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Non-covalent stabilization of a β-hairpin peptide into liposomes

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An oligopeptide modified on both the N- and C-termini with hydrophobic moieties was prepared on a solid phase and anchored into a liposome, stabilizing the fold of the peptide into a β-hairpin, which would otherwise be a random coil.

Much effort has been put in the design of peptide-based molecules that are able to interact selectively with proteins by mimicking a natural binding partner.[1] This design has proven to be a non-trivial task and the enormous diversity of potential targets requires a flexible approach. Moreover, it has been clearly demonstrated that such interactions do not only depend on the primary amino acid sequence, but also on the three-dimensional structure that sequence adopts. Additionally, often multiple epitopes are involved in binding, in which they are usually displayed at the turn of β-hairpin structures.[2] Therefore, many researchers have recognized the importance not only to introduce the specific recognition site for a template, but also to mimic the structural environment in which this oligopeptide is incorporated. Since β-hairpins are ubiquitous in molecular recognition events and biological activity of proteins, much research has focused on the stabilization of these structures in oligopeptides. Various approaches have been described in the literature to achieve this goal: *e.g.* variations in primary amino acid sequence,[3] introduction of stabilizing linkages such as cysteine bridges,[4] amide bonds,[5] metal complexation,[6] double bonds[7] using non-natural amino acids,[8] or attachment to a scaffold.[9,10] These methods have been successful in introducing the desired fold, however, their limitation is that they require the positioning of certain amino acids at specific sites in the sequence. Additionally, the covalent approaches might restrict the peptide's flexibility to such an extent that it could impair its biological activity!

The goal of our current research is to stabilize short β-hairpin peptides onto a dynamic liposome scaffold by means of a non-covalent approach. Oligopeptides capable of forming β-hairpins can be modified on both the N- and C-termini with hydrophobic moieties,[11,12] allowing the peptides to be anchored at both ends in a dynamic bilayer, as shown schematically in Fig. 1. This in our view simple but novel approach, results in stabilization of the folding pattern without much interference with the dynamic character of the peptide, contrary to covalent methods used to impose secondary structure.[13] Furthermore, the method presented here does not require the incorporation of specific amino acid residues nor any specialized chemistry but conventional solid-phase peptide synthesis. Finally, modification of the liposome periphery by non-covalent interactions and exploiting the fluidic nature of the bilayer allows for the preparation of combinatorial libraries of epitopes.

In our initial experiments, the β-turn sequence from the CS protein of the malaria parasite, *plasmodium falciparum*,[14] was chosen as an ideal candidate for stabilization by anchoring into a lipid bilayer. This fold has already been carefully studied by Robinson and coworkers using covalent attachment to a synthetic template. They demonstrated that a short peptide based on the NPNA sequence of the CS protein could be stabilized in

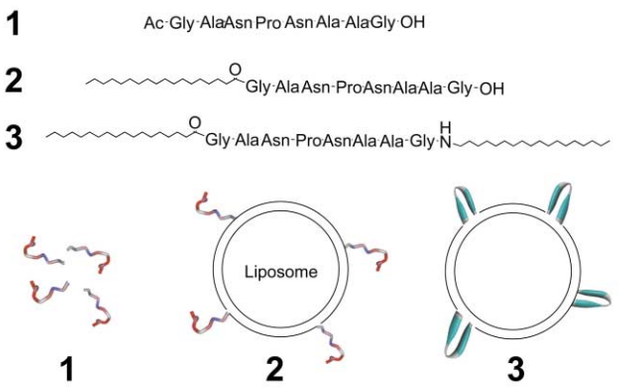

Fig. 1 Stabilization of a β-hairpin by attachment of terminal alkyl tails.

the β-turn conformation on an L-Pro–D-Pro scaffold creating a cyclic peptide.[10,12,15]

In order to study the effect of N- and C-terminal alkyl chains on peptide conformation, peptides **1**–**3** (Fig. 1) were synthesized. Peptides **1** and **2** were prepared on a Wang resin using standard Fmoc chemistry[16] and, in the case of peptide **2**, a final coupling with stearic acid was performed before cleavage.

The synthesis of peptide **3**, which required the incorporation of lipophilic tails at both termini of a peptide, was accomplished as shown in Scheme 1. The preparation commenced with a reductive amination reaction on a commercially available aldehyde modified resin,[17] followed by standard Fmoc synthesis of the peptide and subsequent capping with stearic acid, to afford the peptide modified at both termini. This generic solid phase strategy allows one to prepare the amphiphilic peptides completely on a solid phase, enabling quick production of a variety of lipidated peptides.

The folding characteristics of the peptides were studied with several techniques. First, the behaviour of peptide amphiphiles **2** and **3** was investigated at the air–water interface using a Langmuir trough.[18] The isotherms of peptides **2** and **3** and those of stearic acid and distearoyl phosphatidyl choline (DSPC) are depicted in Fig. 2. Comparing the behaviour of peptide **2** with that of stearic acid, we conclude the behaviour of **2** not to be determined by its alkyl chain but by the peptide head group. However, no further conclusion could be drawn since a stable monolayer was not formed, probably due to the partial solubility of the peptide in water. In contradistinction, peptide **3**, containing two alkyl chains, formed a stable monolayer. From the observed plot a molecular area was extrapolated of just over double the size of a stearic acid molecule, as can be seen in Fig. 2. The first rise of the curve compared favorably with DSPC, a neutral amphiphile with two stearoyl tails. It is surprising to see that the molecular area occupied by **3** is solely determined by the lipophilic alkyl chains. Therefore, we conclude the peptide must be able to adopt such a conformation that it is possible for the alkyl chains to closely pack in a similar fashion as for the model phospholipid. A molecular model of compound **3**, as shown in Fig. 3a, indicates that such a

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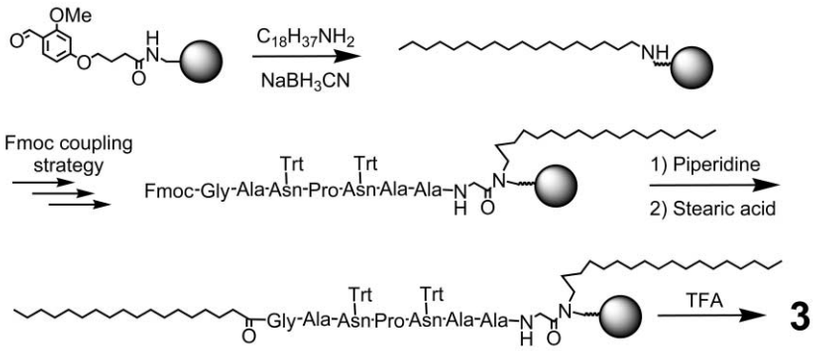

Scheme 1 Solid-phase synthesis of amphiphilic peptide **3**.

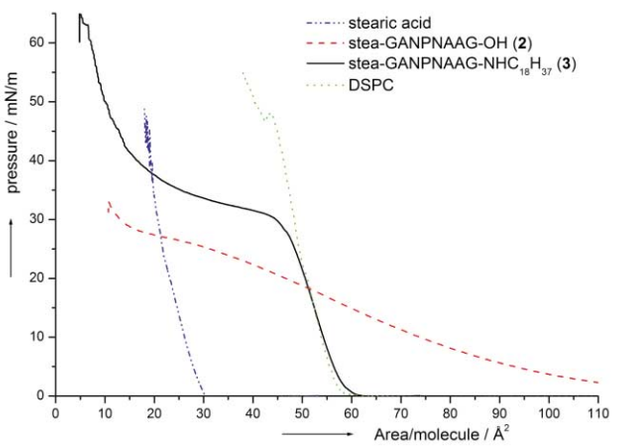

Fig. 2 Pressure–area isotherms of stearic acid (–··–··), peptides **2** (- - -) and **3** (—), and DSPC (···) on a pure water subphase.

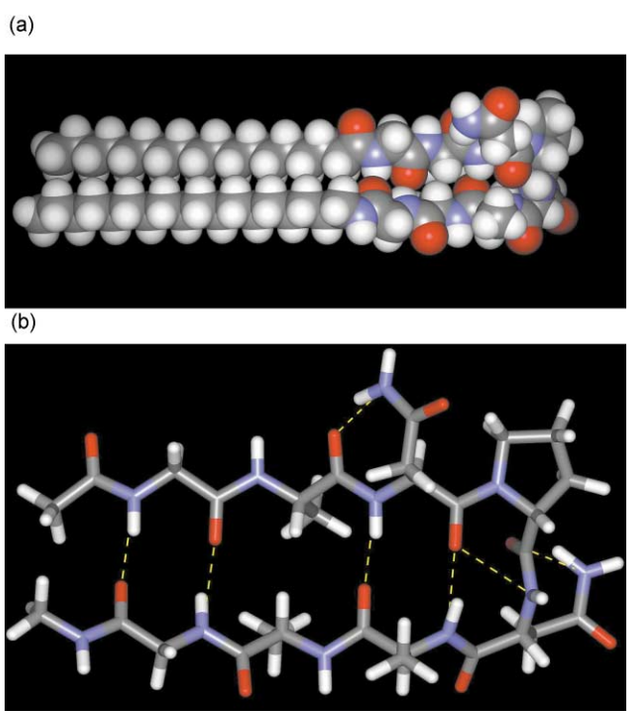

Fig. 3 (a) A CPK model of peptide **3** in a fully compressed state; (b) An energy minimized structure of the peptide head group showing intramolecular hydrogen bonds.[19]

fold is possible. This conformation is stabilized by internal hydrogen bonds forming a short antiparallel β-hairpin (Fig. 3b).

More definite evidence for the folding behaviour of the peptides was obtained by CD spectroscopy. This technique is a useful tool for the determination of protein and peptide secondary structure.[20] Various methods have been developed to extract structural information from CD spectra. In the simplest approach, the spectrum is deconvoluted using a linear combination of the spectra of the common structural elements a peptide or protein consists of. Fig. 4 shows CD spectra that were recorded from peptides **1**–**3**. The spectrum of an aqueous solution of the N-acetylated peptide **1** is clearly indicative of a random coil fold with a strong negative band near 200 nm and a very weak band around 220 nm.[21] To obtain a CD spectrum of peptide **2**, a liposomal dispersion consisting of 20% (w/w) of **2** in DSPC had to be prepared, due to the low solubility of the amphiphilic peptide in water. Langmuir isotherms of mixtures of the amphiphilic peptide and DSPC indicated that they behave as a non-ideal mixture;[18] *i.e.* although the molecular areas of the mixtures deviate positively from additivity, no phase separation with Brewster-angle microcopy was observed. Electron microscopy showed that the sonicated and subsequently extruded dispersions consisted of small unilaminar liposomes with an average diameter of 100 nm.[22] Such dispersions afforded a CD spectrum almost identical to that of peptide **1**, as depicted in Fig. 4. This implies that peptide **2** exists in the same structurally unordered form. Contrary to the aforementioned examples, an analogous 20% (w/w) dispersion in DSPC of peptide **3**, modified on both the N- and the C-termini, showed a drastically different spectrum. The strong positive Cotton effect at 197 nm and a small negative band around 223 nm, now indicate a β-pleated sheet that is comparable to that of poly(Lys-Leu-Lys-Leu).[23] Moreover, a compressed monolayer of peptide **3** at the air–water interface was transferred onto a quartz slide and studied by CD spectroscopy. The CD spectrum of this Langmuir–Blodgett monolayer was identical to that of the liposomal bound peptide, which substantiates the results

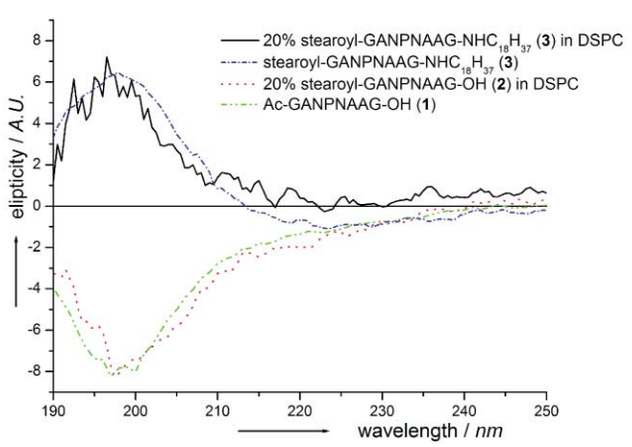

Fig. 4 Circular dichroism traces of peptides **1**–**3**. — a liposomal dispersion of a mixture of 20% (w/w) **3** in DSPC; –·–· a Langmuir–Blodgett film of peptide **3** on a quartz substrate; ···· a liposomal dispersion of a mixture of 20% (w/w) **2** in DSPC; –··–·· an aqueous solution of peptide **1**. The spectra have been normalized for comparison.

from the surface–pressure diagrams. To exclude the possibility that the structure of the peptide was altered by interaction with the quartz surface, the monolayer was also transferred to quartz that had first been modified with a monolayer of stearic acid. This resulted in a monolayer in which the alkyl chains interact with the surface. Once more, the same CD spectrum was obtained. Finally, the possibility was eliminated that the observed changes in the CD spectra could be ascribed to the fact that the (neutral) peptide was absorbed into the bilayer. The conformational change would then be due to the organic medium within the membrane rather than β-turn folding of the peptide. However, transfer of peptide **1** from its aqueous to an organic (acetonitrile) environment did not result in formation of a β-hairpin but rather increased the helical content and completely eradicated any β-hairpin character, suggesting this not to be the preferred fold in an organic environment like a membrane.

Finally, lyophilized samples of peptides **1**–**3** were analyzed by infrared spectroscopy. The amide I and II bands are indicative of the fold of a peptide.[24] The amide II vibration was located at 1535 cm^{-1} for all compounds, however, the amide I band of peptide **3** had shifted to 1630 cm^{-1} compared to 1647 and 1643 cm^{-1} for peptides **1** and **2**, respectively. This is in agreement with a random fold for the latter two structures and an antiparallel-chain pleated sheet[25] for peptide **3**. It can be concluded that the two alkyl chains stabilize the fold to such an extent that it even occurs in the freeze-dried sample of peptide **3**. The amide I band of a compressed layer of peptide **3** transferred to a polystyrene substrate was determined and found to be at 1625 cm^{-1}. This substantiates the evidence for a β-hairpin conformation for this peptide and further supports the monolayer and CD experiments. As the liposomal dispersion of **3** yields an almost identical CD spectrum to that of a compressed monolayer of **3**, both must possess the same β-hairpin secondary structure.

In conclusion, we have shown that amphiphilic peptides with an apolar N- and/or C-terminus can easily be prepared using an entirely solid-phase strategy. The apolar moieties allow us to anchor the attached peptides into liposomes or form monolayers at the water–air interface. Additionally, we demonstrated from the combined monolayer, CD and IR results, that anchoring a peptide at both termini forces it to adopt a hairpin conformation, whereas unmodified or partially alkylated it would exist in an unordered fold. Thus, this simple but novel supramolecular approach enables one to very efficiently stabilize secondary structure. We are currently investigating the scope of this strategy, trying to impose folds on peptides regardless of their sequence and further explore their behaviour in peptide–protein interactions.

Notes and references

1 (*a*) W. F. Degrado, *Adv. Prot. Chem*, 1988, **39**, 51; (*b*) G. Tuchscherer, L. Scheibler, P. Dumy and M. Mutter, *Biopolymers*, 1998, **47**, 63; (*c*) M. W. Peczuh and A. D. Hamilton, *Chem. Rev.*, 2000, **100**, 2479; (*d*) H. A. Klok, *Angew. Chem., Int. Ed.*, 2002, **41**, 1509.

2 (*a*) A. Giannis and T. Kolter, *Angew. Chem., Int. Ed. Engl.*, 1993, 1244; (*b*) W. E. Stites, *Chem. Rev.*, 1997, **97**, 1233; (*c*) R. E. Babine and S. L. Bender, *Chem. Rev.*, 1997, **97**, 1359; (*d*) P. Y. Chou and G. D. Fasman, *J. Mol. Biol.*, 1977, **115**, 135; (*e*) G. D. Rose, L. M. Gierasch and J. A. Smith, *Adv. Prot. Chem*, 1985, **37**, 1.

3 (*a*) T. Kortemme, M. Ramírez-Alvarado and L. Serrano, *Science*, 1998, **281**, 253; (*b*) M. S. Searle, *J. Chem. Soc., Perkin Trans. 2*, 2001, 1011; (*c*) C. Das, S. C. Shankaramma and P. Balaram, *Chem. Eur. J.*, 2001, **7**, 840.

4 (*a*) A. R. B. V. Prasad and P. Balaram, *J. Am. Chem. Soc.*, 1983, **105**, 105; (*b*) J. Venkatraman, G. A. N. Gowda and P. Balaram, *J. Am. Chem. Soc.*, 2002, **124**, 4987.

5 (*a*) B. Charpentier, A. Dor, P. Roy, P. England, H. Pham, C. Durieux and B. P. Roques, *J. Med. Chem.*, 1989, **32**, 1184; (*b*) A. Miranda, S. L. Lahrichi, J. Gulyas, S. C. Koerber, G. Craig, A. Corrigan, C. Rivier, W. Vale and J. Rivier, *J. Med. Chem.*, 1997, **40**, 3651.

6 G. Platt, C.-W. Chung and M. S. Searle, *Chem. Commun.*, 2001, 1162.

7 (*a*) S. J. Miller, H. E. Blackwell and R. H. Grubbs, *J. Am. Chem. Soc.*, 1996, **118**, 9606; (*b*) J. Pernerstorfer, M. Schuster and S. Blechert, *Chem. Commun.*, 1997, 1949; (*c*) E. R. Jarvo, G. T. Copeland, N. Papioannou, P. J. Bonitatebus and S. J. Miller, *J. Am. Chem. Soc.*, 1999, **121**, 11638; (*d*) J. R. Reichwein, C. Versluis and R. M. J. Liskamp, *J. Org. Chem.*, 2000, **65**, 6186; (*e*) S. Hanessian and M. Angioloini, *Chem. Eur. J.*, 2002, **8**, 111.

8 (*a*) H. N. Gopi, R. S. Roya, S. R. R. Raghothama, I. L. Karle and P. Balaram, *Helv. Chim. Acta*, 2002, **85**, 3313; (*b*) S. H. Gellman, *Acc. Chem. Res.*, 1998, **31**, 173.

9 (*a*) J. S. Nowick, J. M. Cary and J. H. Tsai, *J. Am. Chem. Soc.*, 2001, **123**, 5176; (*b*) J. P. Schneider and J. W. Kelly, *Chem. Rev.*, 1995, **95**, 2169; (*c*) E. Drakopoulou, S. Zinn-Justin, M. Guenneugues, B. Gilquin, A. Ménez and C. Vita, *J. Biol. Chem.*, 1996, **271**, 11979; (*d*) B. G. Aguilera, G. Siegal, H. S. Overkleeft, N. J. Meeuwenoord, F. P. J. T. Rutjes, J. C. M. van Hest, H. E. Schoemaker, G. A. van der Marel, J. H. van Boom and M. Overhand, *Eur. J. Org. Chem.*, 2001, 1541.

10 J. A. Robinson, *Synlett.*, 1999, **4**, 429.

11 (*a*) K. Yamada, H. Ihara, T. Ide, T. Fukumoto and C. Hirayama, *Chem. Lett.*, 1983, 1713; (*b*) C. Cescato, P. Walde and P. L. Luisi, *Langmuir*, 1997, **13**, 4480; (*c*) Y.-C. Yu, M. Tirrell and G. B. Fields, *J. Am. Chem. Soc.*, 1998, **120**, 9979; (*d*) K. Ariga and T. Kunitake, *Acc. Chem. Res.*, 1998, **31**, 371; (*e*) Q. Huo, G. Sui, P. Kele and R. M. Leblanc, *Angew. Chem., Int. Ed.*, 2000, **39**, 1854; (*f*) C. M. Paleos, Z. Sideratou and D. Tsiourvas, *ChemBioChem*, 2001, **2**, 305; (*g*) V. Marchi-Artzner, B. Lorz, U. Hellerer, M. Kantlehner, H. Kessler and E. Sackmann, *Chem. Eur. J.*, 2001, **7**, 1095; (*h*) J. D. Hartgerink, E. Beniash and S. I. Stupp, *Science*, 2001, **294**, 1684; (*i*) F. Eisele, J. Kuhlmann and H. Waldmann, *Chem. Eur. J.*, 2002, **8**, 3362.

12 R. Moreno, L. Jiang, K. Moehle, R. Zurbriggen, R. Glück, J. A. Robinson and G. Pluschke, *ChemBioChem*, 2001, **2**, 838.

13 Compare *e.g.* ref. 1*b*.

14 C. Cerami, U. Frevert, P. Sinnis, B. Takacs, P. Clavijo, M. J. Santos and V. Nussenzweig, *Cell*, 1992, **70**, 1021.

15 C. Bisang, L. Jiang, E. Freund, F. Emery, C. Bach, H. Matile, G. Pluschke and J. A. Robinson, *J. Am. Chem. Soc.*, 1998, **120**, 7439.

16 (*a*) E. Atherton and R. C. Sheppard, *Solid phase peptide synthesis*, IRL Press, Oxford, 1989; (*b*) G. B. Fields and R. L. Noble, *Int. J. Pept. Protein Res.*, 1990, **35**, 161.

17 (*a*) J. Alsina, K. J. Jensen, F. Albericio and G. Barany, *Chem. Eur. J.*, 1999, **5**, 2787; (*b*) S. Caddick, D. Hamza and S. N. Wadman, *Tetrahedron Lett.*, 1999, **40**, 7285.

18 (*a*) G. Roberts, *Langmuir-Blodgett Films*, Plenum Press, New York, 1990; M. S. Aston, *Chem. Soc. Rev.*, 1993, 67; (*b*) M. Puggelli and G. Gabrielli, *Colloid Polym. Sci.*, 1983, **261**, 82.

19 Structures were generated in Macromodel. F. Mohamadi, N. G. J. Richards, W. C. Guida, R. Liskamp, M. Lipton, C. Caufield, G. Chang, T. Hendrickson and W. C. Still, *J. Comput. Chem.*, 1990, **11**, 440.

20 (*a*) J. T. Yang, C.-S. C. Wu, H. M. Martinez and J. T. Yang, *Methods Enzymol.*, 1986, **130**, 208; (*b*) N. Sreerama, S. Y. Venyaminov and R. W. Woody, *Prot. Sci.*, 1999, **8**, 370.

21 W. C. Johnson Jr., *Proteins: Struct., Funct. Genet.*, 1990, **7**, 205.

22 All dispersions were sonicated and subsequently extruded through 100 nm polycarbonate membranes at 60 °C to form small unilaminar liposomes. The approximately 100 nm diameter was confirmed with electron microscopy on negatively stained samples and dynamic light scattering.

23 S. Brahms and J. Brahms, *J. Mol. Biol.*, 1980, **138**, 149.

24 (*a*) H. Susi and D. M. Byler, *Methods Enzymol.*, 1986, **130**, 290; (*b*) J. Bandekar and S. Krimm, *Biopolymers*, 1988, **27**, 909.

25 B. T. Miyazawa and E. R. Blout, *J. Am. Chem. Soc.*, 1961, **83**, 712.