

# Critical Self-assembly Concentration of Bolaamphiphilic Peptides and Peptide Hybrids Determined by Fluorescence Measurements

Marco Martari\*<sup>‡</sup> and Ronald D. Sanderson

*Department of Chemistry and Polymer Science, Stellenbosch University, Private Bag X1, Matieland, 7602 South Africa.*

Received 6 November 2007, revised 20 February 2008, accepted 20 February 2008.

## ABSTRACT

The study of the self-assembly properties of peptides and proteins is important for the understanding of molecular recognition processes and for the rational design of functional biomaterials. Novel bolaamphiphilic peptides and peptide hybrids incorporating non-natural aminoacids were designed around a model lysine/leucine-rich peptide with the intention to study their self-assembly behaviour. Steady-state fluorescence measurements using pyrene as fluorescent probe were adapted to the determination of the critical self-assembly concentrations (CSACs) of these amphiphilic peptides and peptide hybrids. Different experimental conditions were studied. The morphology of the peptide aggregates was evaluated by scanning electron microscopy (SEM). Concentration and pH have been revealed to play a key role in the control of the process. Peptides presented different three-dimensional supramolecular arrangements that were correlated with their aminoacid compositions (specifically considering the presence of tyrosine and proline) and CSAC values.

## KEYWORDS

Peptide aggregation, self-assembly, critical self-assembly concentration, fluorescence, pyrene.

## 1. Introduction

Nature provides scientists with many examples of self-organization processes, from protein folding and unfolding to DNA and RNA expression, from lipid vesicles to membrane formation.<sup>1</sup> Self-assembly is an energetically-favoured process in which different molecules interact with non-covalent bonds: hydrogen bonds, ionic bonds, water mediated hydrogen bonds, hydrophobic interactions and van der Waals forces.<sup>2</sup> Efforts have been made in the last decade to mimic natural self-assembly processes and create macroscopic structures starting from the atoms from which they are formed (the so-called 'bottom-up' approach).<sup>3</sup> Many molecules are studied for their self-assembly properties, such as surfactants and lipids, proteins and peptides, amphiphilic and bolaamphiphilic compounds. In many cases the interest is in how to regulate the process itself and in the understanding of the key elements that control it. The possibility to trigger the aggregation, e.g. by using pH modification,<sup>4–6</sup> metal ions,<sup>7</sup> light,<sup>8</sup> or temperature,<sup>9</sup> allows one to create smart materials which can only be formed in response to precise stimuli.<sup>1</sup>

In the present study we focused our attention on the self-assembly behaviour of bolaamphiphilic peptides and peptide hybrids in water (where the term 'bolaamphiphile' refers to molecules that are formed by two hydrophilic groups connected by a hydrophobic skeleton).<sup>10</sup> Several years ago, peptide bolaamphiphiles with antimicrobial activity were developed from the active core of an insect defensin, sapecin B.<sup>11</sup> The general structure was made of different combinations of lysine and leucine, according to the following motif: H-KLK(L)<sub>n</sub>KLK-NH<sub>2</sub>, where  $n = 3, 4, 5$ .<sup>12</sup> To enhance the amphiphilic character, the poly-leucine chain was exchanged with  $\omega$ -aminoacids (AAs) that

have an aliphatic chain between the two functional groups. In particular, 6-aminohexanoic acid (6-Ahx) and 9-aminononanoic acid (9-Anc) were used and four peptides derived from this modification (KL1, KL2, KL3 and KL4).<sup>13</sup> The library was extended to study the influence of different AAs on the aggregation behaviour. Other structures were generated starting from the same pattern, using the non-natural AA ornithine instead of lysine (OL1) and the aromatic AA tyrosine instead of leucine (OY1). Both OL1 and OY1 included 9-Anc. To assess the importance of the secondary structure two other peptides were designed (OL2 and OY2) presenting similarities to OY1 and OL1 except for a glycyl-prolyl-glycine tripeptide instead of the linear 9-Anc.<sup>14</sup> The compounds used in this study are reported in Table 1.

Several techniques are commonly used to study peptide aggregation in water. Well known are surface tension measurements by axisymmetric drop shape analysis-profile (ADSA-P),<sup>15</sup> thioflavine T<sup>16</sup> and Congo red binding<sup>17</sup> together with microscopy observations. Steady-state fluorescence measurements are generally used to determine the critical micelle concentrations (CMCs) of surfactants but they are not very common in peptide chemistry. Their use in peptide chemistry was therefore investigated by using the abovementioned series of compounds with the aim of determining their critical self-assembly concentrations (CSACs) in water. These measurements are based on the use of pyrene as fluorescence probe. Pyrene is a very hydrophobic molecule with low water solubility.<sup>18</sup> In the presence of micelles and other macromolecular assemblies, the pyrene molecule is preferentially solubilized in the interior hydrophobic regions of these aggregates. The relative intensity of each peak comprising the fluorescence spectrum of pyrene depends strongly on the polarity of the microenvironment. The relative intensity of the third band ( $I_3$ , at 384 nm) increases with increased polarity of the

\* To whom correspondence should be addressed. E-mail: marco.martari@gmail.com

<sup>‡</sup>Present address: Johns Hopkins University, School of Medicine, Baltimore, MD, USA.

**Table 1** Peptides and peptide hybrids used in this study.

| Sequence <sup>a</sup>                                 | Ref | Net charge <sup>b</sup> | Mass (calcd.)/Da | Mass (found)/Da |
|-------------------------------------------------------|-----|-------------------------|------------------|-----------------|
| H-Lys-Leu-Lys-6-Ahx-Lys-Leu-Lys-OH                    | KL1 | +4                      | 870.65           | 870.51          |
| H-Lys-Leu-Lys-9-Anc-Lys-Leu-Lys-OH                    | KL2 | +4                      | 912.70           | 912.63          |
| H-Lys-Leu-Lys-6-Ahx-Lys-Leu-Lys-NH <sub>2</sub>       | KL3 | +5                      | 869.67           | 869.63          |
| H-Lys-Leu-Lys-9-Anc-Lys-Leu-Lys-NH <sub>2</sub>       | KL4 | +5                      | 911.71           | 911.32          |
| H-Orn-Leu-Orn-9-Anc-Orn-Leu-Orn-NH <sub>2</sub>       | OL1 | +5                      | 855.65           | 855.56          |
| H-Orn-Tyr-Orn-9-Anc-Orn-Tyr-Orn-NH <sub>2</sub>       | OY1 | +5                      | 955.61           | 955.61          |
| H-Orn-Leu-Orn-Gly-Pro-Gly-Orn-Leu-Orn-NH <sub>2</sub> | OL2 | +5                      | 911.15           | 911.52          |
| H-Orn-Tyr-Orn-Gly-Pro-Gly-Orn-Tyr-Orn-NH <sub>2</sub> | OY2 | +5                      | 1011.57          | 1011.52         |

<sup>a</sup> Lys, lysine; Leu, leucine; Orn, ornithine; Tyr, tyrosine; Gly, glycine; Pro, proline.<sup>b</sup> Net charge at pH 7.

environment (aggregated molecules) while the relative intensity of the first band ( $I_1$ , at 373 nm) is not affected by increased polarity. The ratio  $I_1/I_3$  is therefore used to monitor the changes in environmental polarity of aggregating molecules in water,<sup>19,20</sup> i.e. their status as aggregates or disaggregated peptides.

CSAC determination by steady-state fluorescence measurements was combined with scanning electron microscopy (SEM) and cryo-fracture SEM (cryo-SEM) observations to study the morphology of the peptide aggregates. SEM micrographs were used to confirm the results obtained by the fluorescence measurements and define a correlation between morphology and several structural elements (such as the presence of tyrosine and proline).

## 2. Results and Discussion

The CSAC values for the synthesized molecules were determined by using steady-state fluorescence measurements and they are reported in Table 2. The changes of pyrene  $I_1/I_3$  ratios with peptide concentration were followed both in water (pH 6.5) and in 0.1 % triethylamine (TEA) (pH 10). The pH measurements performed during the experiment showed that the pH was independent of the peptide concentration in the range of concentrations used. Additionally, analytical reversed-phase high-pressure liquid chromatography (RP-HPLC) on the purified peptides revealed no detectable impurities and ruled out the presence of contaminants as the force that activated the aggregation (data not shown). Important differences in the aggregation profile were found within the series of peptides and peptide hybrids and they were related to the different aminoacid compositions.<sup>21</sup> A four-parameter logistic model was used to curve-fit the  $I_1/I_3$  ratios plotted against the logarithms of the concentrations and a correlation factor ( $R^2$ ) varying between  $0.95 \pm 0.01$  and  $0.99 \pm 0.01$  was found for each curve. Because not enough material was available, it was not possible to determine the CSAC values in water for some compounds. Only for compounds OY1 and OY2 was it possible accurately to establish the CSAC values under both conditions. For the other compounds the CSAC in water was determined to be much higher than the starting concentration, which corresponded to high  $I_1/I_3$  ratios ( $\sim 1.6$ , related to the presence of disaggregated peptides in solution) thus precluding the establishment of the CSAC in that environment. The concentration of the peptides/peptide hybrids in solution (both in water and 0.1 % TEA) was the driving force for the aggregation and played a primary role in the aggregation process in both environments.<sup>15,22</sup>

A second important factor in determining the propensity towards aggregation was the pH. Because of the presence of primary amino groups on the side-chains of several AA (such as Orn and Lys), an acidic/neutral pH could be translated into a net

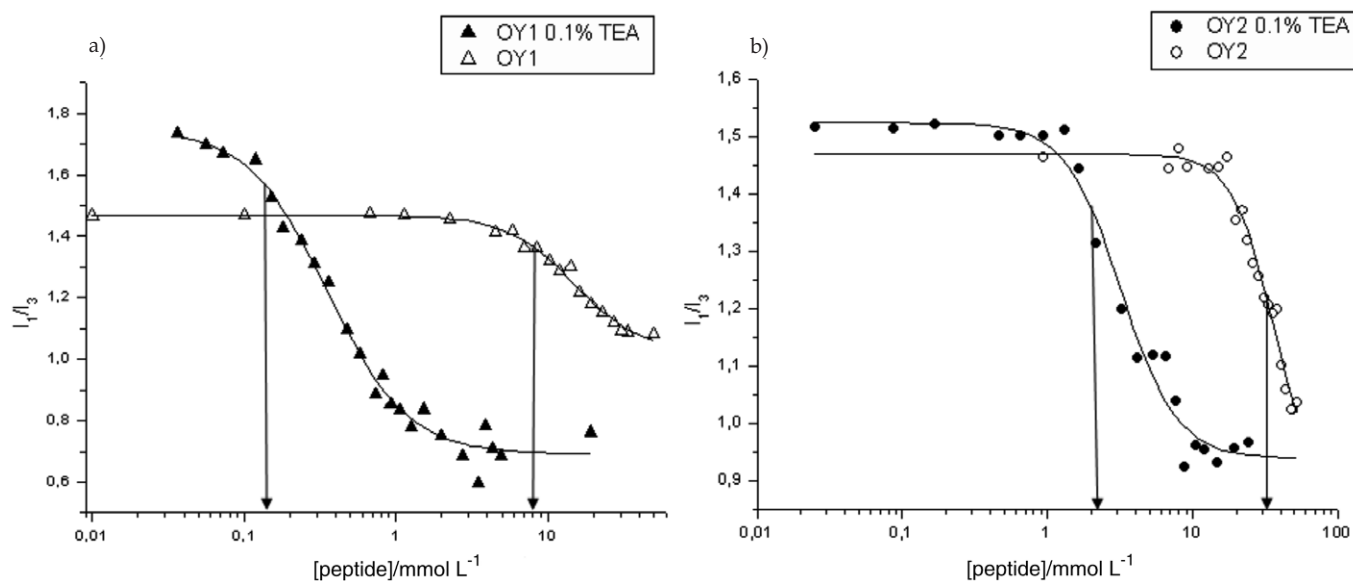
**Table 2** CSAC values in water and 0.1 % TEA determined by steady-state fluorescence measurements.

| Peptide | CSAC/mmol L <sup>-1</sup> | CSAC (0.1 % TEA)/mmol L <sup>-1</sup> |
|---------|---------------------------|---------------------------------------|
| KL1     | >45.00                    | 6.50                                  |
| KL2     | >45.00                    | 8.16                                  |
| KL3     | >45.00                    | 5.84                                  |
| KL4     | >45.00                    | 7.45                                  |
| OL1     | >45.00                    | 3.30                                  |
| OY1     | 8.64                      | 0.14                                  |
| OL2     | >45.00                    | 7.60                                  |
| OY2     | 30.02                     | 2.10                                  |

positive charge on the molecules. This positive charge could ultimately lead to strong repulsive electrostatic forces with a resultant high CSAC. On the other hand, a basic environment facilitates the formation of hydrogen bonds and hydrophobic interactions, lowering the CSAC.<sup>23</sup> This tendency was clearly pointed out by the comparison of the aggregation profiles in water and 0.1 % TEA of OY1 and OY2 (see Fig. 1). For OY1, the shift to higher CSAC values was very striking, and the CSAC in water is more than 60 times greater than in 0.1 % TEA (8.64 mmol L<sup>-1</sup> and 0.14 mmol L<sup>-1</sup>, respectively). For OY2 the difference was smaller, but there was still a decrease of 15 times in the CSAC value going from water to 0.1 % TEA (from 30.02 mmol L<sup>-1</sup> to 2.10 mmol L<sup>-1</sup>).

The effect of pH was also considered to be fundamental in the aggregation process of KL1/KL2 and KL3/KL4. They present different C terminus functionalities, acid in KL1/KL2 and amide in KL3/KL4. In a basic environment, KL1 and KL2 have negative charges that KL3 and KL4 do not possess. Moreover, they also lose the possibility of creating intermolecular H bonds at the C terminus. KL1 and KL2 were therefore expected to show higher CSAC values than KL3 and KL4. Nonetheless, a comparison of the aggregation behaviours of peptides KL1, KL2, KL3 and KL4 attested to only small differences in their CSACs (between 6 and 8 mmol L<sup>-1</sup>). These small differences may indicate that the functionality at the C terminus is not a critical factor for the aggregation (see Fig. 2).

By contrast, the structure of the compounds in solution (e.g. in OL2 and OY2) and the presence of hydrophobic AAs (e.g. in OY1 and OY2) gave much stronger indications of the significant parameters governing the self-assembly. Proline strongly influenced the aggregation of the peptides. The OY2 and OL2 sequences are similar to those of the corresponding hybrid peptides OY1 and OL1, except for the presence of the glycyl-prolyl-glycine tripeptide between the two charged heads, which replaces the 9-aminononanoic acid. OY2 and OL2 are



**Figure 1** Effect of pH on the aggregation process of OY1 (a) and OY2 (b). The arrows graphically indicate the CSAC values.

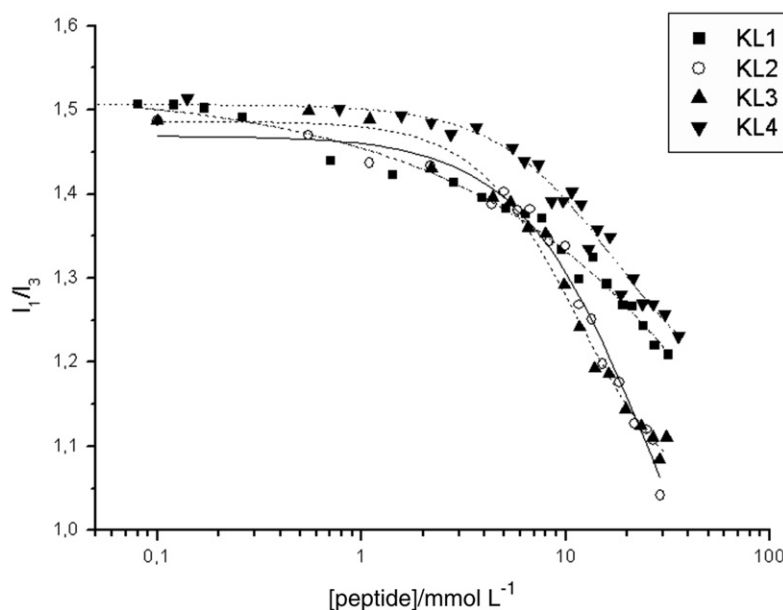
characterized by high CSAC values (the CSAC of OY2 is 15 times higher than that of OY1 and the CSAC of OL2 is twice as large as that of OL1). Because of the introduction of a  $\beta$ -turning AA (Pro), the three-dimensional arrangement of the molecules in solution is altered and peptides are forced into a structure that decreases the possibility of intermolecular interactions. Differences in CSAC values and aggregation behaviour between OY1 and OY2, and between OL1 and OL2, are shown in Fig. 3a and Fig. 3b.

The use of more hydrophobic AAs tends to enhance the self-organization process of aggregating molecules.<sup>21,24,25</sup> This trend was clearly noticed in some of the compounds studied. OY1 and OY2 are derived from OL1 and OL2 and differ only in the presence of tyrosine instead of leucine (Leu  $\rightarrow$  Tyr substitution). The presence of a more hydrophobic AA caused a decrease in their CSAC values compared with leucine-containing molecules. The tyrosine-containing compounds did aggregate at lower concentrations than the leucine-containing homologues (more than 23 times lower for OY1 and about 4 for OY2) (Fig. 3c

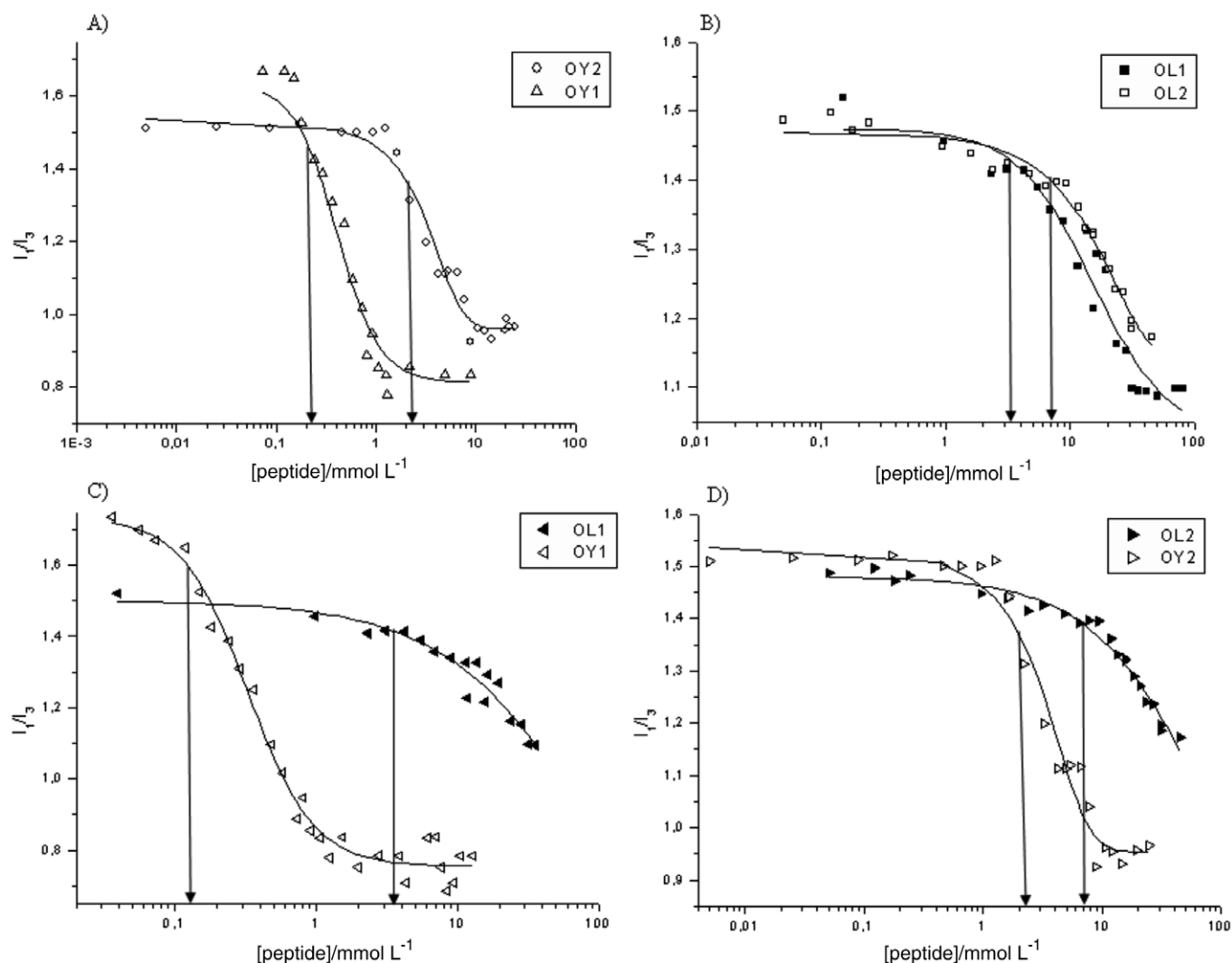
and Fig. 3d). The use of a more hydrophobic backbone (going from 6-Ahx to 9-Anc) did not seem to cause an increase in the aggregation behaviour and KL1, KL2, KL3 and KL4 had similar CSAC values.

The fluorescence emission spectrum of pyrene was also observed around 470 nm, where the excimer formation can be detected by the presence of a broad unstructured emission. No emission was observed, indicating that the aggregates could only accommodate the inclusion of monomeric pyrene and not  $\pi$ -stacked pyrene dimers.

The microscopic study that was carried out confirmed that these bolaamphiphilic compounds self-assemble in response to a pH increase (of 3.5 pH units), when the net charge of the peptide molecules is near zero.<sup>23</sup> It has already been shown that amino acid composition strongly influences the morphology of peptide assemblies. The type of non-polar AA and the size of the side-chains both influence the self-assembly process.<sup>26</sup> In the present study peptide assemblies presented different dimensions and morphologies (see Fig. 4). OY1 and OY2 (peptides with low



**Figure 2** Comparison of the aggregation behaviour of KL1, KL2, KL3 and KL4 in 0.1 % TEA.



**Figure 3** The role of three-dimensional structure on the aggregation behaviour of OY1/OY2 (a) and OL1/OL2 (b) in 0.1 % TEA, and the effect of Leu  $\rightarrow$  Tyr substitution on the aggregation behaviour of OL1/OY1 (c) and OL2/OY2 (d) in 0.1 % TEA. The arrows graphically indicate the CSAC values.

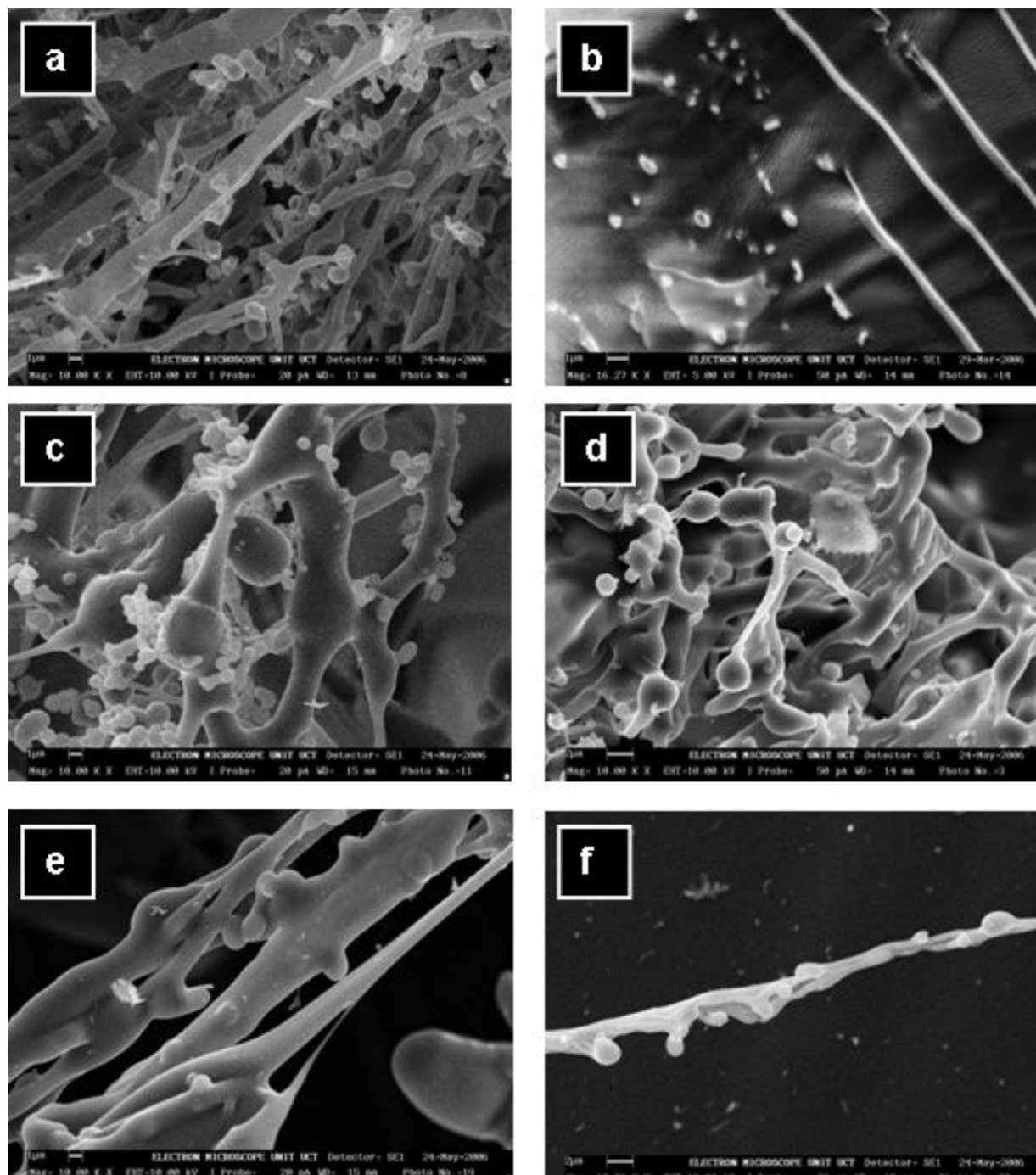
CSACs) showed a network of fibres with uniform longitudinal growth and distribution, diameter smaller than 1  $\mu\text{m}$  and few three-way junctions (branches) (Figs. 4a and 4b). Compounds OL1 and OL2 (intermediate CSACs) presented larger structures (around 1–2  $\mu\text{m}$ ) and many three-way junctions and connections creating a sponge-like structure (Figs. 4c and 4d). Additionally, KL1, KL2, KL3 and KL4 (with the highest CSACs), presented microtubular structures with similar dimensions to OL1 and OL2, often associated with vesicles and entangled rod-like micelles (Figs. 4e and 4f). It appears from the SEM micrographs that the presence of tyrosine had a significant role in driving the aggregation towards the formation of uniform tubular structures (compare OY1 and OL1 in Fig. 4) most likely for the  $\pi$ -stacking of the side chains.<sup>7,25</sup> Additionally, the disordering influence of Gly-Pro-Gly is also evident, especially in OY1 and OY2. The SEM micrograph of OY2 showed an increased number of vesicles forming from the microtubes compared with OY1, vesicles that are related to more dynamic three-dimensional arrangements.<sup>27</sup> The difference between OL1 and OL2 is not as clear as in the previous example (OY1/OY2). OL2 presented larger supramolecular structures and numerous vesicles.

### 3. Conclusions

Steady-state fluorescence measurements using pyrene as fluorescent probe were used to determine CSAC values of the

synthesized bolaamphiphilic peptides in aqueous solutions (water and water/0.1 % TEA). The bolaamphiphilic peptides were shown to assemble in response to a pH modification<sup>23</sup> and the self-assembly was clearly driven by the concentration of the molecules in solution.<sup>15,22</sup> The use of steady-state fluorescence measurements permitted small differences in the aggregation profiles of the synthesized compounds to be underlined and related to their aminoacid compositions. As expected, the presence of hydrophobic aminoacids was shown to be an important factor in enhancing the self-assembly process even at low concentration.<sup>29</sup> Interestingly, the three-dimensional structure (in this case represented by the presence or absence of proline) also played a key role in regulating the process. Proline introduced a turn in the structure and the packaging of the bolaamphiphilic molecules was negatively affected.

As previously stated, pyrene is commonly used as fluorescence probe to determine the CMCs of surfactants. Surfactant micelles are rather homogeneous structures compared with the diversity of structures formed by these bolaamphiphilic peptides. Pyrene could therefore have differential sensitivity for the different peptide assemblies. As far as is known, to date no other studies on peptides have been done using pyrene, and some method dependence in the fluorescence measurements on the synthesized molecules cannot be excluded. In any event, it must be remembered that CMC determination commonly displays a



**Figure 4** SEM images of dried peptide assemblies from OY2 (a), OL2 (c), OL1 (d), KL1 (e) and KL4 (f). Cryo-SEM picture of assemblies from OY1 (b). The differences in size and morphology among the series of peptide assemblies were related to the presence of tyrosine/proline and to the CSAC values.

certain degree of method dependence and CMC values for a given molecule can differ slightly according to the method used.<sup>28</sup> Additional work could be done to address this issue for the present set of molecules by using a different technique (such as conductivity measurements) and correlating the CSAC values with a new set of data.

Cryo-SEM and SEM micrographs showed the complex three-dimensional organization of the peptide assemblies. Peptides and peptide hybrids appeared to assemble into structures with different morphologies. These differences were correlated with the presence of tyrosine ( $\pi$ -stacking of the side chains that leads to tubular structures)<sup>7,25</sup> and proline (vesicles because of the disorganizing effect), two AAs which were shown

to influence the general self-assembly process of these bolaamphiphilic compounds.

## 4. Experimental

### 4.1. Materials

Resins,  $N^9$ -fluorenylmethoxycarbonyl (Fmoc)-protected aminoacids and coupling reagents for solid phase peptide synthesis (SPPS) were supplied by Novabiochem (Laufelfingen, Switzerland). Pyrene (99 %) was supplied by Fluka (Buchs, Switzerland). Other chemicals and solvents were from Aldrich (Steinheim, Germany). Analytical grade water was obtained by filtering glass-distilled water with a Millipore Milli-Q® system (Bedford, MA, USA).

## 4.2. Methods

### 4.2.1. Peptide Synthesis

Peptide bolaamphiphiles (0.75 mmol) were synthesized by manual SPPS using the Fmoc (*N*<sup>9</sup>-fluorenylmethoxycarbonyl) polyamide protocol.<sup>29,30</sup> Peptides were synthesized as amides by using Novasyn TGR resin or as acids by using Novasyn TGA resin. The coupling step was performed by using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (2.25 mmol)/N-hydroxybenzotriazole (HOBt) (2.25 mmol)/diisopropylethylamine (DIEA) (4.50 mmol) in dimethylformamide (DMF) for 60 min. Deprotection of the loaded amino acid was achieved by treating the resin with 20 % (v/v) piperidine in DMF for 30 min.<sup>31</sup> Coupling and deprotection were monitored by the ninhydrin test<sup>32</sup> or by the chloranil test (for proline).<sup>33</sup> Cleavage of the peptides from the resin and deprotection of the side chains were carried out in trifluoroacetic acid (TFA)/H<sub>2</sub>O/triisopropylsilane (TIS) (95/2.5/2.5) for 4 h or TFA/H<sub>2</sub>O/TIS/phenol (92.5/2.5/2.5/2.5) for 6 h for tyrosine-containing peptides.<sup>30</sup> Peptides were purified by semi-preparative RP-HPLC (A = 95 % H<sub>2</sub>O/5 % acetonitrile (ACN)/0.1 % TFA and B = 95 % ACN/5 % H<sub>2</sub>O/0.1 % TFA, from 0 % B to 50 % B over 30 min) and characterized by electrospray-ionization mass spectrometry (ESI-MS) and analytical RP-HPLC. Peptides were freeze-dried after purification and stored at -20 °C.

### 4.2.2. Steady-state Fluorescence Measurements

Peptides were analytically weighed, dissolved in pyrene/water (pH 6.5 ± 0.1) or pyrene/water/0.1 % TEA (pH 10.0 ± 0.1) at a starting concentration of about 45 mmol L<sup>-1</sup>. The pyrene concentration was 0.4 μmol L<sup>-1</sup>. Samples were diluted with the same solvent directly inside the cuvette reaching a final concentration of about 1 × 10<sup>-3</sup> mmol L<sup>-1</sup>. Fluorescence emission was recorded in a 10 mm pathlength 100 μL quartz cuvette (Hellma, Mülheim, Germany) using a LS 50B luminescence spectrometer (Perkin Elmer, Waltham, MA, USA). The excitation wavelength was set at 334 nm and the emission wavelengths recorded at 373 and 384 nm (I<sub>1</sub> and I<sub>3</sub>, respectively). Excitation and emission slitwidths were set at 2.5 nm. Experiments were carried out at 25 °C. The ratio I<sub>1</sub>/I<sub>3</sub> was plotted against the logarithm of the concentration and a four-parameter logistic model was used for the curve fitting. The maximum of the first derivative of the function was taken as CSAC.<sup>34</sup>

### 4.2.3. pH Measurements

A CyberScan pH-510 pH meter (Eutech Instruments, Nijkerk, the Netherlands) was used for pH monitoring.

### 4.2.4. SEM and Cryo-SEM

Bolaamphiphilic peptides and peptide hybrids were dissolved in water/0.1 TEA (concentration above their CSACs) and left undisturbed for up to two weeks. (A) Samples were freeze-dried, coated with a thin palladium/gold layer and observed with a Leo S440 scanning electron microscope (Zeiss, Göttingen, Germany) with an accelerating voltage of 10 kV. (B) Alternatively, the aged solutions of bolaamphiphiles were frozen in liquid nitrogen in glass capillaries. The capillaries were fractured with a blade and samples observed with the same microscope (accelerating voltage of 5 kV).

## Acknowledgements

M.M. would like to thank Dr M. Rautenbach (Department of

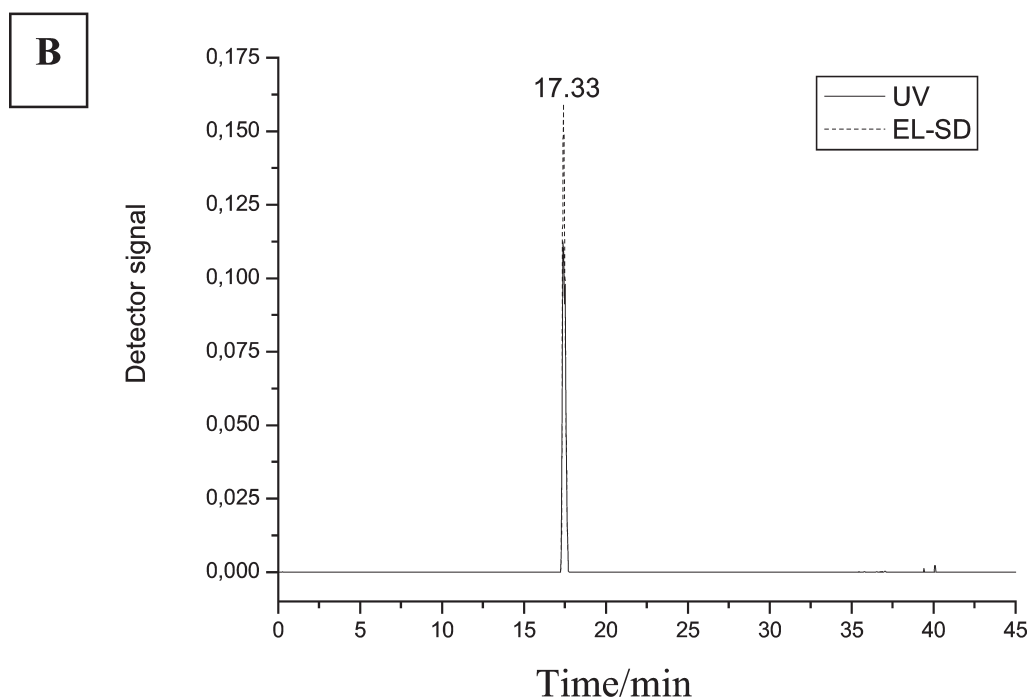
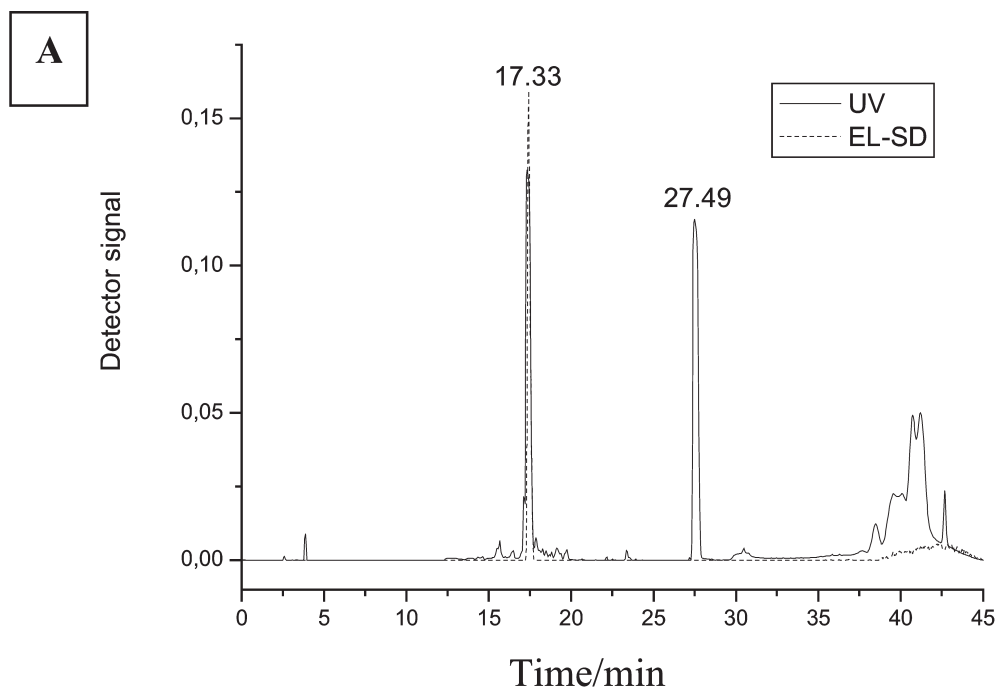
Biochemistry, Stellenbosch University) and Dr J. Juodaityte (Department of Chemistry and Polymer Science, Stellenbosch University) for helpful discussions.

## References

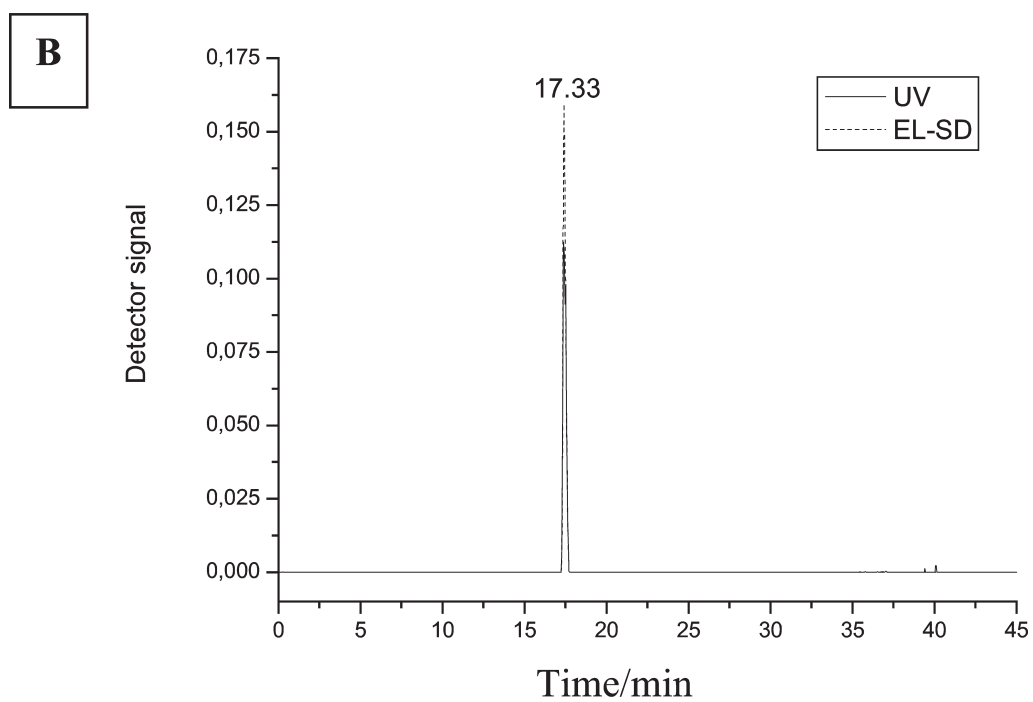
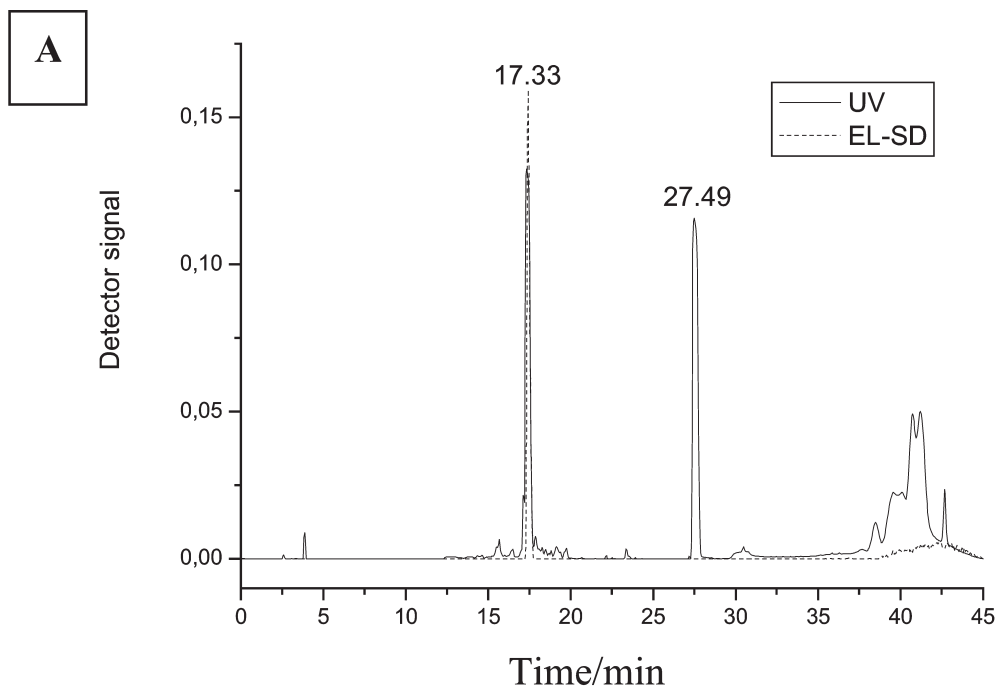
- 1 K. Rajagopal and J.P. Schneider, *Curr. Opin. Struct. Biol.*, 2004, **14**, 480–486.
- 2 S. Zhang and M. Altman, *React. Funct. Polym.*, 1999, **41**, 91–102.
- 3 S. Zhang, *Nature Biotechnol.*, 2003, **21**, 1171–1178.
- 4 A. Aggeli, M. Bell, L.M. Carrick, C.W.G. Fishwick, R. Harding, P.J. Mawer, S.E. Radford, A.E. Strong and N. Boden, *J. Am. Chem. Soc.*, 2003, **125**, 9619–9628.
- 5 R.C. Claussen, B.M. Rabatic and S.I. Stupp, *J. Am. Chem. Soc.*, 2003, **125**, 12680–12681.
- 6 K.L. Niece, J.D. Hartgerink, J.J.J.M. Donners and S.I. Stupp, *J. Am. Chem. Soc.*, 2003, **125**, 7146–7147.
- 7 H. Matsui and G.E. Douberly, *Langmuir*, 2001, **17**, 7918–7922.
- 8 J.H. Collier, B.H. Hu, J.W. Ruberti, J. Zhang, P. Shum, D.H. Thompson and P.B. Messersmith, *J. Am. Chem. Soc.*, 2001, **123**, 9463–9464.
- 9 D.J. Pochan, J.P. Schneider, J. Kretsinger, B. Ozbaz, K. Rajagopal and L.B. Haines, *J. Am. Chem. Soc.*, 2003, **125**, 11802–11803.
- 10 J.H. Fuhrhop and J. Mathieu, *Angew. Chem., Int. Ed. Engl.*, 1984, **23**, 100–113.
- 11 K. Yamada and S. Natori, *Biochem. J.*, 1994, **298**, 623–628.
- 12 J. Alvarez-Bravo, S. Kurata and S. Natori, *Biochem. J.*, 1994, **302**, 535–538.
- 13 V.B. Naidoo, *Supramolecular Structures of Biomacromolecular Assemblies*, Ph.D. thesis, University of Stellenbosch, Stellenbosch, South Africa, 2004.
- 14 M. Martari, *Structure–Function Relationships of Bolaamphiphilic Peptides and Peptide Hybrids*, Ph.D. thesis, University of Stellenbosch, Stellenbosch, South Africa, 2006.
- 15 P. Chen, *Colloids Surf. A*, 2005, **261**, 3–24.
- 16 H. LeVine III, *Protein Sci.*, 1993, **2**, 404–410.
- 17 M.R. Nilsson, *Methods*, 2004, **34**, 151–160.
- 18 R. Ninomiya, K. Matsuoka and Y. Moroi, *Biochim. Biophys. Acta*, 2003, **1634**, 116–125.
- 19 M.S. Bakshi and G.J. Kaur, *J. Colloid Interface Sci.*, 2005, **289**, 551–559.
- 20 D. Yin, W. Yang, Z. Ge and Y. Yuan, *Carbohydr. Res.*, 2005, **340**, 1201–1206.
- 21 S. Zhang, T. Holmes, C. Locksmith and A. Rich, *Proc. Natl. Acad. Sci. U.S.A.*, 1993, **90**, 3334–3338.
- 22 S.Y. Fung, C. Keyes, J. Duhamel and P. Chen, *Biophys. J.*, 2003, **85**, 537–548.
- 23 M.R. Caplan, P.N. Moore, S. Zhang, R.D. Kamm and D.A. Lauffenburger, *Biomacromolecules*, 2000, **1**, 627–631.
- 24 M.R. Caplan, E. Schwartzfarb, S. Zhang, R.D. Kamm and D.A. Lauffenburger, *Biomaterials*, 2002, **23**, 219–227.
- 25 E. Gazit, *FASEB J.*, 2002, **16**, 77–83.
- 26 K. Wang, J.D. Keasling and S.J. Muller, *Int. J. Biol. Macromol.*, 2005, **36**, 232–240.
- 27 S. Vauthey, S. Santoso, H. Gong, N. Watson and S. Zhang, *Proc. Natl. Acad. Sci. U.S.A.*, 2002, **99**, 5535–5560.
- 28 P. Messina, M.A. Morini, P.C. Schulz and G. Ferrat, *Colloid Polym. Sci.*, 2002, **280**, 328–335.
- 29 E. Atherton, M.J. Gait, R.C. Sheppard and B.J. Williams, *Bioorg. Chem.*, 1979, **8**, 351–370.
- 30 E. Atherton and R.C. Sheppard, *Solid-phase Synthesis: A Practical Approach* (D. Rickwood and B.D. Hames, eds.), 1st edn., Oxford University Press, Oxford, UK, 1989.
- 31 L.A. Carpino and G.Y. Han, *J. Am. Chem. Soc.*, 1970, **92**, 5748–5749.
- 32 E. Kaiser, R.L. Colescott, C.D. Bossinger and P.I. Cook, *Anal. Biochem.*, 1970, **34**, 595–598.
- 33 T. Christensen, *Acta Chem. Scand.*, 1979, **33B**, 763–766.
- 34 Y. Fang and F. Cheng, *Proceedings of the 2006 AIChE Annual Meeting, Session 162ag, San Francisco, CA, USA, 12–17 November 2006*, American Institute of Chemical Engineers, New York, NY, USA, 2006.

## Supplementary material to:

M. Martari and R.D. Sanderson, *S. Afr. J. Chem.*, 2008, **61**, 47–52.



**A**, RP-HPLC chromatogram of crude OY2 on a C<sub>12</sub> column. **B**, RP-HPLC chromatogram of pure OY2 under the same conditions.



A) RP-HPLC chromatogram of crude OL1 on a C<sub>12</sub> column. B) RP-HPLC chromatogram of pure OL1 under the same conditions.

**RP-HPLC system characteristics:**

A Kontron 500 HPLC System (Kontron Instruments, Milan, Italy) comprising a Kontron Bio-Tek 522 dual solvent pump, a Kontron HPLC 560 autosampler, a Kontron degasser 3493, a Kontron HPLC 535 dual wavelength UV detector and a PL-ELS 2100 EL-SD (Polymer Laboratories, Church Stretton, UK). The column was eluted at 30 °C. The UV detector was set at 220 nm. The parameters for the EL-SD detector were set as follows: nebuliser 70 °C, evaporator 40 °C, gas flow (N<sub>2</sub>) 1 L min<sup>-1</sup>. The system was controlled by Geminix software (Goebel-Instrumentelle Analytik, Au, Germany).

Analytical RP-HPLC was performed on a C<sub>12</sub> Proteo Jupiter column (250 x 4.6 mm, 4 µm particle size, 90 Å pore size) (Phenomenex, Torrance, CA, USA).

Elution profile: A= 95% H<sub>2</sub>O/5% ACN/0.1% TFA and B= 95% ACN/5% H<sub>2</sub>O/0.1% TFA, from 0% B to 50% B over 30 min.