

## Amphiphilic CCK peptides assembled in supramolecular aggregates: structural investigations and *in vitro* studies

Antonella Accardo, Anna Morisco, Pasquale Palladino, Rosanna Palumbo, Diego Tesaro and Giancarlo Morelli\*

Received 18th October 2010, Accepted 16th November 2010

DOI: 10.1039/c0mb00238k

Supramolecular aggregates obtained by self-aggregation of five new cationic amphiphilic CCK8 peptides have been obtained in water solution and characterized for: (i) aggregate structure and stability; (ii) CCK8 peptide conformation and bioavailability on the external aggregate surface; and (iii) for their cell binding properties. The cationic amphiphilic CCK8 peptides self-aggregate giving a combination of liposomal and micelle structures, with radii ranging between ~60 nm and ~90 nm, and between ~5 and ~10 nm, respectively. The presence of CCK8 peptide well-exposed on the aggregate surface is demonstrated by fluorescence measurements. Peptide conformation changes in the five supramolecular aggregates: the CCK8 conformational behaviour is probably induced by the presence of three charged lysine residues close to the bioactive peptide sequence. Only aggregates in which the CCK8 peptide presents a structural arrangement similar to that found for the same peptide in DPC micelles give promising binding properties to CCK2-R receptors overexpressed by transfected A431 cells. Chemical modifications on the CCK8 N-terminus seem to play an important role in stabilizing the peptide active conformation, either when the peptide derivative is in monomeric or in aggregate form. For their easy preparation procedures and their binding properties, supramolecular aggregates based on cationic peptide amphiphiles can be considered as promising candidates for target selective drug carriers on cancer cells.

### 1. Introduction

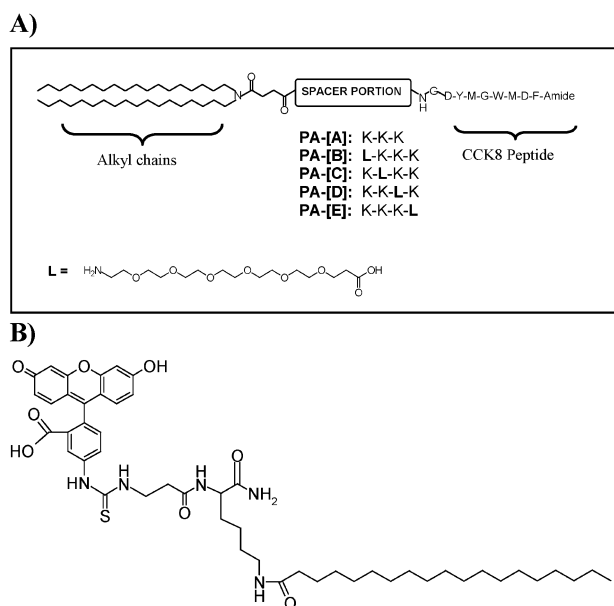
A major goal in current medical treatment is the selective delivery of drugs and other active compounds directly to a target tissue.<sup>1</sup> This would allow the reduction of undesired effects on non target organs and the increase of therapeutic efficacy on the desired tissues. Drug delivery procedures, aimed at achieving this goal, have been investigated particularly for the treatment of cancer.<sup>2</sup> Several receptors overexpressed on tumour cells can provide homing properties for therapeutic or diagnostic molecules containing binding motifs for the specific receptor.<sup>3,4</sup> Peptides able to recognize in a selective way these receptors may be exploited to address a simple entity, such as an anticancer drug, to target cells at higher concentrations compared to non-target cells.<sup>5</sup>

A more complex and challenging objective would be to adopt a similar scheme to deliver peptide-exposing supramolecular aggregates, such as micelles or liposomes, to a tissue of interest.<sup>6,7</sup> Such aggregates could be utilized as nanocarriers for large amounts of drugs or other substances contained in the inner cavity or exposed on the external surface of the supramolecular

aggregate.<sup>8–10</sup> Examples of target specific supramolecular aggregates, employed as carriers of drugs or metal complexes, include micelles and liposomes loaded with poorly soluble anticancer agents or bearing Gd(III) complexes.<sup>11–14</sup> To allow the selective target of the above described supramolecular aggregates on cancer cells, it is mandatory that the covering peptide remains well-exposed on the external surface of the aggregates, preserving the right conformation for receptor binding. Moreover the aggregates should have high *in vivo* stability and the appropriate size and shape to remain in the blood before target binding.

Recently, we reported the synthesis and the structural characterization of (C18)<sub>2</sub>L5CCK8 Peptide Amphiphile (PA) in isolated or aggregated form. This molecule contains a dioctadecyl moiety as a hydrophobic segment, a spacer consisting of five units of 8-amino-3,6-dioxaoctanoic acid and glycine residue, and the carboxyl terminal sequence of the peptide hormone cholecystokinin, CCK8 (Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-Amide), as a bioactive hydrophilic peptide.<sup>15</sup> (C18)<sub>2</sub>L5CCK8 is able to self-assemble, spontaneously, in well-ordered nanostructures in aqueous solution. The reported supramolecular aggregates based on PA, could be employed for target-selective delivery of anticancer drugs, such as the cytotoxic doxorubicin drug, on a biological target, through the bioactive peptide exposed on the external surface. In fact, the bioactive CCK8 peptide is able to bind, with high affinity, both CCK<sub>1</sub>-R and

Department of Biological Sciences, CIRPeB University of Naples "Federico II", & IBB CNR, Via Mezzocannone 16, 80134 Naples, Italy. E-mail: [gmorelli@unina.it](mailto:gmorelli@unina.it); Fax: +39 081-2536642



**Scheme 1** Panel A: schematic representation of cationic PAs. The amino acid sequence of CCK8 peptide is reported by using the one-letter amino acid code. The hexaoxahene linker (21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid), L, is also reported. Panel B: schematic representation of C18-FITC.

CCK<sub>2</sub>-R cholecystinin receptors over-expressed by cancer cells in several human tumours.<sup>16</sup>

In this way the entrapped drug is mainly delivered in proximity of the tumour, thus reducing the well-expected toxic side effects on non-target organs. Although short peptides tend to be flexible in solution,<sup>17</sup> CCK8 has been reported to have some interchanging turn conformations stabilized in the presence of the membrane-like surfactant.<sup>18</sup> In contrast, (C18)<sub>2</sub>L5CCK8 PA, when self-assembled in nanostructures, presents a much more defined conformation likely due to the interactions of peptide side chains that remain very close to each other in the nanostructure hydrophilic shell.<sup>19</sup> However, this folding induced by the aggregation process could represent a trouble in preserving the peptide biological activity and its accessibility to the interacting receptor. In fact, according to the theory of membrane-bound pathway, CCK8 needs to assume a pseudo- $\alpha$ -helix conformation for the interaction with its G-protein coupled receptors.<sup>20–24</sup>

Here we report a complete characterization of supramolecular aggregates composed of five novel amphiphilic peptides based on CCK8. In these new compounds, reported in Scheme 1, the hydrophobic and the hydrophilic moieties are unchanged with respect to (C18)<sub>2</sub>L5CCK8, while new spacer moieties, represented by several lysine residues and by a Ahoh linker, are introduced in order to stabilize peptide conformations that are able to guarantee receptor binding also in the aggregate forms.

## 2. Materials and methods

### 2.1 Chemicals

Protected N<sup>z</sup>-Fmoc-amino acid derivatives, coupling reagents, and Rink amide MBHA resin were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). The Fmoc-21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid (Fmoc-Ahoh-OH)

was purchased from Neosystem (Strasbourg, France). The *N,N*-dioctadecylsuccinamic acid was synthesized according to published methods.<sup>25</sup> FITC isomer I, BSA, PBS were purchased from Sigma-Aldrich (Milan, Italy). DMEM, FCS, L-glutamine were purchased from Lonza (Milano, Italy). All other chemicals were commercially available from Sigma-Aldrich or Fluka (Buchs, Switzerland) or LabScan (Stillorgan, Dublin, Ireland) and were used as received unless otherwise stated. Preparative RP-HPLCs were carried out on a LC8 Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV lambda-Max Model 481 detector using a Phenomenex (Torrance, CA) C4 (300 Å, 250 × 21.20 mm, 5 μm) column eluted with H<sub>2</sub>O/0.1% TFA (A) and CH<sub>3</sub>CN/0.1% TFA (B) from 20% to 95% over 20 minutes at 20 mL min<sup>-1</sup> flow rate. Purity and identity were assessed by analytical LC-MS analyses by using Finnigan Surveyor MSQ single quadrupole electrospray ionization (Finnigan/Thermo Electron Corporation San Jose, CA), column: C4-Phenomenex eluted with H<sub>2</sub>O/0.1% TFA (A) and CH<sub>3</sub>CN/0.1% TFA (B) from 20% to 95% over 20 minutes at 1 mL min<sup>-1</sup> flow rate. Mass spectra were carried out on a MALDI-TOF Voyager-DE Perseptive Biosystem (Framingham, MA) apparatus using the  $\alpha$ -cyano-4-hydroxycinnamic acid as the matrix and bovine insulin as the internal reference. Concentrations of solutions containing peptide surfactant were determined by absorbance measurement on a UV-vis Jasco V-5505 spectrophotometer equipped with a Jasco ETC-505T Peltier temperature controller with a 1 cm quartz cuvette (Hellma). The monodimensional <sup>1</sup>H NMR and <sup>13</sup>C-NMR spectra were performed on a Varian (Palo Alto, CA) 400 MHz spectrometer. Fluorescence spectra were recorded at room temperature on a Jasco Model FP-750 spectrofluorophotometer in 1.0 cm path length quartz cell. Equal excitation and emission bandwidths were used throughout experiments, with a recording speed of 125 nm min<sup>-1</sup> and automatic selection of the time constant. Dynamic light scattering measurements were carried out by miniDAWN TREOS Wyatt Technology (CA, USA).

### 2.2 Synthesis of C<sub>18</sub>H<sub>37</sub>CONHLYs-(FITC)CONH<sub>2</sub> (C18-FITC)

The fluorescent monomer was synthesized on solid phase using Rink-amide (MBHA) resin (0.85 mmol g<sup>-1</sup>; 0.2 mmol, 0.235 g) as the polymeric support. After the swelling of the resin in 3 mL of DMF for 1 h, the Fmoc protecting group was removed by adding a mixture of piperidine/DMF 30/70. The carboxylic function of Fmoc-Lys(Mtt)-OH (0.8 mmol, 0.500 g) was activated by 1 equiv. of PyBop, HOBt and 2 equiv. of DIPEA in DMF. The solution was added to the resin and the slurry suspension was stirred for 1 h. The coupling of the lysine residue was performed twice and after filtration, the coupling was checked by the Kaiser colorimetric test. The solution was filtered and the resin washed with three portions of DMF and three portions of DCM. The Mtt protecting group was removed by treatment of the resin with DCM/TIS/TFA (94 : 5 : 1) mixture, stirring for 5 min. This procedure was repeated several times until the solution became colorless. The resin was washed three times by DCM and three times by DMF. Nonadecanoic acid (2 equiv., 0.252 g) was

coupled under standard conditions (HOBT/PyBop/DIPEA 1/1/2), and the reaction monitored by Kaiser test. Then, after the removal of N-terminal Fmoc protecting group Fmoc- $\beta$ -Ala-OH and FITC were coupled in sequence. The FITC coupling was performed overnight at room temperature by using HATU/DIPEA (1/2 with respect to FITC) as activating agents. The cleavage from the resin and deprotection of the *t*Bu protecting groups were obtained stirring for 2 h in TFA containing 2.5% (v/v) water and 2.0% (v/v) TIS as scavengers at room temperature. The crude product was slowly precipitated at 0 °C by adding water drop-wise. The crystalline solid was washed several times with small portions of cold water. The product was identified by mass spectra (electrospray ionization ESI) and NMR spectroscopy.

C18-FITC: MS (ESI<sup>+</sup>):  $m/z$  (%): 886 (100) [M - H<sup>+</sup>]

<sup>1</sup>H-NMR (CDCl<sub>3</sub>/CD<sub>3</sub>OD 50/50) (chemical shifts in  $\delta$ , CD<sub>3</sub>OD as an internal standard 3.55) = [8.4 (d,  $J$  = 14 Hz, 1H), 7.90 (s, 1H), 7.41 (d,  $J$  = 9 Hz, 1H), 7.12 (s, 1H), 7.02 (s, 1H), 6.90 (s, 1H), 6.85 (d,  $J$  = 12 Hz, 1H), 6.78 (s, 1H) aromatic signals] 4.75–4.65 (br m, 1H, CH Lys $\alpha$ ), 3.65 (t,  $J$  = 8 Hz, 2H, CH<sub>2</sub>NHCO), 3.4–3.2 (m, CH<sub>2</sub> Lys $\epsilon$ ), 2.60 (t,  $J$  = 7 Hz, 2H CH<sub>2</sub>CONH), 2.85–2.75 (br m, 6H, CONHCH<sub>2</sub>), 2.35 (m, COCH<sub>2</sub>), 2.1–2.0, 2.0–1.9 (br m, 2H, CH<sub>2</sub> Lys $\beta$ ), 1.8–1.7 (br m, 2H, CH<sub>2</sub> Lys $\delta$ ), 1.7–1.6 (br m, 2H, CH<sub>2</sub> Lys $\gamma$ ), 1.45–1.40 (m, 32H, CH<sub>2</sub>), 1.12 (t,  $J$  = 6 Hz, 3H, CH<sub>3</sub>).

<sup>13</sup>C-NMR (CDCl<sub>3</sub>/CD<sub>3</sub>OD 50/50) (chemical shifts in  $\delta$ , CD<sub>3</sub>OD as an internal standard 49.3) = 183.0 (COOH), 176.3, 172.3, 172.0 (3CONH<sub>2</sub>), 155.1, 153.0, 152.2 (3C–O), 138.0, 132.1, 132.0, 130.3, 130.2, 128.0, 126.2, 125.6, 123.0, 122.2, 118.0, 116.0, 113.9, 113.7, 103.7 (15 C aromatic carbon), 53.4 (CH-Lys $\alpha$ ), 53.1 (COCH<sub>2</sub>NH), overlapped (NCH<sub>2</sub>CH<sub>2</sub>CO), 45.3 (CH<sub>2</sub>-Lys $\epsilon$ ), 40.1, 37.3 (2 CH<sub>2</sub>CO), 33.6 (CH<sub>2</sub>-Lys $\beta$ ), 33.1 (CH<sub>2</sub>-Lys $\gamma$ ), 31.8, 31.4–30.4, 27.12, 24.4, 24.0 (15 CH<sub>2</sub> aliphatic moiety), 23.7 (CH<sub>2</sub>-Lys $\delta$ ), 18.5 (CH<sub>3</sub>CH<sub>2</sub>), 14.7 (CH<sub>3</sub>CH<sub>2</sub>).

### 2.3 Peptide synthesis

The synthesis of Fmoc-G-CCK8-NH<sub>2</sub> peptide was carried out in solid-phase under the standard Fmoc strategy,<sup>26</sup> by using 433A Applied Biosystems automatic synthesizer. Rink-amide MBHA resin (0.78 mmol g<sup>-1</sup>, 0.7 mmol scale, 0.900 g) was used. The elongation of peptides was achieved by sequential addition of Fmoc-AA-OH with PyBOP/HOBt and DIPEA (1 : 1 : 2) as coupling reagents, in DMF in pre-activation mode. The mixture was stirred for 1 h and after filtration. All couplings were performed twice for 1 hour, by using an excess of 4 equivalents for the single amino acid derivative. Fmoc deprotections were obtained by 30% solution of piperidine in DMF. The peptide-resin was shared in 7 reactors (each one containing 0.1 mmol of peptide-resin), and syntheses were manually brought to a close. Two reactors were used to obtain the peptide amide derivatives: H-G-CCK8 and H-K-K-K-G-CCK8, while the other five reactors to obtain the peptide amphiphiles (PA-[A]), PA-[B], PA-[C], PA-[D] and PA-[E]). Fmoc-Lys(Boc)-OH residues and the Fmoc-Ahoh-OH linker were coupled manually in DMF by using an excess of 4 and 2 equivalents, respectively. *N,N*-Diocetadecylsuccinamic acid (0.4 mmol, 0.249 g) coupling was performed twice for 1 h in the DMF/DCM (50/50) mixture.

The lipophilic moiety was activated *in situ* by the standard HOBT/PyBop/DIPEA procedure. The cleavage from the resin and deprotection of the protecting groups were obtained stirring for 2 h in TFA containing 2.5% (v/v) water and 2.0% (v/v) TIS, as scavengers, at room temperature. The crude products were slowly precipitated at 0 °C by adding diethylether drop-wise. The solids were washed several times with small portions of cold water, and lyophilized. Purification of the crude mixtures was carried out by RP-HPLC ( $\lambda$  = 280 nm). The final products were recovered at a purity higher than 95%, as indicated by LC-MS analysis, and final yields ranged between 20 and 40%. MALDI-TOF mass spectra confirm the identities of the five peptide derivatives.

PA-[A]: (C18)<sub>2</sub>-K-K-K-G-CCK8-NH<sub>2</sub>:  $t_R$  = 24.66 min, (MW = 2107)  $m/z$  = 2108

PA-[B]: (C18)<sub>2</sub>-L-K-K-K-G-CCK8-NH<sub>2</sub>:  $t_R$  = 25.38 min, (MW = 2443)  $m/z$  = 2444

PA-[C]: (C18)<sub>2</sub>-K-L-K-K-G-CCK8-NH<sub>2</sub>:  $t_R$  = 25.46 min, (MW = 2443)  $m/z$  = 2444

PA-[D]: (C18)<sub>2</sub>-K-K-L-K-G-CCK8-NH<sub>2</sub>:  $t_R$  = 25.48 min, (MW = 2443)  $m/z$  = 2444

PA-[E]: (C18)<sub>2</sub>-K-K-K-L-G-CCK8-NH<sub>2</sub>:  $t_R$  = 25.32 min, (MW = 2443)  $m/z$  = 2444

H-G-CCK8:  $t_R$  = 15.26 min, (MW = 1119)  $m/z$  = 1120

H-K-K-K-G-CCK8:  $t_R$  = 13.65 min, (MW = 1521)  $m/z$  = 1522

### 2.4 Aggregate formulation and DLS characterization

All solutions were prepared by weight, buffering the samples at pH values in 5.0–12.0 range. Three different buffer solutions were used: 0.10 M phosphate buffer (pH 12.0); 0.10 M HBS buffer (pH 7.0 and 5.0). pH measurements were made by using pH-meter MeterLab PHM 220. The pH-meter was calibrated with three standards at pH 4.00, pH 7.00 and pH 10.00. In most cases the samples to be measured were prepared from stock solutions. Concentrations of all solutions were determined by absorbance on a UV-vis Jasco (Easton, MD) Model 440 spectrophotometer with a path length of 1 cm using a molar absorptivity ( $\epsilon_{280}$ ) of 6845 M<sup>-1</sup> cm<sup>-1</sup> for CCK8. This value was calculated according to the Edelhoich method,<sup>27</sup> taking into account contributions from tyrosine and tryptophan present in the primary structure, which amount to 1215 and 5630 M<sup>-1</sup> cm<sup>-1</sup>, respectively.<sup>28</sup> Fluorescent aggregates of PA, containing 1% mol mol<sup>-1</sup> of C18-FITC fluorescent probe (FITC-PA aggregates), were prepared for *in vitro* uptake assays. Confirmation of incorporation of the fluorescent probe (1%) into the aggregates was obtained by gel filtration on Sephadex G-50 pre packed columns (Pharmacia Biotech). For DLS measurements self-assembled aggregates solution in isotonic HBS solution were prepared at the final concentration of 1 × 10<sup>-4</sup> M. Samples were centrifuged at room temperature at 13000 rpm for 5 min. The measurement

was performed at 25 °C. Scattered light intensities were measured at a fixed scattering angle ( $\theta = 90^\circ$ ). The results were processed with the program Qels (Wyatt Technology).

## 2.5 Fluorescence measurements

Critical micellar concentrations of PA aggregates were obtained by fluorescence spectroscopy. Emission spectra were recorded at room temperature using a Jasco Model FP-750 spectrofluorimeter in 1.0 cm path length quartz cell. Equal excitation and emission bandwidths were used throughout experiments, with a recording speed of 125 nm min<sup>-1</sup> and automatic selection of the time constant. The CMC were measured by using ANS as the fluorescent probe. Small aliquots of  $1 \times 10^{-5}$  M aggregate solution, dissolved in 10 mM HEPES at pH 7.0, were added to a fixed volume (1.00 mL) of fluorophore ( $1 \times 10^{-5}$  M ANS) directly in the quartz cell. CMC values were determined by linear least-squares fitting of the fluorescence emission at 480 nm, upon excitation at 350 nm *versus* the amphiphile concentration, as previously reported.<sup>29,30</sup> To analyse the tryptophan fluorescence, emission spectra were recorded between 290 and 450 nm at an excitation wavelength of 280 nm. The spectra of PA-[A]-PA-[E] were collected at a monomer concentration  $2.0 \times 10^{-4}$  M in 10 mM HEPES at pH 7.0. Fluorescence emission spectra were also collected for H-G-CCK8 in buffered solution and for H-G-CCK8 in the presence of DPC. In these experiments the H-G-CCK8 peptide concentration was  $2 \times 10^{-4}$  M, while DPC concentration was 10 mM and buffer was 10 mM HEPES at pH 7.0.

## 2.6 Circular dichroism measurements

Far-UV CD spectra were recorded from 195 to 260 nm on a Jasco J-810 spectropolarimeter equipped with a NesLab RTE111 thermal controller unit using a 1 mm quartz cell at 25 °C. Circular dichroism measurements were carried out on solutions containing peptide or peptide amphiphile at concentrations of  $2 \times 10^{-4}$  M at several pH values (5.0, 7.0, 12.0). Solutions of H-K-K-K-G-CCK8 peptide were prepared in 10 mM HEPES buffer at pH 7.0, and in the presence of 20, 40, 60 or 80% of TFE. Other experimental settings were: scan speed, 10 nm min<sup>-1</sup>; sensitivity, 50 mdeg; time constant, 16 s; bandwidth, 1 nm. Each spectrum was obtained averaging three scans, subtracting contributions from other species in solution, and converting the signal to mean residue ellipticity in units of deg cm<sup>2</sup> dmol<sup>-1</sup> res<sup>-1</sup>.

## 2.7 Cell culture and flow cytometry

Cellular uptake of PA aggregates was studied by flow cytometry on A431 cells overexpressing the CCK<sub>2</sub>-R by stable transfection.<sup>31</sup> Cells were cultured as an exponential growing subconfluent monolayer on 100 mm plates in DMEM supplemented with 10% FCS, 2 mM L-glutamine and 250 µg mL<sup>-1</sup> G418 in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C. Cell aliquots ( $1 \times 10^5$ ) were incubated at 4 °C in 100 µL final volume. At the end of the incubation, cells were pelleted, washed, resuspended in PBS/BSA at 400 µL and analyzed with a flow cytometer equipped with a 488 nm argon laser (FACScan, Becton Dickinson, CA, USA). A total of 20 000 events per sample were collected. Values of fluorescence intensity were obtained from histogram statistic of CellQuest

software. For binding experiments, cells were incubated for 1 h with 226 µM FITC-PA aggregates. The fluorescence intensity of cells treated with different unlabeled aggregates was comparable to untreated cells. Therefore, cells treated with PA-[C] were used as negative control. For competitive binding experiments, cells were pre-incubated with 2.26 mM H-G-CCK8 peptide (100 fold excess with respect to PA concentration) for 30 min followed by the addition of FITC-PA aggregates and an additional incubation of 1 h.

## 3. Results and discussion

### 3.1 PA design, synthesis and aggregate formulation

The CCK8 peptide amphiphile (PA-[A]), reported in Scheme 1, contains the CCK8 sequence, a glycine residue on the N-terminus, three lysine residues and a hydrophobic moiety with two C18 alkyl chains. The insertion of the three positively charged lysine residues between the peptide portion and the alkyl chains should induce an electrostatic repulsion effect, thus breaking the inter-peptide hydrogen bonds established between the amino acid side chains during the aggregation process. Actually, other charged amino acids, such as Glu or Asp, could be used with the same aim. In this study, lysine has been chosen because, at the physiological pH, the lysine  $\epsilon$ -amino functions are protonated and the entire amphiphiles result positively charged. As it is well known, cationic aggregates are usually employed as gene delivery systems due to their low toxicity and immunogenicity.<sup>32</sup> The other four CCK8 peptide amphiphiles (PA-[B]-PA-[E]) contain an additional hexaohaxene linker. The presence of the hexaohaxene linker should help in the exposition of the bioactive peptide sequence on the aggregate external surface. Moreover, the idea to alternate the lysine residues with the linker allows studying as to whether the positions of the positive charged residues influence the peptide structure and its activity. The CCK8 peptide modified with the glycine residue on the N-terminus (H-G-CCK8) or with the glycine and three lysine residues on the N-terminus (H-K-K-K-G-CCK8) have been also synthesized for CD and fluorescence comparative studies.

All the peptide derivatives were synthesized according to standard solid-phase-peptide-synthesis protocols, using Fmoc/*t*Bu chemistry and Rink-amide MBHA resin as the polymeric support.<sup>26</sup> In the synthesis of amphiphilic peptides PA-[A]-PA-[E], as already experienced with similar compounds,<sup>33</sup> the hexaohaxene linker and lysine residues were coupled, in solid phase, by using the Fmoc-protected starting reagents (Fmoc-Ahoh-OH and Fmoc-Lys(Boc)-OH), with a two-fold and four-fold molar excess, respectively, and PyBop/HOBt/DIPEA as activators. The hydrophobic *N,N*-dioctadecylsuccinamic acid was efficiently coupled in solid-phase in a DMF/DCM mixture. All the peptide derivatives were collected in good yields after RP-HPLC chromatography purification. Analytical liquid chromatography and mass spectrometry data (LC-MS and MALDI-TOF) confirmed the compound identities and their high purities. Notwithstanding, variation of the position of the hexaohaxene linker in the peptide sequence of PA-[B]-PA-[E] amphiphiles occurs, the HPLC retention times do not change, significantly.

Self-assembled aggregates were prepared by simply dissolving monomers in buffered solutions at different pH values (5.0, 7.0, 12.0) at room temperature. C<sub>18</sub>H<sub>37</sub>CONHLys-(FITC)CONH<sub>2</sub> (C18-FITC) fluorescent probe was also synthesized in solid-phase and crystallized by adding water drop-wise to the TFA cleavage solution, and used without further purification. The β-alanine residue was introduced before the coupling step of FITC chromophore to avoid fluorescence elimination by the Edman degradation mechanism.<sup>34</sup> This product was also characterized by NMR spectroscopy (<sup>1</sup>H and <sup>13</sup>C). Fluorescent aggregates of PA, containing 0.1% mol mol<sup>-1</sup> of C18-FITC fluorescent probe, were prepared for *in vitro* cellular uptake assays. Incorporation of C18-FITC fluorescent probe into PA aggregates was monitored by gel filtration using Sephadex G-50 pre-packed column.

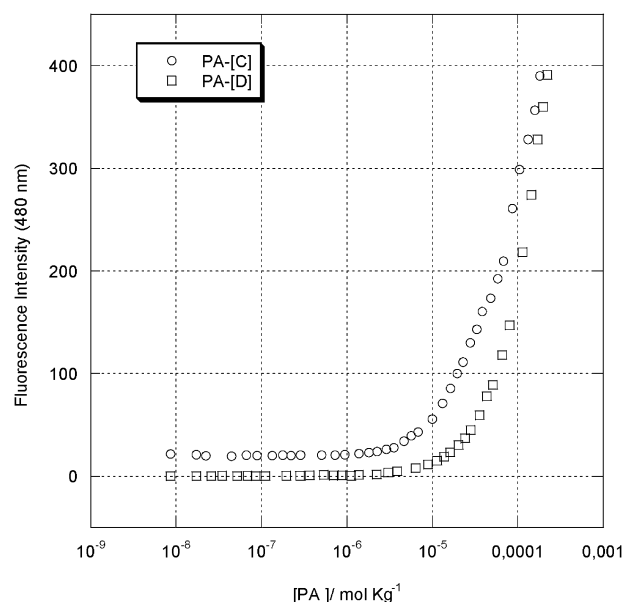
### 3.2 Structural characterization

Dynamic Light Scattering measurements were performed on PA aggregates in 10 mM HEPES buffer at pH 7.0. Aggregates show a bimodal distribution, with well-separated modes. Both modes are due to translational diffusion processes, which could be attributed to two different aggregate structures, with apparent translational diffusion coefficients  $D_{\text{fast}}$  and  $D_{\text{slow}}$  (see Table 1). The Stokes-Einstein equation (1) is used to evaluate the hydrodynamic radius,  $R_{\text{H}}$ , at infinite dilution

$$R_{\text{H}} = \frac{K_{\text{B}}T}{6\pi\eta D_0} \quad (1)$$

where  $D_0$  is the translational diffusion coefficient at infinite dilution,  $K_{\text{B}}$  is the Boltzmann constant,  $T$  is the absolute temperature, and  $\eta$  is the solvent viscosity. Due to the high solution dilution ( $C = 1 \times 10^{-4}$  M) of the studied systems, we have approximately  $D \approx D_0$ , and eqn (1) can be reasonable used to estimate the hydrodynamic radius of the aggregates. The  $R_{\text{H}}$  values obtained for slow and fast modes, reported in Table 1, are compatible with liposome and micelle structures, respectively.<sup>35</sup> All liposome hydrodynamic radii range between 63.40 nm and 92.32 nm. On the other hand, the radii of the micelle aggregates are between 4.2 and 12.3 nm. From a structural point of view, these values are similar to those observed in systems we already studied.<sup>33</sup> In order to verify if the aggregate preparation mode could influence the aggregate distribution in micelles and liposomes, samples were also prepared according to well-assessed sonication and extrusion procedures. DLS results indicate a non-significant deviation from data collected on untreated samples (data not shown).

CMC values of micelle aggregates were determined by a fluorescence-based method using ANS as the probe.<sup>29,30</sup> ANS



**Fig. 1** Fluorescence intensity of the ANS probe at 480 nm *versus* amphiphiles concentration for micellar aggregates. Graphical break-points indicate the CMC values. For clearness, only the curves concerning PA-[C] and PA-[D] are reported. Similar behaviour is found for the other amphiphilic compounds (see Table 1).

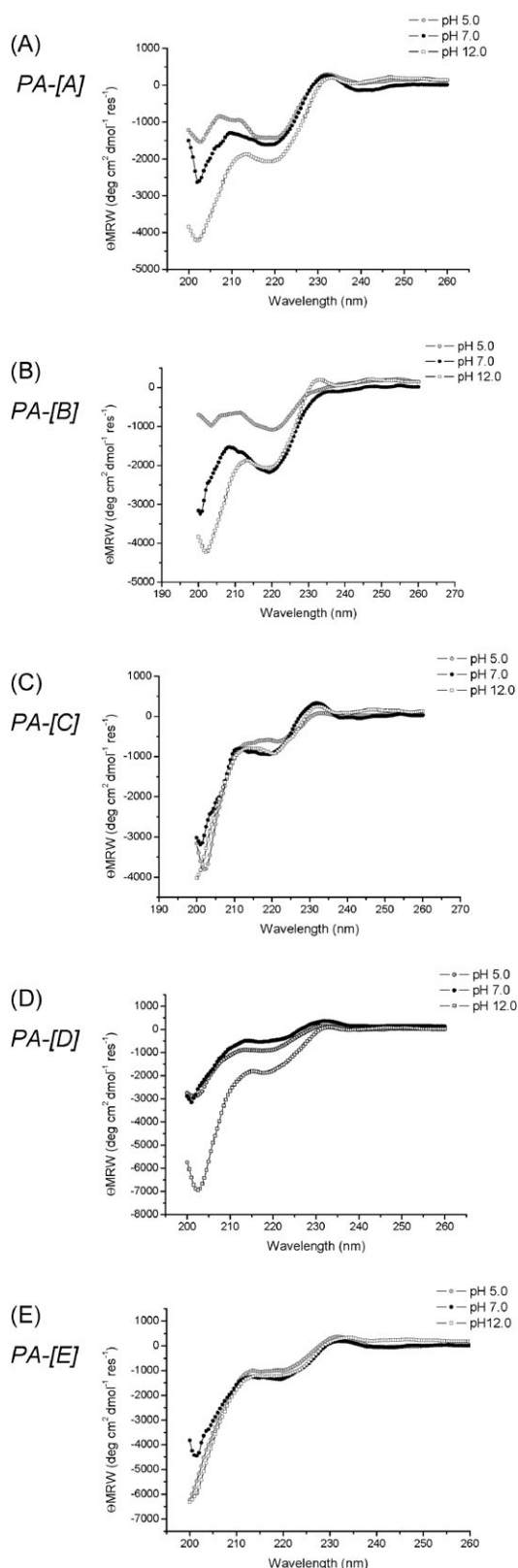
is weakly fluorescent or non fluorescent in water, but strongly fluorescent when bound to membranes in water. An increase in probe fluorescence intensity due to aggregate formation was observed for all the studied samples. The fluorescence intensities at 480 nm, corresponding to the maximum of spectrum, on function of the PA concentration are reported in Fig. 1 for selected amphiphiles. CMC values of all samples determined by the graphical break-points and reported in Table 1, range between  $9.70 \times 10^{-6}$  and  $2.53 \times 10^{-5}$  mol kg<sup>-1</sup>. The CMC values of the new PA aggregates are higher with respect to the CMC of (C18)<sub>2</sub>L5CCK8 ( $2.0 \times 10^{-6}$  mol kg<sup>-1</sup>), indicating micelle destabilization due to the presence of positive charges in these PA monomers. As expected, the destabilization effect increases as the positive charges are closer to the hydrophobic moiety that drives the aggregation process.

### 3.3 Circular dichroism (CD) studies

The secondary peptide structure in PA aggregates was evaluated by circular dichroism (CD) spectroscopy. CD studies (Fig. 2) were carried out on samples at monomer concentration above the CMC values. The CD spectra of PA-[C], PA-[D] and PA-[E] indicate a random coil conformation of CCK8, similar to that

**Table 1** Critical Micellar Concentration (CMC) values determined by fluorescence and diffusion coefficients and hydrodynamic radii obtained from Dynamic Light Scattering measurements for the systems studied. The terms fast and slow refer to micelles and liposomes, respectively

Systems	CMC/mol kg <sup>-1</sup>	$D_{\text{fast}} \times 10^{-11}/\text{m}^2 \text{ s}^{-1}$	$R_{\text{H}}/\text{nm}$	$D_{\text{slow}} \times 10^{-12}/\text{m}^2 \text{ s}^{-1}$	$R_{\text{H}}/\text{nm}$
PA-[A]	$1.70 \times 10^{-5}$	$4.75 \pm 0.10$	$4.18 \pm 0.08$	$2.6 \pm 0.1$	$76.24 \pm 1.52$
PA-[B]	$9.70 \times 10^{-6}$	n.m.	n.m.	$3.1 \pm 0.4$	$64.59 \pm 7.75$
PA-[C]	$1.17 \times 10^{-5}$	$4.42 \pm 0.13$	$4.50 \pm 0.13$	$3.1 \pm 0.1$	$63.40 \pm 3.80$
PA-[D]	$2.52 \times 10^{-5}$	$1.62 \pm 0.19$	$12.31 \pm 1.48$	$2.2 \pm 0.3$	$91.79 \pm 11.01$
PA-[E]	$2.53 \times 10^{-5}$	$2.97 \pm 0.45$	$6.70 \pm 0.44$	$2.2 \pm 0.3$	$92.32 \pm 4.45$



**Fig. 2** Far UV CD spectra of PA-[A]–PA-[E] supramolecular aggregates at different pH values; concentrations of amphiphilic peptides are  $2.0 \times 10^{-4}$  M, higher than CMC values, to be certain of the presence of aggregates in solution.

found for H-G-CCK8, while PA-[A] and PA-[B] spectra show a more defined structural arrangement of the CCK peptide.

CD spectra were also carried out at different pH values (5.0, 7.0 and 12.0) in order to investigate how the protonation grade of acid or basic residues influences the secondary structure of the monomer (Fig. 2). Interestingly, an increase of the peptide folding is observed for PA-[A] and PA-[B] as pH increases, while variations in pH do not modify CD spectra of PA-[C], PA-[D] and PA-[E].

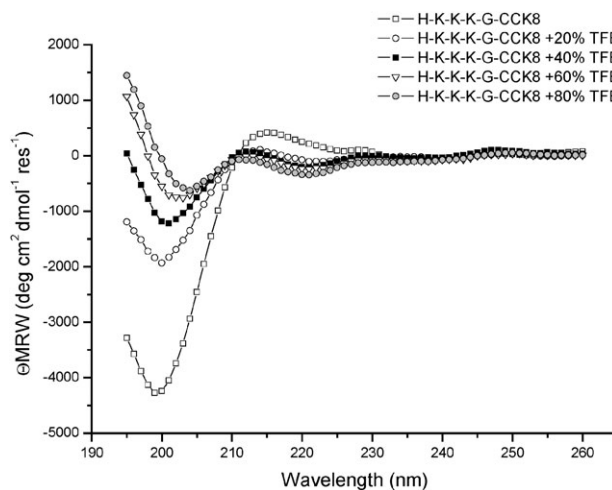
The CD spectra suggest that in PA-[A] and PA-[B] amphiphiles the three charged lysine residues, very close to the CCK8 peptide sequence, induce the peptide folding. This behaviour is confirmed by comparison with the conformational arrangement of the reference compound H-K-K-K-G-CCK8. As reported in Fig. 3, H-K-K-K-G-CCK8 peptide, in the presence of TFE (a solvent widely used in conformational studies for its properties to unmask peptides folding propensities), shows CD spectra similar to that of PA-[A] and PA-[B].

Furthermore, PA-[A] and PA-[B] CD spectra look like the reference compound H-G-CCK8 spectrum in the presence of the membrane-like surfactant DPC (Fig. 4A).<sup>36</sup> These results suggest that both PA-[A] and PA-[B] peptides, adopt a conformation similar to that found for the membrane-bound CCK8: this indication allows us to hope that CCK8 peptide on the external surface of PA-[A] and PA-[B] could be able to interact with CCK receptors, delivering the entire aggregates on the cellular target.

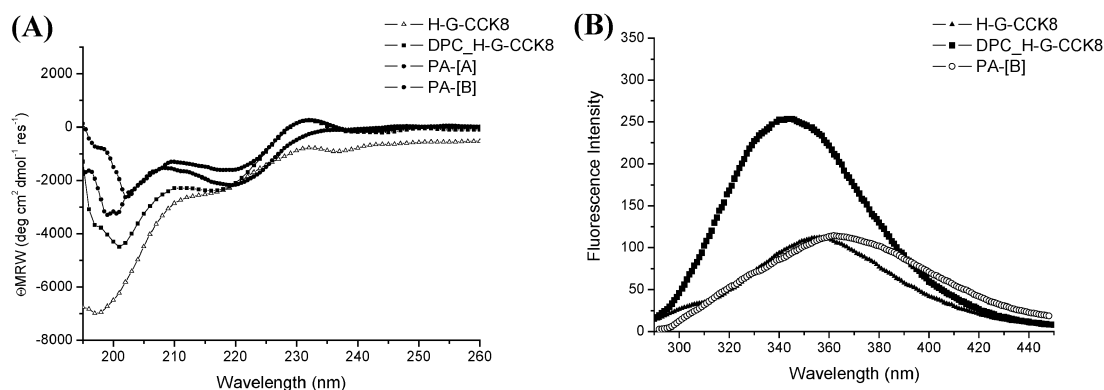
### 3.4 Fluorescence of tryptophan residue

A comparison between the fluorescence behaviour of the free H-G-CCK8 peptide in HEPES buffered water solution, of H-G-CCK8 in the presence of DPC micelles, and of PA-[B] aggregates is reported in Fig. 4B. PA-[A], PA-[C], PA-[D] and PA-[E] show fluorescence spectra super imposable with PA-[B] and are omitted from Fig. 4B. This study aims to examine the effect of the different solution conditions on the environment of the tryptophan residue in the peptide sequence.

The spectra were collected on samples in which the H-G-CCK8 peptide concentration was  $2 \times 10^{-4}$  M, DPC concentration was 10 mM in 10 mM HEPES at pH 7.0, and spectra of PA-[B] were collected at a monomer concentration of  $2.0 \times 10^{-4}$  M in 10 mM



**Fig. 3** CD spectra of H-K-K-K-G-CCK8 in HEPES buffered water solution containing different amounts of TFE (from 0 to 80%).



**Fig. 4** (A) Plot of the CD molecular ellipticity from 195 to 260 nm of H-G-CCK8 peptide in the presence of 10 mM DPC micelle and of PA-[A] and PA-[B] amphiphiles. (B) Plot of the fluorescence emission spectra from 290 to 450 nm after excitation at 280 nm of H-G-CCK8 peptide in the presence of 10 mM DPC micelle and of PA-[B] aggregates. CD and fluorescence spectra of the free H-G-CCK8 are also reported for comparison. Peptide concentration in all samples is  $2.0 \times 10^{-4}$  M; buffer is 10 mM HEPES at pH 7.0.

HEPES at pH 7.0. The  $2 \times 10^{-4}$  M concentration used for all systems investigated was chosen at the aim to assure that amphiphilic monomers remain in their aggregate form. At this concentration fluorescence quenching phenomena could occur and therefore any comparison between fluorescence intensity can be evaluated. On the other hand, fluorescence wavelengths for the indole moiety can be related to the tryptophan environment.

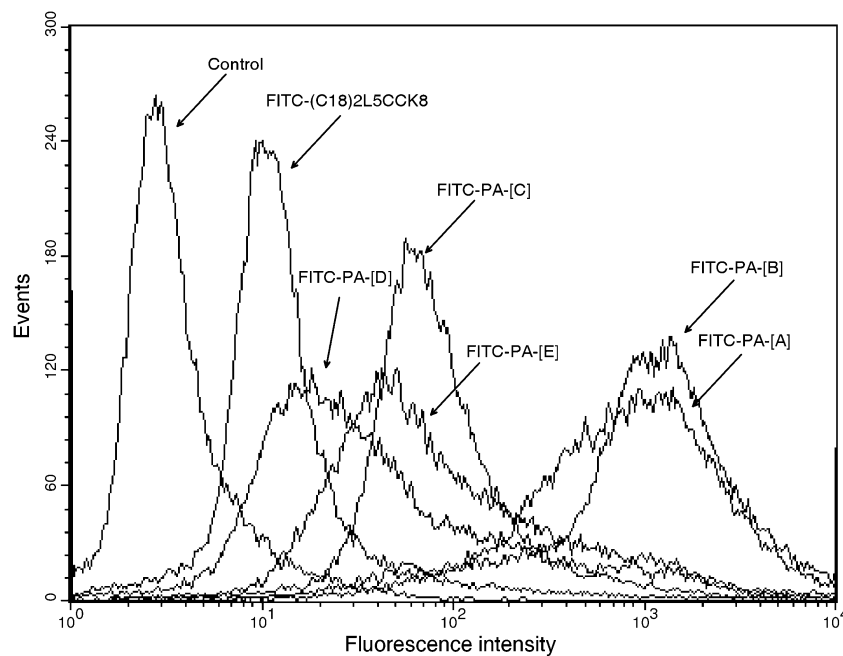
H-G-CCK8 in water solution and PA-[B] aggregates show an emission maximum at 356 and 362 nm, respectively. These wavelengths are diagnostic of the hydrophilic environment of the indole group on tryptophan side-chain.<sup>37</sup> Tryptophan exposition on the aggregate surface suggests the bioavailability of CCK8 peptide to the receptor binding site. On the other hand, in the presence of DPC micelles, the emission maximum for H-G-CCK8 is blue-shifted to 342 nm.

This wavelength is typical of a tryptophan contacting water molecules of low mobility at the protein surface.

### 3.5 Flow cytometric analysis

The cell labelling efficiency of each PA formulation was assessed by using flow cytometry analysis based on FITC fluorescence. PA formulations were made fluorescent by insertion of a small amount of C18-FITC fluorescent probe into the aggregates. The previously reported aggregates based on (C18)<sub>2</sub>L5CCK8 were also studied for comparative analysis.

Assays were performed on A431 cell line at 4 °C, in order to block non-specific internalization; incubating cells with PA fluorescent aggregates for 1 h. Cells treated with unlabeled aggregates were used as negative control. Binding results are shown in Fig. 5. Fluorescent FITC-PA-[A] and FITC-PA-[B]



**Fig. 5** Representative flow cytometry histogram illustrating FITC-PA aggregates binding to A431 cells. A431 tumour cells ( $10^6$  cells  $\text{mL}^{-1}$ ) were treated with 226  $\mu\text{M}$  FITC-PA aggregates for 1 h at 4 °C. Cells treated with not fluorescent PA-[C] were used as negative control. Measurements were performed at least three times and typical result is showed.

**Table 2** Competitive binding assay between FITC-PA and H-G-CCK8 peptide

	Fluorescence intensity
H-G-CCK8	4.21
FITC-PA-[A]	606.45
FITC-PA-[B]	1635.34
FITC-PA-[A] + H-G-CCK8	4.07
FITC-PA-[B] + H-G-CCK8	4.18

Values are expressed as relative fluorescence intensity *i.e.* the mean of fluorescence minus the mean of fluorescence of untreated cells.

aggregates are found at higher fluorescence values, while FITC-(C18)<sub>2</sub>L5CCK8 is present at low fluorescence values; and FITC-PA-[C], FITC-PA-[D] and FITC-PA-[E] are found in intermediate field. This behaviour indicates that PA-[A] and PA-[B] aggregates are a more efficient cell-labelling vehicle than PA-[C], PA-[D] and PA-[E]. On the other hand, (C18)<sub>2</sub>L5CCK8 does not show any effective binding properties towards A431 cells. Moreover, competitive binding experiments on FITC-PA-[A] and FITC-PA-[B] were also performed after pre-incubation of cells with a large excess of H-G-CCK8, in order to demonstrate that the uptake is receptor mediated. Results, reported in Table 2, were compared with the binding behaviour of fluorescent FITC-PA aggregates alone. When the FITC-PA-[A] and FITC-PA-[B] fluorescence is monitored in the presence of the unlabeled H-G-CCK8, less binding occurs, indicating a competition between the two ligands for the receptor.

#### 4. Conclusions

Supramolecular aggregates are obtained by self-aggregation of cationic peptide amphiphiles in buffered water solution. They have been characterized for peptide conformation and bioavailability on the external aggregate surface, and for their cell binding properties. The presence of CCK8 peptide well-exposed on the aggregate surface has been assessed by fluorescence measurements. Anyway the peptide availability on aggregates hydrophilic shell is not the unique requirement for receptor binding: the peptide conformation is a fundamental task to assure high affinity and selectivity in ligand/protein binding processes. This is particularly true in the case of CCK8 peptide. CCK8 needs to assume a pseudo- $\alpha$ -helix conformation to give high affinity interaction to the CCK1-R and CCK2-R receptors, according to the membrane-bound pathway theory. In fact, the supramolecular aggregates obtained by self-aggregation of (C18)<sub>2</sub>L5CCK8, in which CCK8 peptide is present in a  $\beta$ -sheet conformation, probably due to the inter-peptide hydrogen bonds between amino acid side chains, does not show *in vitro* cellular binding. Similar negative results are observed for aggregates based on the cationic amphiphiles PA-[C]–PA-[E]: the introduction of positive charges in the amphiphilic monomers induces an electrostatic repulsion between peptides giving a CCK8 unfolding process. Otherwise, as clearly indicated by cytofluorimetric studies, PA-[A] and PA-[B] aggregates present promising binding properties to CCK<sub>2</sub>-R receptors overexpressed by transfected A431 cells. In these active aggregates, CCK8 peptide presents a structural

arrangement similar to that found for the same peptide in DPC micelles: *i.e.* the peptide conformation required for receptor binding. This conformational behaviour could be probably induced by the presence of three charged lysine residues close to the bioactive peptide sequence, as also supported by the conformational behaviour of the reference compound H-K-K-K-G-CCK8. Chemical modifications on the CCK8 N-terminus seem to play an important role in stabilizing the peptide active conformation, either when the peptide derivative is in monomer or in aggregate form. Supramolecular aggregates based on PA-[A] and PA-[B] can be considered as promising candidates for target selective drug carriers on cancer cells.

#### Abbreviations

PA	peptide amphiphile
FITC	Fluorescein 5-isothiocyanate
BSA	bovine serum albumin
PBS	phosphate buffered saline
DMEM	Dulbecco eagle medium modified
FCS	fetal calf serum
TFA	trifluoroacetic acid
DLS	Dynamic Light Scattering
DMF	dimethylformamide
PyBop	benzotriazol-1-yl-oxytris(pyrrolidino) phosphonium
HOBt	1-hydroxybenzotriazole
DIPEA	<i>N,N</i> -diisopropylethylamine
DCM	dichloromethane
Mtt	4-methyltrityl
TIS	triisopropylsilane
HATU	<i>O</i> -(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium
<i>t</i> Bu	<i>tert</i> -butyl
HBS	4-2-hydroxyethyl-piperazino-ethanesulfonic acid
CMC	Critical micellar concentration
ANS	8-anilinonaphthalene-1-sulfonate
DPC	dodecylphosphocholine
TFE	trifluoroethanol

#### Acknowledgements

The authors thank the European Molecular Imaging Laboratories Network (EMIL) for financial support. The authors thank Mr Leopoldo Zona for NMR experimental assistance and M. L. De Luca for his assistance in editing the manuscript.

#### References

- 1 R. K. Jain, The next frontier of molecular medicine: delivery of therapeutics, *Nat. Med.*, 1998, **4**, 655–657.
- 2 R. Langer, Drug delivery and targeting, *Nature*, 1998, **392**, 5–10.
- 3 J. C. Reubi, Peptide receptors as molecular targets for cancer diagnosis and therapy, *Endocr. Rev.*, 2003, **24**(4), 389–427.
- 4 D. Kwekkeboom, E. P. Krenning and M. De Jong, Peptide Receptor Imaging and Therapy, *J. Nucl. Med.*, 2000, **41**, 1704–1713.
- 5 J. C. Reubi, Neuropeptide receptors in health and disease: the molecular basis for *in vivo* imaging, *J. Nucl. Med.*, 1995, **36**, 1825–1835.
- 6 V. P. Torchilin, Targeted polymeric micelles for delivery of poorly soluble drugs, *Cell Mol. Life Sci.*, 2004, **61**, 2549–2559.
- 7 M. Ferrari, Cancer nanotechnology: opportunities and challenges, *Nat. Rev. Cancer*, 2005, **5**, 161–171.



- 8 V. P. Torchilin, Structure and design of polymeric surfactant based drug delivery systems, *J. Controlled Release*, 2001, **73**, 137–172.
- 9 J. W. Park, Liposome-based drug delivery in breast cancer treatment, *Breast Cancer Res.*, 2002, **4**, 95–99.
- 10 J. P. Andre, E. Tóth, H. Fischer, A. Seelig, H. R. Maecke and A. E. Merbach, High relaxivity for monomeric Gd(DOTA)-based MRI contrast agents, thanks to micellar self-organization, *Chem.–Eur. J.*, 1999, **5**, 2977–2983.
- 11 C. P. L. King and M. D. Bednarski, Vascular-targeted molecular imaging using functionalized polymerized vesicles, *J. Magn. Reson. Imaging*, 2002, **16**, 388–393.
- 12 T. M. Allen, Ligand-targeted therapeutics in anticancer therapy, *Nat. Rev. Cancer*, 2002, **2**, 750–763.
- 13 A. Accardo, D. Tesauero, P. Roscigno, E. Gianolio, L. Paduano, G. D'Errico, C. Pedone and G. Morelli, Physicochemical Properties of Mixed Micellar Aggregates Containing CCK Peptides and Gd Complexes Designed as Tumor Specific Contrast Agents in MRI, *J. Am. Chem. Soc.*, 2004, **126**, 3097–3107.
- 14 A. Morisco, A. Accardo, E. Gianolio, D. Tesauero, E. Benedetti and G. Morelli, Micelles Derivatized with Octreotide as Potential Target Selective Contrast Agents in MRI, *J. Pept. Sci.*, 2009, **15**, 242–250.
- 15 A. Accardo, D. Tesauero, G. Mangiapia, C. Pedone and G. Morelli, Nanostructures by Self-Assembling Peptide Amphiphile as Potential Selective Drug Carriers, *Biopolymers*, 2007, **88**, 115–121.
- 16 M. Dufresne, C. Seva and D. Fourmy, Cholecystokinin and gastrin receptors, *Physiol. Rev.*, 2006, **86**(3), 805–847.
- 17 S. Underfriend and J. Meienhofer, in *The Peptides*, ed. V. J. Hruby, Academic press, vol. 7, 1985.
- 18 M. C. Fournie-Zaluski, J. Belleney, B. Lux, C. Durieux, D. Gerard, G. Gacel, B. Maigert and B. P. Roques, Conformational analysis of cholecystokinin CCK26-33 and related fragments by proton NMR spectroscopy, fluorescence-transfer measurements, and calculations, *Biochemistry*, 1986, **25**, 3778–3787.
- 19 R. C. Claussen, B. M. Rabatic and S. I. Stupp, Aqueous Self-Assembly of Unsymmetric Peptide Bolaamphiphiles into Nanofibers with Hydrophilic Cores and Surfaces, *J. Am. Chem. Soc.*, 2003, **125**, 12680–12681.
- 20 M. Pellegrini and D. F. Mierke, Molecular Complex of Cholecystokinin-8 and N-Terminus of the Cholecystokinin A Receptor by NMR Spectroscopy, *Biochemistry*, 1999, **38**(45), 14775–14783.
- 21 L. Moroder, R. Romano, W. Guba, D. F. Mierke, H. Kessler, C. Delporte, J. Winand and J. Christophe, New evidence for a membrane-bound pathway in hormone receptor binding, *Biochemistry*, 1993, **32**(49), 13551–13559.
- 22 J. Lutz, R. Romano-Goetsch, C. Escrieut, D. Fourmy, B. Mathae, G. Mueller, H. Kessler and L. Moroder, *Biopolymers*, 1997, **41**(7), 799–817.
- 23 R. Schwyzer, In search of the 'bio-active conformation'—Is it induced by the target cell membrane?, *J. Mol. Recognit.*, 1995, **8**(1/2), 3–8.
- 24 R. Schwyzer, 100 Years lock-and-key concept: Are peptide keys shaped and guided to their receptors by the target cell membrane?, *Biopolymers*, 1995, **37**(1), 5–16.
- 25 L. Schmitt, C. Dietrich and R. Tampe, Synthesis and characterization of chelator-lipids for reversible immobilization of engineered proteins at self-assembled lipid interfaces, *J. Am. Chem. Soc.*, 1994, **116**, 8485–8491.
- 26 W. C. Chang and P. D. White, *Fmoc solid phase peptide synthesis*, Oxford University Press, New York, 2000.
- 27 H. Edelhoch, Spectroscopic Determination of Tryptophan and Tyrosine in Proteins, *Biochemistry*, 1967, **6**, 1948–1954.
- 28 C. N. Pace, F. Vajdos, L. Fee, G. Grimsley and T. Gray, How to measure and predict the molar absorption coefficient of a protein, *Protein Sci.*, 1995, **4**, 2411–2423.
- 29 K. S. Birdi, H. N. Singh and S. U. Dalsager, Interaction of Ionic Micelles with the Hydrophobic Fluorescent Probe 1—Anillno-8-naphthalenesulfonate, *J. Phys. Chem.*, 1979, **83**, 2733–2737.
- 30 E. De Vendittis, G. Palumbo, G. Parlato and V. Bocchini, A fluorimetric method for the estimation of the critical micelle concentration of surfactants, *Anal. Biochem.*, 1981, **115**, 278–286.
- 31 L. Aloj, C. Caraco, M. Panico, A. Zannetti, S. del Vecchio, D. Tesauero, S. De Luca, C. Arra, C. Pedone, G. Morelli and M. Salvatore, *In vitro* and *in vivo* evaluation of <sup>111</sup>In-DTPAGlu-G-CCK8 for cholecystokinin-B receptor imaging, *J. Nucl. Med.*, 2004, **45**(3), 485–494.
- 32 K. Kostarelos and A. D. Miller, Synthetic, self-assembly ABCD nanoparticles; a structural paradigm for viable synthetic non-viral vectors, *Chem. Soc. Rev.*, 2005, **34**, 970–994.
- 33 A. Accardo, D. Tesauero, G. Morelli, E. Gianolio, S. Aime, M. Vaccaro, G. Mangiapia, L. Paduano and K. Schillen, High-relaxivity supramolecular aggregates containing peptide and Gd complexes agents in MRI, *J. Biol. Inorg. Chem.*, 2007, **12**, 267–276.
- 34 M. Jullian, A. Hernandez, A. Maurras, K. Puget, M. Amblard, J. Martinez and G. Subra, N-Terminus FITC labeling of peptides on solid support: the truth behind the spacer, *Tetrahedron Lett.*, 2009, **50**, 260–263.
- 35 M. Jonstromer, B. Johnsson and B. Lindman, Self-diffusion in nonionic surfactant–water systems, *J. Phys. Chem.*, 1991, **95**, 3293–3300.
- 36 M. Kumar, J. R. Jr Reeve, W. Hu, L. J. Miller and D. A. Keire, The Micelle-associated 3D structures of Boc-Y(SO<sub>3</sub>)-Nle-G-W-Nle-D-2-phenylethylester (JMV-180) and CCK-8(s) share conformational elements of a calculated CCK1 receptor-bound model, *J. Med. Chem.*, 2008, **51**, 3742–3754.
- 37 E. A. Permyakov, *Luminescent Spectroscopy of Proteins*, CRC Press, Boca Raton, FL, 1993.