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Micelles by self-assembling peptide-conjugate amphiphile: synthesis and structural characterization

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Abstract: The chemical synthesis by solid-phase methods of a novel amphiphilic peptide, peptide-conjugate amphiphile (PCA), containing in the same molecule three different functions: (i) the *N*,*N*-bis[2-[bis(carboxy-ethyl)amino]ethyl]-L-glutamic acid (DTPAGlu) chelating agent, (ii) the CCK8 bioactive peptide, and (iii) a hydrophobic moiety containing four alkyl chains with 18 carbon atoms each, is reported. In water solution at pH 7.4, PCA self-assembles in very stable micelles at very low concentration [critical micellar concentration (cmc) values of 5×10^{-7} mol kg^{-1}] as confirmed by fluorescence spectroscopy. The structural characterization, obtained with small-angle neutron scattering (SANS) measurements, indicates that the aggregates are substantially represented by ellipsoidal micelles with an aggregation number of 39 ± 2 and the two micellar axes of about 52 and 26 Å. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide-conjugate amphiphile (PCA); CCK8 peptide; micelles; small-angle neutron scattering

INTRODUCTION

During the past years, several supramolecular aggregates, such as micelles, vesicles and liposomes, containing a high number of stable gadolinium(III) complexes and derivatized on their external surface with bioactive molecules (peptides or antibodies) have been proposed as target selective contrast agents in magnetic resonance imaging (MRI) [1–4]. An ideal target selective MRI contrast agent should increase the signal intensity at the target site and in the meantime should lower the signal intensity in the vascular space, so as to eliminate the disadvantageous value of the background, giving well-solved images of the cellular target and of the surrounding tissues [5].

On this topic, we developed a first example of mixed micelles containing a large number of gadolinium complexes and bioactive peptides, conjugated to C_{18} single-chain hydrophobic moieties. These aggregates present a spherical shape with a diameter in the range of 70–80 Å. They were characterized by high relaxivity, due to the large number of paramagnetic gadolinium complexes, and by target specificity due to the bioactive peptide [6].

More recently, we reported the synthesis, the structural characterization and the relaxometric behavior of a new monomer with an 'upsilon' shape (*Mon*Y) in which a lysine residue is derivatized on its three reactive functions with: the chelating agent DTPAGlu(*N*,*N*-bis[2-[bis(carboxyethyl)amino]ethyl]-L-glutamic acid), a DTPA derivative able to give very stable complex of Gd(III) ions; the bioactive *C*-terminal octapeptide of cholecystokinin (CCK8) able to give receptor recognition; and a hydrophobic moiety containing two alkyl chains with 18 carbon atoms each. As indicated by dynamic light scattering (DLS) and small-angle neutron scattering (SANS) measurements, *Mon*Y, and its gadolinium complex *Mon*Y(Gd), aggregates in water solution giving ellipsoidal micelles with the ratio between the micellar axis of ~1.7 and the aggregation number N_{agg} of ~30. Self-assembling micelles of *Mon*Y(Gd) present high relaxivity values ($r_{1p} = 15.03$ mM^{-1}s^{-1}) for each gadolinium complex in the aggregate and could be considered as new promising selective MRI contrast agents [7].

Although in this latter monomer, the hydrophobic moiety is represented by two long hydrocarbon chains (18 carbon atoms each) vesicular aggregates have not been detected by structural characterization. A plausible explication can be found in the well-known empirical model developed by Ninham and coworkers [8] to predict aggregate type based on the steric relationship of head-group and side-arm size. A surfactant parameter, $P = v/a^{\bullet} l$, was proposed (a is head-group surface area, v and l are the volume and length of the side chains) to predict the aggregation behavior in solution. A value $0.5 < P < 1$ predicts vesicles and $P < 0.5$ predicts micelle formation. To favor vesicular aggregation the head-group surface area (a parameter) should be decreased or the volume of the hydrophobic moiety (v parameter) should be increased. In any case, the first one cannot be modified because both functions (chelating agent and bioactive peptide) are crucial for

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the complex and to address gadolinium ions to the biological target. Then, the only part of the molecule that can be re-designed is the hydrophobic moiety.

In this article, we report, as natural evolution of the previously described compounds, the synthesis in solid-phase and the characterization of a novel peptide-conjugate amphiphile (PCA) in which the hydrophobic moiety is formed by four hydrocarbon tails with 18 carbon atoms, while the hydrophilic head still contains the DTPAGlu chelating agent and the CCK8 bioactive peptide (Figure 1). The structural characterization, by SANS and fluorescence spectroscopy of the resulting supramolecular aggregates is reported.

RESULTS AND DISCUSSION

Monomer Synthesis

The PCA was synthesized by solid-phase methods using the Rink-amide 4-methylbenzhydrylamine (MBHA) resin as polymeric support and the Fmoc/tBu chemistry. The CCK8 peptide was synthesized according to standard SPPS protocols [9]. At the Asp *N*-terminal residue of the CCK8 peptide, a glycine residue and five units of 8-amino-3,6-dioxaoctanoic acid, acting as spacers, were consecutively added. The five ethoxylic spacers were introduced in order to increase the hydrophilicity of the CCK8 peptide and to improve the exposition of the peptide on the external surface of the aggregate. The synthesis of the asymmetric peptidic compound was carried out by the introduction of two orthogonally protected amino acids: Fmoc-Lys(Mtt)-OH and Fmoc-Asp-ODmab. The scheme of synthesis is briefly reported in Figure 2. After the Fmoc protecting group removal from the last ethoxylic spacer, the Fmoc-Lys(Mtt)-OH residue was condensed on the primary chain of the peptide derivative; the *N*-4-methyltrityl (Mtt) protecting group was removed in a selective way in acido-labile conditions. On the lysine side chain a Fmoc-Asp-ODmab residue was condensed. The Dmab group was removed using brief treatment with 2% hydrazine in DMF, giving the free acid [10]; then, DTPALys-pentaester was condensed by activating the aspartic carboxylic function with HATU. The four alkyl chains were introduced in one step at the *N*-terminal end, after removing the two Fmoc groups from the primary chains. The crude product was obtained by using standard cleavage procedure; anyway, reaction time was prolonged up to 12 h to allow complete removal of protecting groups from the carboxylic functions present on the DTPALys chelating agent. After RP-HPLC purification on C4 column, the desired compound was obtained at HPLC purity higher than 95% in 10% yield. The self-assembling behavior of the PCA monomer was evidenced by critical micellar concentration (cmc) measured using fluorescence and SANS measurements.

Structural Characterization

Fluorescence studies. The 8-anilino-1-napthalene sulfonic acid ammonium salt (ANS) is one of the most frequently used fluorescence probe to determine the relative hydrophobicities of the binding sites in a number of proteins and to detect the protein conformational changes induced by ligand binding [11]. The properties of ANS fluorescence, such as quantum yield, lifetime, and position of fluorescence maximum, are sensitive to the polarity of the immediate environment surrounding the probe (the micropolarity). In solution, surfactants and dyes can interact and form micellar complexes which have characteristic features [12–14]. ANS is an anionic probe which is essentially nonfluorescent in water, but highly fluorescent in nonpolar environments or macromolecules (micelles) [14]. Its wavelength of maximum fluorescence intensity varies with its environment, but usually occurs between 450 and 480 nm. In aqueous solutions, ANS is almost nonfluorescent. The addition of the amphiphilic surfactant, to the aqueous ANS solution caused an increase in the fluorescence intensity, which reaches a *plateau* at higher

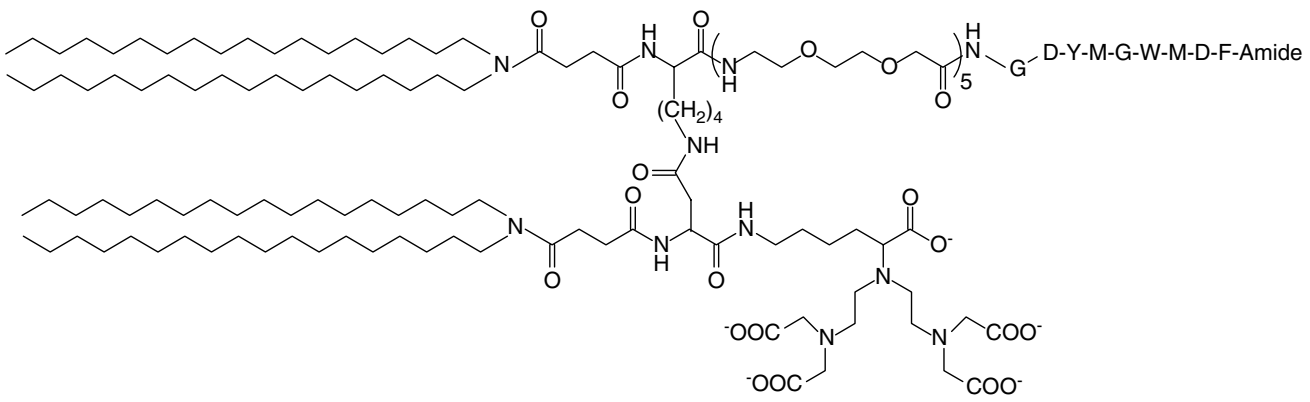

Figure 1 Schematic representation of $(C18)_4$-(Asp(DTPALys))Lys-L5-G-CCK8 (PCA). The amino acid sequence of CCK8 peptide is reported by using the one-letter amino acid code.

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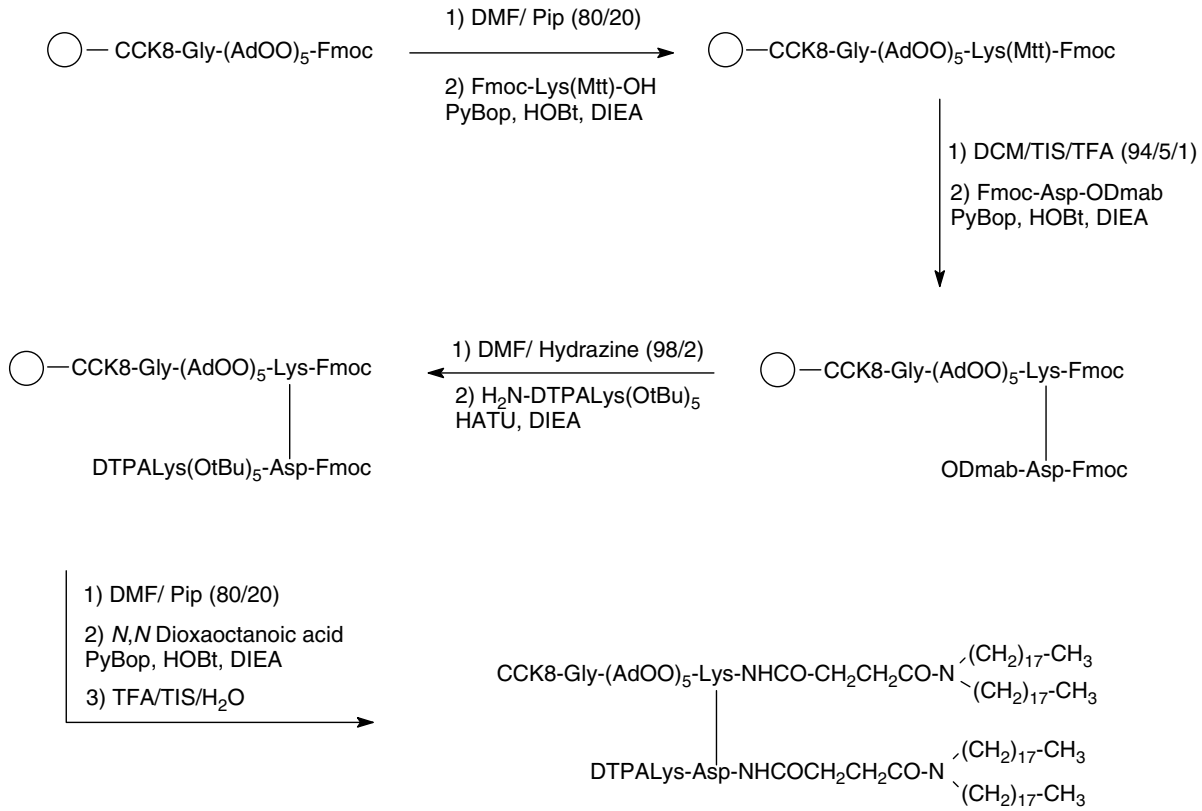

Figure 2 Peptide synthesis, reagents and conditions according to the Fmoc solid-phase protocol. Wang resin is schematically represented as an empty circle.

concentrations. The *plateau* indicates that all the dye is interacting with the surfactant micelles and occurs at only at sufficiently high concentrations of surfactant.

The experimental measure of the ANS fluorescence intensity at 480 nm, corresponding to the maximum of spectrum, on the function of the PCA concentration, provides the cmc. The break-point in Figure 3(a) indicates a cmc value of 5×10^{-7} mol kg^{-1} that confirms the high stability of the resulting supramolecular aggregates. As expected, this value is much lower than those found for mixed aggregates in which monomers contain only one C18 tail (5×10^{-5} mol kg^{-1}) [6] and slightly lower than that found for self-assembling aggregates of *Mon*Y monomer in which the monomeric molecule contains two C18 tails in the hydrophobic region (8×10^{-7} mol kg^{-1}) [7]. The high tendency of PCA monomer to self-assemble in water solution at low concentration can be ascribed to the presence of four C18 hydrocarbon tails for each monomer; the hydrocarbon tails strongly interact forming a well-stabilized hydrophobic inner region.

Aggregation number of surfactants into the micelles is generally evaluated by fluorescence quenching of pyrene. In the present case, having in the sequence of the PCA a Trp residue, electron transfer phenomena between pyrene and Trp indole side chains can occur [15]. Recently, a new method had been reported to evaluate the aggregation number by starting from the ANS fluorescence behavior in a surfactant solution [16]. The validity of this method is demonstrated by the similar value of the aggregation numbers obtained to those that have been reported in literature. It is based on the assumption that ANS dye interacts with the micelle, forming a complex, with a well-defined constant K_M:

$$K_M = \frac{F_{MAX} - F_0}{[D_{complex}]} \tag{1}$$

Where $[D_{\text{complex}}]$ is the concentration of the dye in the complex form, F_{MAX} and F_0 are the maximum and initial fluorescence intensities, respectively. The F values at any value other than F_{MAX} can be related similarly to the complexed dye $[D^1{}_{\text{complex}}]$ as:

$$K_M = \frac{F - F_0}{[D^1_{complex}]} \tag{2}$$

By combining Eqns (1) and (2), the following equation is obtained:

$$Fr = \frac{F - F_0}{F_{MAX} - F0} = \frac{D^1_{complex}}{D_{complex}} \tag{3}$$

In accordance with the complexation equilibrium in the excited state of ANS, the fluorescence intensity can

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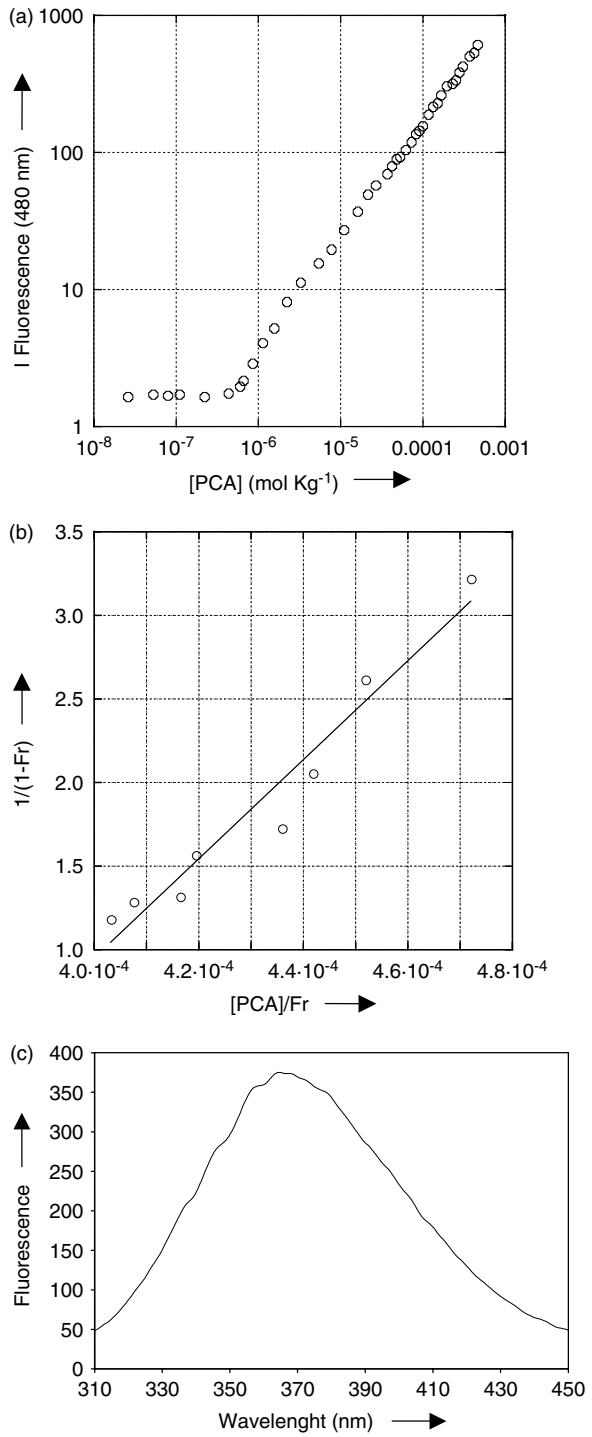

Figure 3 (a) Fluorescence intensity of ANS fluorophore at 480 nm *versus* PCA concentration. The cmc value of 5×10^{-7} mol kg^{-1} is established from graphical break-point; (b) The linear plots of $1/(1-Fr)$ *versus* $[ANS]/Fr$; (c) fluorescence spectrum of tryptophan residue in $1.0 \cdot 10^{-4}$ M PCA. The spectrum was excited at 280 nm.

be used to evaluate K_M. On the basis of Eqns 1 to 3, the equilibrium constant is given by:

$$\frac{1}{1-Fr} = \frac{K_M}{N_{agg}} \frac{[S]}{Fr} - K_M[ANS] \tag{4}$$

The linear plots of $1/(1-Fr)$ *versus* $[ANS]/Fr$ is reported in Figure 3(b). The values of binding constant K_M and aggregation number N_{agg} are determined from the slope and intercept of the linear plot.

The aggregation number was found to be 38. This result is in good agreement with the aggregation number calculated by SANS measurement (see below).

The fluorescence spectroscopy was also utilized to localize the position of the CCK8 peptide moiety in the aggregate. The presence of a fluorescence maximum at 354 nm, diagnostic of the hydrophilic environment of the indole group on tryptophan side chain [17], confirms the well-exposition of the peptide on the hydrophilic external shell of the micelle (Figure 3(c)). This result suggests the use of the CCK8 peptide to drive the entire aggregate toward CCK receptors present on cell membranes.

Small-angle neutron scattering. SANS measurements have been carried out on binary system PCA/D_2O. The collected data are reported in Figure 4, where in order to make the discussion of the results easier, data have been multiplied for a scale factor, as specified. Inspection of the data reveals the presence of a typical form factor of scattering aggregates, i.e. spherical or ellipsoidal micelles, with weak or negligible interactions.

The scattering cross section $d\Sigma/d\Omega$ of a collection of monodisperse bodies is described by the equation [18].

$$\frac{d\Sigma}{d\Omega} = n_p P(q) S(q) + \left(\frac{d\Sigma}{d\Omega}\right)_{incoh} \tag{5}$$

where n_p is the number density of scattering bodies, $P(q)$ and $S(q)$ are the form factor and the structure factor of the bodies, respectively, while $(d\Sigma/d\Omega)_{inch}$ is the incoherent scattering cross section, mainly due to the presence of hydrogen atoms in the molecules studied. The form factor $P(q)$ contains information on the shape of the scattering objects, while the structure factor $S(q)$ accounts for interparticle correlations and is normally important for concentrated or charged systems. Provided that the solutions are quite dilute, the structure function $S(q)$ can be approximated to unity, and the scattering cross section is reduced to

$$\frac{d\Sigma}{d\Omega} = n_p P(q) + \left(\frac{d\Sigma}{d\Omega}\right)_{incoh} \tag{6}$$

In order to extract quantitative information from the scattering data, the system has been modeled as a collection of ellipsoids with an inner core and an outer shell having two different scattering length densities, ρ_1 and ρ_2 respectively. If the three main semiaxis of the inner core are labeled $a = b = c$ and δ is the outer shell thickness, the form factor can be written as [19].

$$P(q) = \int_0^1 \left[\frac{4}{3}\pi ab^2(\rho_1 - \rho_2)\frac{3j_1(u_1)}{u_1} + \frac{4}{3}\pi(a+\delta)(b+\delta)^2(\rho_2 - \rho_0)\frac{3j_1(u_2)}{u_2}\right] d\mu \tag{7}$$

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where ρ_0 is the solvent scattering length density, and

$$u_1 = q\sqrt{a^2\mu^2 + (1-\mu^2)b^2} \quad (8)$$

$$u_2 = q\sqrt{(a+\delta)^2\mu^2 + (1-\mu^2)(b+\delta)^2} \quad (9)$$

whereas $j_1(x) = (\sin x - x\cos x)/x^2$ is the spherical Bessel function of the first kind. By fitting Eqn 6 to 9 to the experimental data, it has been possible to get information about the micellar aggregates, treating the parameters a, b and δ as unknown parameters. Information on the aggregation number N_{agg} has also been obtained by the equation $N_{agg} = 4\pi ab^2/(3\nu_{surf})$ where ν_{surf} is the volume of a surfactant molecule.

The scattering profile reported in Figure 4 suggests that the aggregates present in the systems are low-charged objects and no evidence of interaction peak is present in I(q) *versus* q, as expected for charged aggregates. From the structural point of view, the system contains oblate micelles that do not show a growth in their size upon concentration increase, see Table 1. The slight increase of the scattering intensity from 0.86 to 1.02 cm^{-1} at $q = 0$, when the concentration is raised, probably just reflects the increase of the micelles density.

It is interesting to highlight that, notwithstanding the presence of four hydrophobic tails for each monomer, PCA monomers form oblate micelles with a growth along the b, and c axes instead of the expected bilayers or vesicles. This result can be explained by calculating the packing factor P ($P = 0.13$) which is still below the 0.50 value. Anyway, the oblate micelles are larger aggregates with respect to that previously observed, as clearly indicated in Figure 5. Accordingly, the packing factor scales up from one to four hydrophobic chains systems.

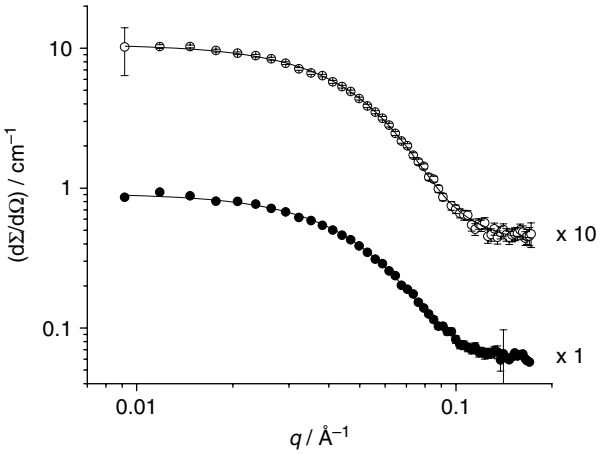

Figure 4 Scattering cross sections obtained at 25 °C for the following systems: ● PCA (0.25 mmol kg^{-1}) – D_2O ○○ PCA (0.50 mmol kg^{-1}) – D_2O Fitting curves to the experimental data through the models reported in the text are reported. For a better comparison, data have been multiplied for a scale factor as indicated in the plot.

Table 1 Fitting parameters obtained from SANS data at 25 °C for the PCA system through the equations reported in the text. The table displays the concentration of the solute, the three main semiaxis of the micellar hydrophobic core (a,b and c), the thickness of the micellar hydrophilic shell δ, and the micellar aggregation number N_{agg}

$\frac{C_{PCA}}{\text{mmol kg}^{-1}}$	$\frac{a}{\text{Å}}$	$\frac{b}{\text{Å}} = \frac{c}{\text{Å}}$	$\frac{\delta}{\text{Å}}$	N_{agg}
0.25	11.4 ± 1.2	38.3 ± 1.0	14.8 ± 0.4	39 ± 2
0.50	11.9 ± 1.4	37.7 ± 1.0	15.1 ± 0.4	39 ± 2

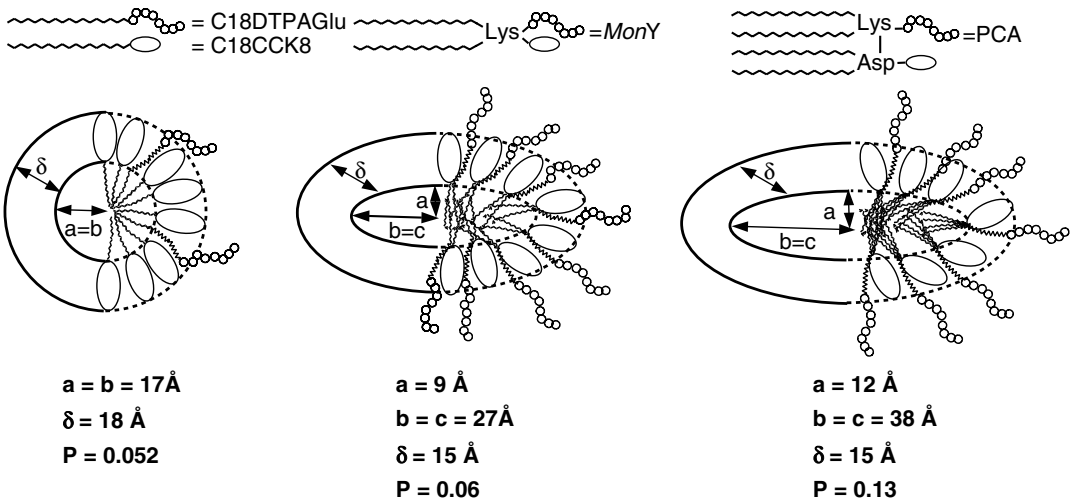

Figure 5 Schematic view of different micellar aggregates with the geometrical parameters: (a) spherical micelles obtained by co-aggregation of monomers bearing a single C18 hydrocarbon tail; (b) ellipsoidal micelles obtained by self-aggregation of monomers bearing a double C18 tail (*MonY*); (c) ellipsoidal micelles obtained by self-aggregation of the four C18 tails PCA monomers.

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CONCLUSIONS

In this article, we have reported the synthesis and the structural characterization of a novel amphiphilic monomer (PCA) containing in the same molecule three different functions: (i) the DTPAGlu chelating agent, (ii) the CCK8 bioactive peptide able to give receptor recognition, and (iii) a hydrophobic moiety containing four alkyl chains with 18 carbon atoms each. In water solution at pH 7.4, the PCA self-assembles at very low concentration as confirmed by fluorescence and SANS measurements. The aggregates are substantially represented by ellipsoidal micelles in which the ratio between the micellar axis is ~2.0. The aggregation behavior of the PCA has been compared to the aggregation behavior of two supramolecular systems previously reported, in which the number of the hydrophobic chains of 18 carbon atoms is one and two, respectively. We investigated how the shape and the size of the aggregates is influenced by the number of the hydrophobic chains [6,7]. As clearly shown in Figure 5 there does not exist a strong dependence between the hydrophobic portion and the aggregate shape. There is only an increase of the ellipticity of the aggregates, as demonstrated by the comparison of the ratio between the micellar axis, which moves from 1.0 to 2.0 from the mono tail to the tetra tail monomers. Moreover, there is a slight increase of the packing factor as expected. This consideration brings to the conclusion that the aggregation behavior is not strongly influenced by the hydrophobic portion, but it is mainly dictated by the steric and electrostatic repulsions between the head-groups of the hydrophilic moiety. The general consideration is that the steric repulsion due to the contemporary presence of the peptide and the chelating agent cannot be eliminated because they are essential for the activity of the aggregates as specific and high relaxivity contrast agent. Instead, there is the possibility to decrease of the electrostatic repulsion replacing the DTPAGlu chelating agent, in which there are five negative charges on the head-group with other chelating agents having a minor number of negative charges such as DOTA, DTPA, DO3A (1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetracetic acid, diethylenetriaminepentaacetic acid, 1,4,7,10-tetraazacyclododecane-1,4,7-triaacetic acid tris(1,1-dimethylethyl) ester). In any case the presence of the CCK8 bioactive peptide well-exposed on the external surface of the aggregates yield these kind of interesting supramolecular compound systems with application in target selective delivery.

EXPERIMENTAL METHODS

Materials and Methods

Protected N^{α}-Fmoc-amino acid derivatives, coupling reagents and Rink-amide MBHA resin were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). The Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-AdOO-OH) was purchased from Neosystem (Strasbourg, France). The DTPALys-pentaester, *N2*, *N2*-bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxoethyl]-amino]ethyl]-L-lysine 1,1-dimethylethyl Ester and the *N*,*N*-dioctadecylsuccinamic acid were synthetized according to the experimental procedure reported in literature [20,21]. All other chemicals were commercially available with Sigma-Aldrich, Fluka (Bucks, Switzerland) or LabScan (Stillorgan, Dublin, Ireland) and were used as received unless otherwise stated. All solutions were prepared by weight using doubly distilled water. Samples to be measured by SANS techniques were prepared using heavy water (Sigma-Aldrich, purity >99.8%). The pH of all solutions was kept constant at 7.4.

Synthesis of $(C18)_4$-(Asp(DTPALys))Lys-L5-G-CCK8 (PCA) Monomer

Peptide synthesis was carried out in solid-phase under standard conditions using Fmoc strategy [9]. Rink-amide MBHA resin (0.78 mmol/g, 0.5 mmol scale, 0.640 g) was used. The peptide chain was elongated by sequential coupling and Fmoc deprotected for the following Fmoc-amino acid derivatives: Fmoc-Phe-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Met-OH, Fmoc-Trp(Boc)-OH, Fmoc-Gly-OH, Fmoc-Met-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH. All couplings were performed twice for 1 h, by using an excess of four equivalents for each amino acid derivative. The α-amino acids were activated *in situ* by the standard HOBt/benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium (PyBop)/DIPEA procedure. DMF was used as a solvent. Fmoc deprotections were obtained by 20% solution of piperidine in DMF. The coupling steps were monitored by the qualitative Kaiser test. When the peptidic synthesis was complete, the Fmoc *N*-terminal protecting group was removed and five residues of Fmoc-AdOO-OH were condensed by using, for each Fmoc-AdOO-OH residue, an excess of two equivalents in a single coupling with a reaction time of 60 min under N_2 flow at room temperature. After Fmoc removal from the *N*-terminal end of the peptide derivative, 1.248 g (2.0 mmol) of Fmoc-Lys(Mtt)-OH, activated by one equivalent of PyBop and HOBt and two equivalents of DIPEA in DMF, were coupled by stirring the slurry suspension of the resin for 1 h. The solution was filtered and the resin washed with three portions of DMF and three portions of DCM. The 4-methyltrityl-protecting group (Mtt) was removed by treatment with DCM/triisopropylsylane (TIS)/TFA mixture (94 : 5 : 1). The peptide resin was stirred with 3.0 ml of this solution for 2 min. The treatment was repeated several times until the solution became colorless. The resin was washed three times by DCM and three times by DMF. A residue of Fmoc-Asp-ODmab (ODmab = 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino} benzyl alcohol) was coupled twice under standard conditions on the ε–NH_2 of the Lysine. ODmab protecting group was removed from the Aspartic residue by a 2% Hydrazine in DMF [10]. Complete ODmab removal was checked by UV measurements (at $\lambda = 290$ nm) [12]. Then the DTPALys-pentaester chelating agent was linked, through its free ε-NH_2 amino function, to the free carboxylic group of the Aspartic residue. This coupling step was performed using two equivalents of DTPALys-pentaester and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium (HATU), and four equivalents of DIPEA in

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DMF as solvent. The coupling time, compared with the classical SPPS protocol, was increased up to 2 h and the reaction was tested for completion by Kaiser test. After removal of the two *N*-terminal Fmoc protecting groups 2.488 g (4.0 mmol) of *N*,*N*-dioctadecylsuccinamic acid in DMF/DCM (1/1) mixture were condensed. Coupling was repeated twice under N_2 flow for 1 h. The liphophilic moiety was activated *in situ* by the standard HOBt/PyBop/DIPEA procedure. The peptide derivative was cleaved from the solid support by suspending the resin in 10 ml of TFA/TIS/H_2O (94.5/2/2.5) mixture of for 12 h. During this step, all the amino acid side chains and the five carboxylic groups of DTPALys were simultaneously freed from their protecting groups. The crude compound was purified by RP-HPLC. Preparative RP-HPLC was carried out on a Shimadzu 8A apparatus equipped with an UV Shimadzu detector using a Phenomenex (Torrance, CA) C4 column, 22 × 250 mm with a flow rate of 20 ml min^{-1}. The single peaks were analyzed by HPLC and mass spectrometry. Analytical RP-HPLC runs were carried out on a HP Agilent Series 1100 apparatus using a Phenomenex (Torrance, CA) C18 column, 4.6 × 250 mm with a flow rate of 1.0 ml min^{-1}. For all the RP-HPLC procedures, the solvent system used was H_2O 0.1% TFA (A) and CH_3CN 0.1% TFA (B), with a linear gradient from 5 to 70% B in 30 min followed by 70 to 95% B in 10 min Mass spectral analysis were carried out on MALDI-TOF Voyager-DE mass spectrometer (Perseptive Biosystems) and on ESI ThermoElectron Spectrometer (Milan, Italy); in the latter case, a peak at 934 amu, corresponding to $[M + 4H^+]/4$ was observed. The desired compound [$(C18)_4$-(Asp(DTPALys))Lys-L5-G-CCK8, 180 mg] was obtained with HPLC; purity higher than 95% with a final yield of 10%, $t_R = 15.5$ min; MW = 3733 amu.

Preparation of the Solutions

Stock solutions of PCA were prepared in 0.1 M phosphate buffer at pH 7.4, and filtered through a 0.45 μm filter. Concentrations of all the 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 (ε_{280}) of 6845 M^{-1} cm^{-1} for CCK8. This value was calculated according to Edelhoch method [22], taking into account the contributions from tyrosine and tryptophan present in the primary structure, which amount to 1215 and 5630 M^{-1} cm^{-1}, respectively [23].

Fluorescence Studies

The cmc values of PCA were obtained by fluorescence spectroscopy using 1 cm path length quartz cell. Emission spectra were recorded at room temperature using a Jasco Model FP-750 spectrofluorimeter. Equal excitation and emission bandwidths were used throughout the experiments, with a recording speed of 125 nm/min and an automatic selection of the time constant. The cmc of PCA was measured by using ANS as fluorescent probe. Small aliquots of a self-assembled aggregate solution ($1 \cdot 10^{-4}$ M) were added to a fixed volume of $1 \cdot 10^{-5}$ M ANS dissolved in the same buffer. The cmc was 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 [24,25].

Small-Angle Neutron Scattering (SANS)

SANS measurements were performed at 25 °C, at the LOQ instrument sited at the ISIS facility of the Rutherford Appleton Laboratory (Chilton, United Kingdom). At the ISIS pulsed neutron source, the LOQ instrument uses neutrons of wavelengths ranging between 2.2 and 10 Å detected by a TOF analysis on a 64 cm^2 two-dimensional detector placed at 4.1 m from the sample [26], giving a q range of $0.006 \div 0.24$ $Å^{-1}$. Raw data were corrected for wavelength dependent sample transmissions, incident spectrum, and detector efficiency and then put into absolute scattering cross sections $d\Sigma/d\Omega$ by comparison with scattering from a partially deuterated polystyrene standard.

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