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Aqueous self-assembly of short hydrophobic peptides containing norbornene amino acid into supramolecular structures with spherical shape†

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The preparation and self-assembly of short hydrophobic peptides able to solubilize in water through the formation of supramolecular assembly is reported. The two diastereoisomeric pentapeptides AcAla-NRB-Ala-Aib-AlaNH$_2$ **1** and **2** containing the two enantiomers of non-proteinogenic norbornene amino acid (NRB) were synthesized in an efficient way and in good yields. They were insoluble in organic solvent, except MeOH and DMSO, but completely soluble in water despite that they are made of hydrophobic amino acids. The formation of a supramolecular assembly in water was assessed by Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS) using **1** and **2** individually or in a mixture. Conformational analysis on the two diastereoisomers, performed in CD$_3$CN, CD$_3$OH and in H$_2$O/D$_2$O, indicated the formation of a stable 3$_{10}$-helix structure for both peptides: the helix structure is more stable in CD$_3$CN and CD$_3$OH than in H$_2$O/D$_2$O where a helix/random coil transition was observed. Apparently, the norbornene moiety plays a role in the stabilization, in fact 1R2R3R-norbornene AA present in peptide **2** induces a more stable secondary structure with respect to the 1S2S3S-isomer present in peptide **1**.

Introduction

Self-assembly is a spontaneous process by which unordered systems of monomers organize into ordered structures as the result of non-covalent interactions including van der Waals, electrostatic, hydrogen bonding, and stacking interactions.[1] Self-assembly lies behind a number of biological nanostructures such as DNA double helix, protein's tertiary or quaternary structure, cell membranes upon self-assembly of phospholipids. In this scenario, better insight into the formation of vesicle-like supramolecular structures from small molecules, especially simple peptides, is of particular interest for the understanding of prebiotic organization and life.[2] Moreover, the concept of self-assembly has also been used in many disciplines for constructing useful materials. In particular, a number of papers report on spontaneous assembly of peptides into ordered nanostructures with a variety of morphologies and the number is still increasing.[3] Most of them describe supramolecular assembly from high molecular weight peptides as well as by small- or medium size peptides conjugated with molecules of nonpeptidic nature. Short peptides alone self-organize predominantly into nanotubes and nanofibers.[4] Spherical (micellar and vesicle-like) architectures are less described although their appear very attractive, due to their promising applications in biomedicine and nanotechnology.[3c,3m,5] Besides the numerous advantages of using peptides, some limitations are well-known such as a low stability in biological medium and their unstable conformation especially when they are short or medium-sized. The insertion of unnatural amino acids in the peptide sequences is a well-known tool to overcome these problems.

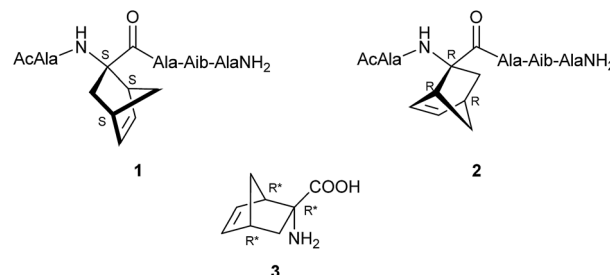In particular, the introduction of Cα-tetrasubstituted α-amino acid residues into peptide backbones generates models

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Fig. 1 Peptides **1** and **2** containing the non-proteinogenic norbornene amino acid **3**.

characterized by reduced conformational flexibility, high tendency to adopt predetermined secondary structures, and enhanced metabolic stability.[6] Both theoretical and experimental studies on this subject have been published and the group of Cα-tetrasubstituted residues in which the quaternary α-carbon forms part of a ring has been the object of extensive investigation.[7] Moreover, the use of non-proteinogenic amino acids allows an expansion in terms of structural diversification.[8] Notwithstanding this interest, studies on the self-assembly of short peptides containing cyclic Cα-tetrasubstituted amino acids are very rare.[9]

Herein we report on the preparation of two diastereoisomeric pentapeptides AcAla-NRB-Ala-Aib-AlaNH$_2$ **1** and **2**, containing the two enantiomers of the unnatural constrained norbornene amino acid (NRB) **3** (Fig. 1). Although not derived from natural sources the bicycloheptane scaffold is known to possess very interesting features.[10a,b] The norbornane amino acid is known to act as an inhibitor of System L-Amino Acid Transporter and several studies were undertaken concerning its ability to suppress cancer cells proliferation.[11a,b] On the other hand the presence of the norbornene scaffold allows an efficient labeling of peptides and proteins.[12]

Despite the hydrophobic nature of the amino acids, the two peptides **1** and **2** resulted completely soluble in water due to formation of supramolecular assemblies of spherical shape.

The formation of supramolecular assembly in water was assessed by Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS) using **1** and **2** individually or in a 1 : 1 mixture. Taking into account the growing interest toward designed peptide-based nanoparticles as candidates for the controlled delivery of drugs, proteins, genes and nucleotides, the stability of the obtained assemblies in blood serum was also studied. Experimental results showed that the supramolecular assembly of peptide **1** and **2** in mixture was stable in serum at 37 °C with unmodified dimension with respect to water.

Results and discussion

Peptide synthesis

The synthesis of the designed peptides **1** and **2** is reported in Scheme 1. Norbornene nitroesters **4** and **5** were prepared on a gram scale using a modified multicomponent protocol [ethyl nitroacetate (1 eq.), formaldehyde (37%, 5 eq.), cyclopentadiene (5 eq.), acetic acid (5 eq.), 50 °C, THF, 12 h] described by Carrol[13] in 88% yields (ratio: 15/85). **4** and **5** were reduced with an excess of Zn dust in H$_3$PO$_4$ (1 M in THF) affording the amines **6** and **7** (80% yields) that were easily separated by flash chromatography. Pure compound **8** (90% overall yields) was obtained through hydrolysis of ester **7** (KOH 6 eq., MeOH, 70 °C for 24 h, then HCl/MeOH, 0 °C) and Boc protection (Boc$_2$O, K$_2$CO$_3$, H$_2$O/MeOH) and purified by precipitation from reaction mixture with HCl 1 M at 0 °C. **8** was successfully coupled using a standard protocol (HOAt 1.1 eq., EDC 1.1 eq., DIPEA 2.2 eq., DCM) with the trifluoroacetic salt of the peptide **9** (H-Ala-Aib-Ala-NH$_2$). This last was previously prepared by a linear Boc chemistry solution strategy recently reported by us (50% overall yield on a gram scale).[10b]

The Boc protected tetrapeptides **10** and **11** were purified by precipitation in DCM/n-hexane in satisfactory yield (85%) as an inseparable diastereoisomeric mixture. The deprotection of **10**/**11** mixture was attempted with TFA but the recovery of the free amines was very troublesome due to their high water solubility. On the other hand the microwave assisted Boc deprotection (150 °C, 20 min, 80 watt)[14] of tetrapeptides **10**/**11** afforded the corresponding amines **12** and **13** (91% yield). The diastereoisomeric mixture of **12** and **13** was then condensed with Ac-Ala-OH. To avoid the high trend of racemization of the acetylated alanine under standard coupling condition, a different protocol, recently assessed by our group, was tested consisting in the use of EEDQ, in absence of base, at the lowest temperature reachable by a conventional compressed air-cooled MW reactor (60 °C, 80 watt).[10b] Diastereoisomeric pentapeptides **1**
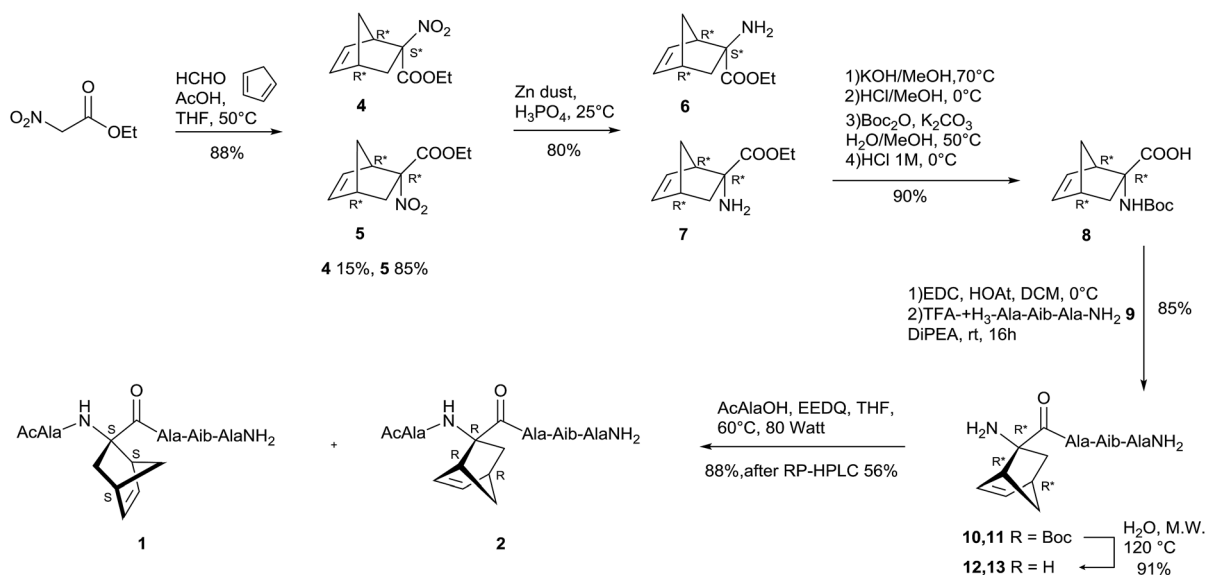
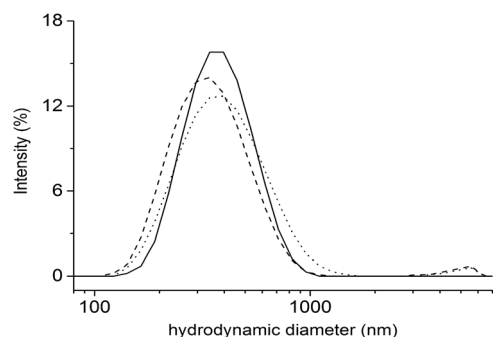**Scheme 1** Synthesis of peptides **1** and **2**.

Fig. 2 DLS results. Size distribution by intensity of **1** (straight) and **2** (dash) oligopeptides, as well as their 1 : 1 mixture (dot). Mean results are given for three different measurements.

and **2** were precipitated from the reaction mixture in pure form with Et$_2$O (88% yield) and separated by RP-HPLC (see ESI† for details). Taking in account future biomedical applications of the mixture of peptide **1** and **2** It is noteworthy that entire protocol required just one chromatographic purification steps at the level of amino acids **6** and **7**.

Morphology and size of supramolecular assemblies of peptides in water

The behavior of **1** and **2** in aqueous environment was investigated by dissolving them individually or in 1 : 1 mixture at the concentration of 5.0 mg mL^{-1} in water. The self-assembly of **1** and **2** was firstly assessed by Dynamic Light Scattering (DLS) that represents a valuable tool to measure the size of particles in the sub-micron region. The DLS profiles of **1** and **2** as well as their mixture are reported in Fig. 2.

All the tested samples showed the capability to self-assemble in aqueous environment and form aggregates whose hydrodynamic diameter values (*i.e.* mean size) ranged from about 320 to 370 nm (Fig. 2). Although the measured size values were quite similar, **1** formed supramolecular structures (357.0 nm) slightly bigger than **2** (322.9 nm). Further, as shown in Fig. 2, the obtained assemblies showed monomodal distributions with polydispersity index (pdI) of 0.117 and 0.195 for **1** and **2**,

respectively. When oligopeptides **1** and **2** were mixed together, monodisperse structures were still detected by DLS (pdI 0.185), whose size (369.1 nm) was similar to that obtained by individual oligopeptides. Accordingly, further characterization was done on the mixture of **1** and **2**. All the DLS measurements were run on freshly prepared oligopeptide solutions and the formation of self-assembled supramolecular structures in water was found to be fully reproducible.

In order to be used as drug delivery carriers, nanoparticles should remain intact and circulate in the blood for a sufficiently long period of time after intravenous injection to accumulate at the target site.[15] The stability of the oligopeptide assemblies (mixture of **1** and **2**) in a complex medium mimicking the *in vivo* conditions, namely fetal bovine serum (FBS), was evaluated and tested by DLS (Fig. 3).

As a control, pure FBS was analyzed by DLS and showed two peaks due to its protein composition. Interestingly, the peak of the supramolecular assemblies of **1** and **2** in mixture in pure FBS was clearly visible. This suggested that the interaction with FBS proteins did not affect the overall structure of the supramolecular assemblies which appeared stable under physiological mimicking conditions without substantial changes in their size. To further investigate the morphology of the supramolecular assemblies, the mixture of **1** and **2** was analyzed by TEM. Notably, the assemblies showed spherical shape with diameters ranging from 30 to 65 nm (Fig. 4).

The discrepancy between TEM and DLS data could arise from the formation of agglomerates (*i.e.* weakly bound aggregates) of the assemblies in suspension to minimize interfacial energy. Finally, the critical aggregation concentrations (CACs) of **1** and **2** in aqueous solution were determined by light
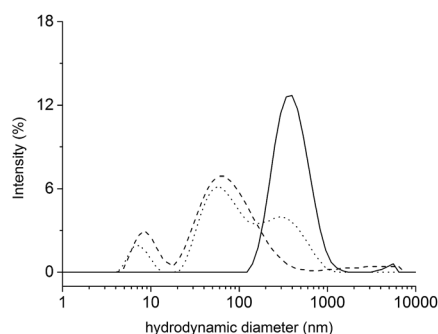

Fig. 3 Size distribution by intensity of the mixture of **1** and **2** suspended in FBS (dot). As a control, FBS (dash) and the mixture of **1** and **2** in water (straight) were analyzed. Mean results are given for three different measurements.
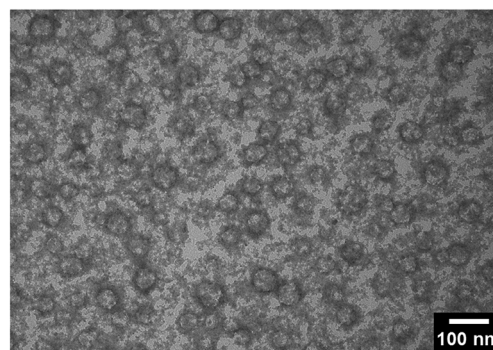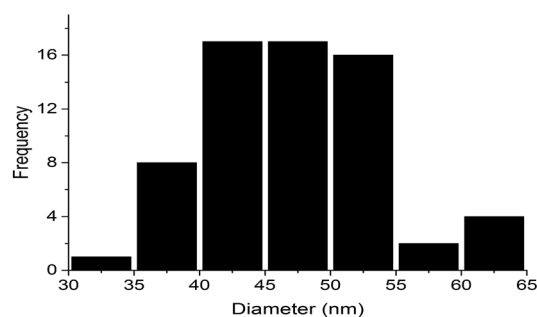

Fig. 4 TEM micrograph of the 1 : 1 mixture of oligopeptides **1** and **2** with uranyl acetate as negative staining agent.
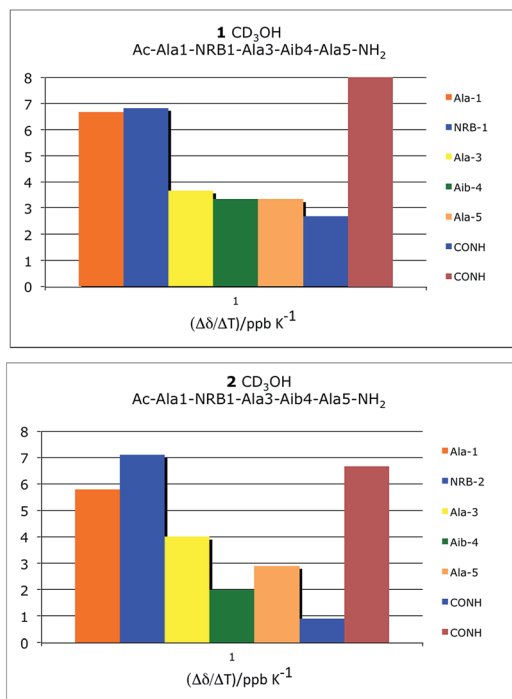
Fig. 5 Temperature dependence of amide chemical shift ($\Delta\delta/\Delta T$ 273–300 K). Analyses in CD_3OH for peptide **1** (32 mg mL^{-1}) and peptide **2** (26 mg mL^{-1}).

scattering.[16] To this aim, different samples with increasing concentration of either **1** or **2** were prepared and their count rate (*i.e.* the intensity of scattered light in DLS) was monitored (see ESI, Tables TS7 and TS8†). According to the experimental data, the count rate was found to increase with increasing the concentration of either **1** or **2**. However, samples with concentration higher than 5.0 and 26.0 mg mL^{-1} for **1** and **2**, respectively, led to a decrease in the count rate value. This suggested that the assemblies' concentration increased up to an optimal value and then decreased, probably due to altered interactions between the involved oligopeptides.

NMR analysis

NMR experiments (^1H, ^{13}C, COSY, NOESY, ROESY, HSQC, HMBC) in CD_3OH and H_2O/D_2O were performed at the concentration of 16 mg mL^{-1} (when not specified differently) and allowed the complete characterization of **1** and **2**.

Evaluation of $^3J_{NH-H\alpha}$. The $^3J_{NH-H\alpha}$ values were evaluated at 300 K° in CD_3OH and H_2O/D_2O for **1** and **2** and are reported in Table TS1 (see ESI†). Ala1 and Ala3 have typical values of helical backbone in CD_3OH in both peptides. In H_2O/D_2O a moderate increase of $^3J_{NH-H\alpha}$ was observed underlining a contribution of the extended unfolded conformation to a helical backbone structure.

NOESY/ROESY. NOESY and ROESY experiments in CD_3OH and in H_2O/D_2O are detailed in the ESI.† A helical structure is present in pentapeptide **1** as confirmed by various NOEs (N,N *i*, *i* + 1, s; Fig. SI1a;† $C^\alpha H_i$–NH_{i+1} (s) and $C^\alpha H_i$–NH_{i+3} (m/w) Fig. SI2a†). The formation of 3_{10}-helix is supported by $C^\alpha H_i$–

NH_{i+2} NOE signals[17] (Fig. SI2a†). Even if several NH signals are overlapped, we found a similar behaviour for peptide **2** with respect to peptide **1** confirming its 3_{10}-helix structure. Further evidence of helical conformation in CD_3OH are given by $C^\alpha H_i$–C^β_{i+3} cross peaks (Ala1-Aib4; Fig. SI5a and b,† respectively for **1** and **2**). The experiment in H_2O/D_2O for peptide **1** confirmed helix structure only at C terminus (Fig. SI3a and SI4a†). On the other hand peptide **2** shows all the typical NOEs characterizing a 3_{10}-helix even if they are less intensive with respect to the same experiment in methanol (Fig. SI3b and SI4b†). Further confirmation of the helical structure are given by $C^\alpha H_i$–C^β_{i+3} cross peaks present only in peptide **2** (Fig. SI5c†).

Temperature dependence of amide chemical shift ($\Delta\delta/\Delta T$). Confirmation of helix structure of peptides **1** and **2** was given estimating the temperature dependence of amide chemical shift ($\Delta\delta/\Delta T$, 273–300 K). In CD_3OH (Fig. 5) both peptides show the presence of four consecutive H-bonded amides at C-terminus being $\Delta\delta/\Delta T$ NH values of Ala3, Aib4, Ala5 lower than 4.0 ppb K^{-1}.[18] Moreover, C-terminus is the most stable part in both peptides with the lowest value of $\Delta\delta/\Delta T$ for one NH of $CONH_2$ (**1**: 2.6 ppb K^{-1}; **2**: 0.8 ppb K^{-1}) highlighting the importance of the primary amide in helix stabilization. On the other hand, the other NHs (Ala5, NBR2 at N-terminus and the other NH proton of the primary amide) are not involved in any intramolecular H-bond ($\Delta\delta/\Delta T$ 6–8 ppb K^{-1}). Interestingly, the ppb K^{-1} NHs values of peptide **2**, are lower with respect to those of peptide **1** indicating a more stable helix secondary structure.

A different behaviour was found in H_2O/D_2O (Fig. 6) where higher $\Delta\delta/\Delta T$ values were detected for all NHs. However, as in

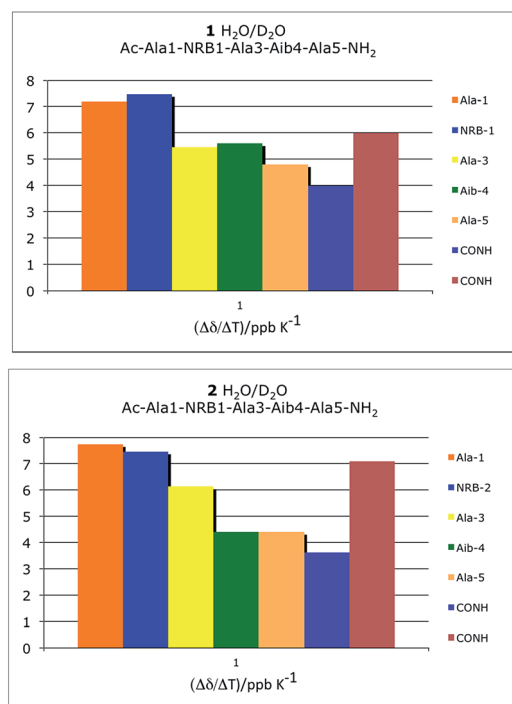Fig. 6 Temperature dependence of amide chemical shift ($\Delta\delta/\Delta T$, 273–300 K). Analyses in H_2O/D_2O for peptide **1** (13 mg mL^{-1}) and peptide **2** (16 mg mL^{-1}).

Table 1 MNE of Aib4 in peptide **1** and peptide **2**

Solvent	**1** MNE ppm δ	**2** MNE ppm δ
CD_3CN	3.38	3.69
CD_3OD	1.94	2.79
D_2O	0.25	0.85

the case of CD_3OH analyses, peptide **2** shows lower NH values at C-termini ($\Delta\delta/\Delta T$ Aib4, Ala5 and NH of the $CONH_2$: 3.8–4.5 ppb K^{-1}), than peptide **1** ($\Delta\delta/\Delta T$ Aib4, Ala5 and NH of the $CONH_2$: 4.0–5.5 ppb K^{-1}). Being known that $\Delta\delta/\Delta T$ for completely random coil peptide in water is ≈ 8.0 ppb K^{-1},[19] we hypothesize an equilibrium between helix/random coil structure in H_2O/D_2O. Moreover is interesting to notice that in water the differences between the two primary amide protons $\Delta\delta/\Delta T$ values are smaller than in methanol.[20] Especially the second primary amide of peptide **1** with a values of 6 ppb K^{-1} seems to be involved in a H-bond but we were not able to determinate the inter- or intra-molecular nature of that bound.

[13]C magnetic nonequivalence. A further confirmation of stable asymmetric secondary structure comes from the evaluation of the ^{13}C magnetic nonequivalence (MNE) of the signals related to the diastereotopic methyl groups of Aib4 (HSQC experiments in CD_3CN, CD_3OD) as shown in Tables 1 and TS2 (see ESI†).[21]

Instead, the absence of a stable helix structure for **1** and a transition helix/random coil structure for **2** were observed in D_2O (see ESI† for discussion).

The comparison with MNE values reported in literature[22] for differently capped (Ala-Aib)$_2$-Ala pentamers (BocNH, COOMe) corroborates the hypothesis that primary amides at C-terminus together with N-terminal acetyl group strongly stabilized helix conformation in organic solvent.

Finally, long range spatial proximity between NH of Ala5 and specific protons of norbornene ring, allows to tentatively assign the absolute stereochemistry of norbornene scaffold (^{1}HNMR in CD_3OH; see Fig. SI6†). As a result, the S- and R- stereochemistry was assigned to norbornene scaffold in peptide **1** and **2**, respectively.

Taking together all these data, we can affirm that the 1R2R3R-norbornene AA present in peptide **2**, induce a more stable 3$_{10}$-helix secondary structure with respect to 1S2S3S-norbornene AA present in peptide **1**. For both peptides, the helix structure is more stable in methanol than in water where a helix/random coil transition was observed. Preliminary experiments of concentration dependence of ^{1}H NMR performed in the range of CAC, did not show any change in NH chemical shifts.[23] Even if this result can suggest the absence of intermolecular H-bond in aggregates formation process it has to be noted that due to experimental limitation experiment were performed only from 5 mM to 10 mM due to the detection limits.

Conclusions

The two oligopeptides **1** and **2** were synthesized and the experimental data collected in CD_3CN and in CD_3OD confirmed

a conformational preference toward a stable 3$_{10}$-helix structure. Norbornene plays a certain role in such stabilization as observed in a previously published computational model[7m] and, basing on the magnitude of MNE in CD_3CN, it seems to be the best helix inducer among the unnatural constrained amino acids synthesized in our group referring to the same model peptide.[6b]

Conformational studies were performed also in H_2O/D_2O where a helix/random coil transition was observed for both peptides.

Moreover, a deep analysis of the NMR experiments allowed to tentatively assign the absolute stereochemistry to peptide **1** and **2**.

Oligopeptides **1** and **2** resulted completely soluble in water due to formation of supramolecular assemblies of spherical shape, despite the hydrophobic nature of the amino acids. Interestingly, the two oligopeptides in mixture gave mono-disperse structures similar to that obtained by individual oligopeptides. This result is of high value because allows the availability of a large amount of the self-assembling peptide material useful for several applications. Finally, the stability of the oligopeptide assemblies in serum at 37 °C was tested showing unmodified dimension opening the way to further studies as candidates for drug delivery.

Acknowledgements

Notes and references

1 G. M. Whitesides and B. Grzybowski, *Science*, 2002, **295**, 2418–2421.

2 J. X. Wang, T. T. Cai, J. L. Li, R. X. Zhuo and X. Z. Zhang, *RSC Adv.*, 2014, **4**, 14993.

3 (*a*) Y. Jin, X. D. Xu, S. Chen, S. X. Cheng, X. Z. Zhang and R. X. Zhuo, *Macromol. Rapid Commun.*, 2008, **29**, 1726; (*b*) A. J. V. Hell, A. Klymchenko, P. P. Burgers, E. E. Moret, W. Jiskoot, W. E. Hennink, D. J. A. Crommelin and E. Mastrobattista, *J. Phys. Chem. B*, 2010, **114**, 11046; (*c*) E. Longo, M. Crisma, F. Formaggio, C. Toniolo and A. Moretto, *Polym. Chem.*, 2013, **45**, 516; (*d*) X. Zhao, F. Pan and J. R. Lu, *Prog. Nat. Sci.*, 2008, **18**, 653; (*e*) D. Mandal, A. N. Shirazi and K. Parang, *Org. Biomol. Chem.*, 2014, **12**, 3544–3561; (*f*) G. Fuks, R. M. Tlom and F. Gauffre, *Chem. Soc. Rev.*, 2011, **40**, 2475; (*g*) Z. Luo and S. Zhang, *Chem. Soc. Rev.*, 2012, 4736; (*h*) R. H. Zha, S. Sur, J. Boekhoven, H. Y. Shi, M. Zhang and S. I. Stupp, *Acta Biomater.*, 2015, **12**, 1; (*i*) P. K. Koumkoua, C. Aisenbrey, E. Salnikov, O. Rifi and B. Bechinger, *J. Pept. Sci.*, 2014, **20**, 526; (*j*) *Peptide materials from nanostructures to applications*, ed. C. Aleman, A. Bianco and M. Venanzi, ISBN: 978-1-119-95373-9, Wiley-VCH, 2013; (*k*) S. Zhang, D. M. Marini, W. Hwang and S. Santoso, *Curr. Opin. Chem. Biol.*, 2002, **6**, 865; (*l*) A. Dehsorkhi, V. Castelletto and I. W. Hamley, *J. Pept. Sci.*,

2014, **20**, 453; (*m*) P. Moitra, K. Kumar, P. Kondaiah and S. Bhattacharya, *Angew. Chem., Int. Ed.*, 2014, **53**, 1113.

4 (*a*) C. M. Rufo, Y. S. Moroz, O. V. Moroz, J. Stöhr, T. A. Smith, X. Hu, W. F. DeGrado and I. V. Korendovych, *Nat. Chem.*, 2014, **6**, 303; (*b*) S. Marchesan, A. V. Vargiu and K. E. Styan, *Molecules*, 2015, 19775; (*c*) C. H. Gçrbitz, *Chem.–Eur. J.*, 2007, 1022; (*d*) A. Bonetti, S. Pellegrino, P. Das, S. Yuran, R. Bucci, N. Ferri, F. Meneghetti, C. Castellano, M. Reches and M. L. Gelmi, *Org. Lett.*, 2015, **17**, 4468–4471.

5 (*a*) J. J. Panda and V. S. Chauhan, *Polym. Chem.*, 2014, **5**, 4418–4436; (*b*) Y. Yang, U. Khoe, X. Wang, A. Horii, H. Yokoi and S. Zhang, *Nano Today*, 2009, 193.

6 (*a*) L. Gentilucci, D. R. Marco and L. Cerisoli, *Curr. Pharm. Des.*, 2010, **16**, 3185–3203; (*b*) S. Pellegrino, A. Contini, F. Clerici, A. Gori, D. Nava and M. L. Gelmi, *Chem.–Eur. J.*, 2012, **18**, 8705.

7 (*a*) C. Toniolo, M. Crisma, F. Formaggio and C. Peggion, *Biopolymers*, 2001, **60**, 396 and references therein; (*b*) P. Balaram, *Curr. Opin. Struct. Biol.*, 1992, **2**, 845; (*c*) C. Toniolo, *Janssen Chim. Acta*, 1993, **11**, 10; (*d*) P. Sudhanand, R. Balaji and P. Balaram, *Biopolymers*, 1995, **35**, 11; (*e*) I. Torrini, M. Paglialunga Paradisi, G. Pagani Zecchini and G. Lucente, *Synth. Commun.*, 1994, **24**, 153; (*f*) M. Cirilli, V. M. Coiro, A. Di Nola and F. Mazza, *Biopolymers*, 1998, **46**, 239; (*g*) C. Cativiela and M. D. Diaz-de-Villegas, *Tetrahedron: Asymmetry*, 1998, **9**, 3517; (*h*) R. Kaul, S. Banumathi, D. Velmurugan, R. Balaji Rao and P. Balaram, *Biopolymers*, 2000, **54**, 159; (*i*) C. Toniolo, *Int. J. Pept. Protein Res.*, 1990, **35**, 287; (*j*) M. Savian, R. Iacovino, V. Menchise, E. Benedetti, G. M. Bonora, M. Gatos, L. Graci, F. Formaggio, M. Crisma and C. Toniolo, *Biopolymers*, 2000, **53**, 200; (*k*) C. Cativiela and M. D. Diaz-de-Villegas, *Tetrahedron: Asymmetry*, 2000, **11**, 645; (*l*) A. Moretto, F. Formaggio, M. Crisma, C. Toniolo, M. Saviano, R. Iacovino, R. M. Vitale and E. Benedetti, *J. Pept. Res.*, 2001, **57**, 307; (*m*) I. Maffucci, S. Pellegrino, J. Clayden and A. Contini, *J. Phys. Chem. B*, 2015, **119**, 1350.

8 S. Pellegrino, A. Contini, M. L. Gelmi, L. Lo Presti, R. Soave and E. Erba, *J. Org. Chem.*, 2014, **79**, 3094.

9 (*a*) N. Zhou, X. Gao, Y. Lv, J. Cheng, W. Zhou and K. Liu, *J. Pept. Sci.*, 2014, **20**, 868; (*b*) M. K. Chung, S. J. Lee, M. L. Waters and M. R. Gagné, *J. Am. Chem. Soc.*, 2012, **134**, 11430; (*c*) D. Zanuy, G. Ballano, A. I. Jiménez, J. Casanovas, N. Haspel, C. Cativiela, D. Curcó, R. Nussinov and C. Alemán, *J. Chem. Inf. Model.*, 2009, **49**, 1623.

10 (*a*) A. Ruffoni, N. Ferri, S. K. Bernini, C. Ricci, A. Corsini, I. Maffuccci, F. Clerici and A. Contini, *J. Med. Chem.*, 2014,

57, 2953; (*b*) A. Ruffoni, A. Contini, R. Soave, L. Lo Presti, I. Esposto, I. Maffucci, D. Nava, S. Pellegrino, M. L. Gelmi and F. Clerici, *RSC Adv.*, 2015, **5**, 32643.

11 (*a*) H. Imai, K. Kaira, N. Oriuchi, K. Shimizu, H. Tominaga, N. Yanagitani, N. Sunaga, T. Ishizuka, S. Nagamori, K. Promchan, T. Nakajima, N. Yamamoto, M. Mori and Y. Kanai, *Anticancer Res.*, 2010, **30**, 4819; (*b*) C. S. Kim, S.-H. Cho, H. S. Chun, S.-Y. Lee, H. Endou, Y. Kanai and D. K. Kim, *Biol. Pharm. Bull.*, 2008, **31**, 1096.

12 (*a*) M. J. Gattner, M. Ehrilch and M. Vrabel, *Chem. Commun.*, 2014, **50**, 12568; (*b*) J. C. Jewett and C. R. Bertozzi, *Chem. Soc. Rev.*, 2010, **39**, 1272; (*c*) S. Datz, C. Argyo, M. Gattner, V. Weiss, K. Brunner, J. Bretzler, C. von Schirnding, A. A. Torrano, F. Spada, M. Vrabel, H. Engelke, C. Bräuchle, T. Carella and T. Bein, *Nanoscale*, 2016, **8**, 8101.

13 P. A. Wade, K. J. Murray Jr, S. Shah-Patela and P. J. Carroll, *Tetrahedron Lett.*, 2002, **43**, 2585.

14 A. Thaqi, A. McCluskey and J. L. Scott, *Tetrahedron Lett.*, 2008, **49**, 6962.

15 J. Lu, S. C. Owen and M. S. Shoichet, *Macromolecules*, 2011, **44**, 6002.

16 E. J. Cho, H. Holback, K. C. Liu, S. A. Abouelmagd, J. Park and Y. Yeo, *Mol. Pharm.*, 2013, **10**, 2093.

17 (*a*) K. Wutricht, *NMR of Protein and Nucleic Acids*, Wiley, New York, 1986; (*b*) G. L. Millhauser, C. J. Stenland, P. Hanson, K. A. Bolin and F. J. M. van de Ven, *J. Mol. Biol.*, 1997, **267**, 963; (*c*) G. L. Millhauser, *Biochemistry*, 1995, **34**, 3873.

18 (*a*) J. D. Augspurger, V. A. Bindra, H. A. Scheraga and A. Kuki, *Biochemistry*, 1995, **34**, 2566; (*b*) N. J. Baxter and M. P. Williamson, *J. Biomol. NMR*, 1997, **9**, 359; (*c*) G. M. Bonora, C. Mapelli, C. Toniolo, R. R. Wilkening and E. S. Stevens, *Int. J. Biol. Macromol.*, 1984, **6**, 179; (*d*) E. K. S. Vijayakumar and P. Balaram, *Biopolymers*, 1983, **22**, 2133.

19 (*a*) R. Banerjee, S. Chattopadhyay and G. Basu, *Proteins: Struct., Funct., Bioinf.*, 2009, **76**, 184; (*b*) G. Merutka, H. J. Dyson and P. E. Wright, *J. Biomol. NMR*, 1995, **5**, 14.

20 Peptide **1** $\Delta CONH_2$ ($\Delta\delta/\Delta T$) = 5.7 ppb K^{-1} in CD_3OH, $\Delta CONH_2$ ($\Delta\delta/\Delta T$) = 2.0 ppb K^{-1} in H_2O/D_2O; peptide **2** $\Delta CONH_2$ ($\Delta\delta/\Delta T$) = 5.8 ppb K^{-1} in CD_3OH, $\Delta CONH_2$ ($\Delta\delta/\Delta T$) = 3.5 ppb K^{-1} in H_2O/D_2O, see ESI: Tables TS3–TS6.†

21 G. Jung, H. Bruckner, R. Bosch, W. Winter, H. Schaal and J. Strhle, *Liebigs Ann. Chem.*, 1983, 1096.

22 R. Oekonomopulos, G. Jung and D. Leibfritz, *Tetrahedron*, 1982, **38**, 2157.

23 S. J. Pike, V. Diemer, J. Raftery, S. J. Webb and J. Clayden, *Chem.–Eur. J.*, 2014, **20**, 15981.