique was lower than the T_m value of Ac-Lys-Ala-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ in pure aqueous buffer, the fact that the alcohol-containing lipopeptide suspension became semi-transparent (as would be observed for a micellar suspension) indicated that the heating was sufficient to disperse the compound. Direct DSC measurement demonstrated that, in fact, Ac-Lys-Ala-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ in HBS containing 46% EtOH has a major transition peak at 29.5°C (vs. 67.6°C in pure HBS, Table 2) and no transition above this value. The influence of EtOH on the thermal behavior of Ac-Lys-Ala-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ is very close to that of $>80\%$ EtOH on $DC_{8,9}PC$ [30].

Finally, we formed well-defined HARMs by addition of Gly-Lys-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ and (Pro)$_3$–Glu($NHC_{12}H_{25}$)$_2$ methanolic solutions to HBS (Fig. 6). Gly-Lys-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$

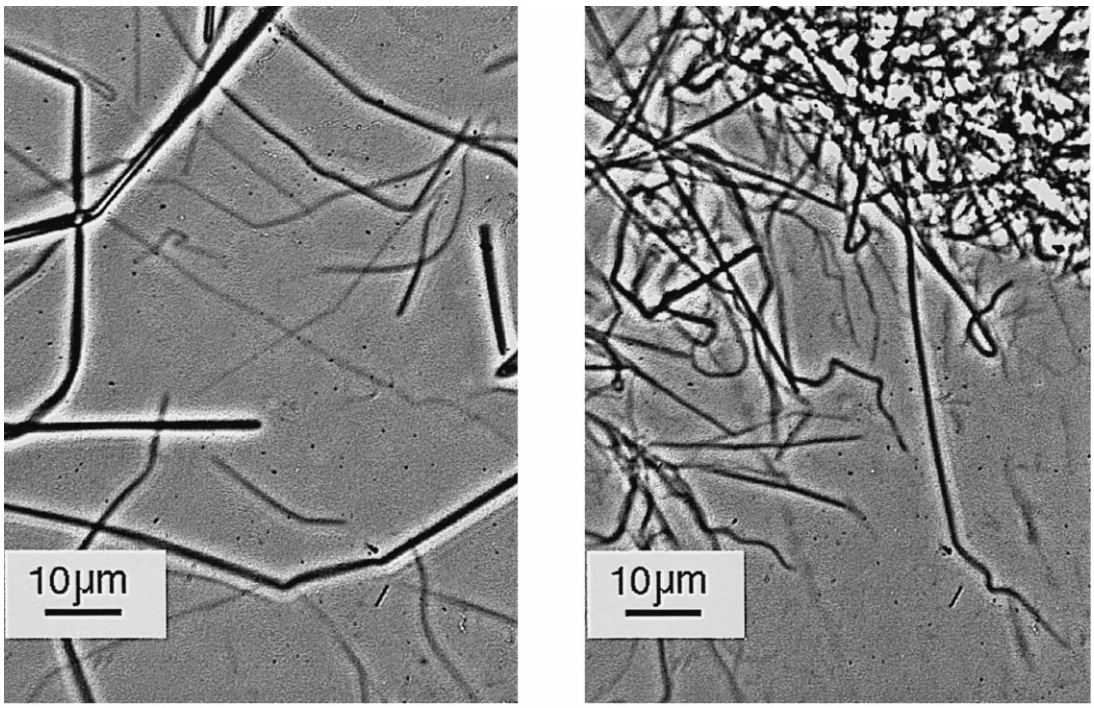

Fig. 5. Optical images of Ac-Lys-Ala-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ (I) and Gly-Lys-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ (II) particles formed after cooling of dispersions of the lipopeptides from 50°C to 20°C in EtOH/HBS mixtures with EtOH concentrations as shown in Table 1.

forms helical ribbons with an average width of 0.12 $\pm$ 0.02 μm and lengths of 3.1 $\pm$ 0.34 μm (Table 3). $(Pro)_3$–Glu($NHC_{12}H_{25}$)$_2$ forms HARMs with morphology and dimensions very close to those obtained by slow cooling (Table 3).

The ability of some intermediate Boc-protected lipopeptides to form HARMs was tested using MeOH precipitation (Table 4, images not shown). All of these compounds form HARMs. Although the HARMs formed were not always uniform in size and

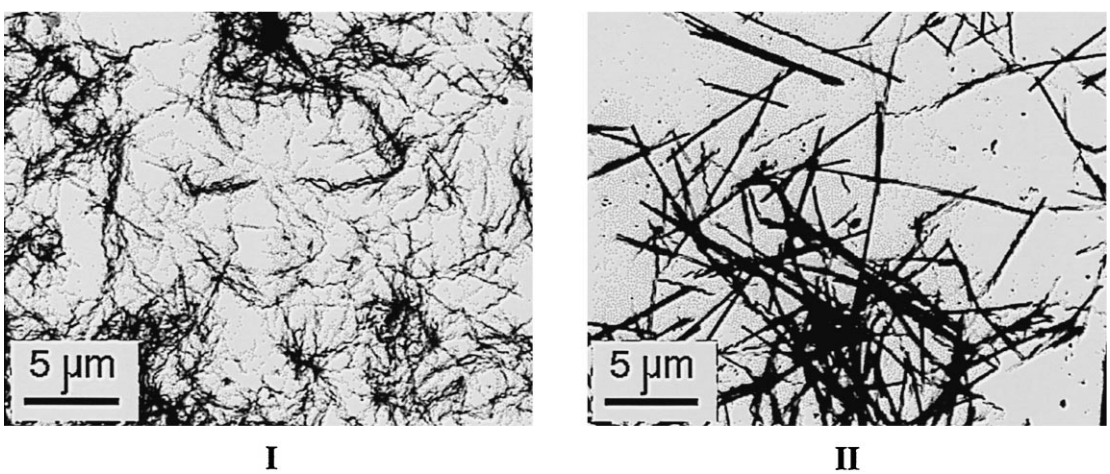

Fig. 6. TEM images of the particles formed by the dilution of MeOH solution method: Gly-Lys-Sar-Pro-Glu($NHC_{12}H_{25}$)$_2$ (I); $(Pro)_3$–Glu($NHC_{12}H_{25}$)$_2$ (II).

were often contaminated with amorphous precipitates, the results obtained strongly suggest that glutamic acid dialkylamides can be used as an universal lipid anchor to construct molecules capable of self-assembling into HARMs from a broad range of peptides.

3.3. Light scattering studies

Ratna et al. reported two distinct mechanisms of tubule formation upon slow cooling of $DC_{8,9}PC$ in MeOH/water mixtures. Light scattering and polarized optical microscopy studies showed that at lower concentrations of lipid, tubules form directly from the isotropic phase of the lipid, whereas at higher concentrations, the formation of tubules is preceded by aggregation of the lipid into an L_α phase [23]. It has been shown later that in MeOH/H_2O mixtures, the first mechanism leads to formation of mostly single bilayer tubules, and the second forms tubules containing 2–4 bilayers [31], thus providing a valuable tool for tailoring the tubule lamellarity for a particular application.

In order to examine if HARM formation proceeds by either of these mechanisms, we studied the light scattering of a $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ HARM suspension in the formation solution (30% MeOH in HBS) as a function of temperature (Fig. 7). Light

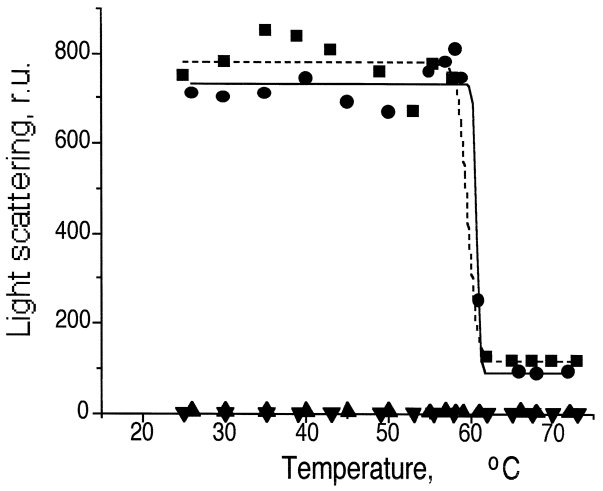

Fig. 7. Light scattering as a function of temperature of 0.4 mM $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ in HBS (squares, dashed line—sigmoidal fit); 0.4 mM $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ in 30% MeOH in HBS (circles, solid line—sigmoidal fit); HBS (down triangles); 30% MeOH in HBS (up triangles).

scattering sharply decreases at about 60°C. This clearly indicates that upon heating to the T_m, HARMs convert into one or more structure(s) of much smaller size. The light scattering of the $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ suspension above the transition temperature is about 45 times higher than the background scattering of 30% MeOH in HBS containing no lipid. This demonstrates that, immediately above the transition temperature, $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ molecules in this solvent do not form an isotropic phase, but are still self-assembled into some kind of aggregate. These aggregates are not visible in the phase contrast microscope (63× magnification) suggesting that they may be micelles. No particles were detected by optical microscopy in samples heated to 65°C and observed at room temperature within approximately 2 min. After that time, the lipidated peptide precipitated as amorphous aggregates. According to Ratna et al. [23], upon heating $DC_{8,9}PC$ to ~20°C above its tubule–non-tubule transition, an isotropic phase forms with light scattering equal to that of the MeOH/water mixture alone. The light scattering of $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ at the concentration studied was the same in the 62–78°C range (Fig. 7), suggesting that smaller aggregates formed from these HARMs immediately above the transition temperature do not change size upon further heating.

The temperatures at which the light scattering of $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ decreases in pure HBS and in 30% MeOH, in general, correlate with the T_m values measured by DSC (Table 2). According to the DSC data, the influence of 30% MeOH on $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ phase transition is consistent with that of $DC_{8,9}PC$ which shows moderate T_m decrease at low concentrations of short chain alcohols [23,30].

3.4. Stability of HARMs

For some potential pharmaceutical applications of lipopeptide HARMs, it would be advantageous if the microstructures do not change morphology on interaction with biological fluids or cell membranes, whether through loss of monomeric surfactants or other mechanisms. One lipopeptide was chosen to test the survival of HARMs in simulated biological fluids. Fig. 8 shows that during incubation for 45 h at 40°C, HARMs made of $(Pro)_3$–$Glu(NHC_{16}H_{33})_2$ do not change significantly in HBS (used as a control),

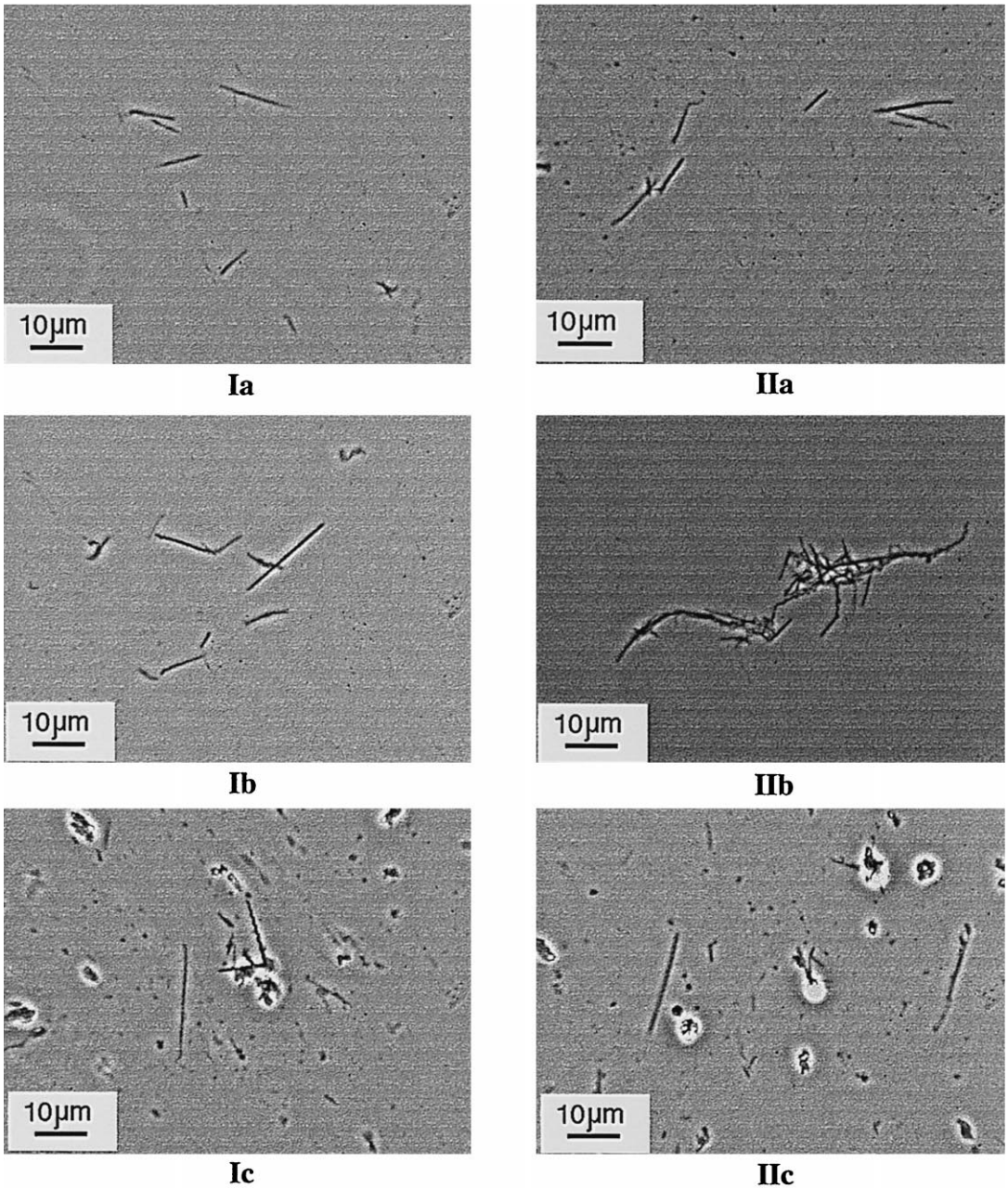

Fig. 8. $(Pro)_3$–Glu$(NHC_{16}H_{33})_2$ HARMs before (Ia–Ic) and after (IIa–IIc) 45 h incubation at 40°C in HBS (a), FCS (b), and DOPC liposomal suspension (c).

in fetal calf serum (modeling extracellular fluids), or in the presence of an excess of DOPC liposomes (used to model cell membranes) at a 1:30 molar lipopeptide/DOPC ratio. Aside from some aggregation of HARMs, these treatment did not detectably alter HARM morphology. This demonstrates that the presence of lipid membranes or components of blood plasma alone at physiological temperature is not immediately detrimental to this type of microstructure. Upon injection into mammals, the morphology of these HARMs are not expected to change dramatically, so they should provide a suitable matrix for extended continuous release in a manner that is characteristic of their shape.

4. Conclusions

Using 10 different amphiphiles, we have shown that lipidation of peptides using glutamic acid dialkylamides is a simple, and perhaps universal method to cause short peptides to self-assemble into HARMs. Despite significant structural differences between the lipopeptides studied and tubule-forming diacetylenic phospholipids, techniques developed for formation of $DC_{8,9}PC$ HARMs were readily adapted for use with the lipopeptides studied, although, in some cases, fabrication of uniform HARMs will require optimization of conditions including buffer composition and cooling rate. Fast, simple, and quantitative formation of uniform HARMs was possible with most compounds described. This provides a basis for further investigation of physico–chemical properties of these particles to determine their molecular conformation and their pharmacological potential.

Acknowledgements

This work was supported by a grant from the Whitaker Foundation to P. Yager and M. Gelb, and by funds from Molecumetics, to K.C. Lee. The authors are grateful to Paul A. Carlson and Dr. Alex S. Goldstein for valuable discussions.

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