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Physicochemical Properties of Mixed Micellar Aggregates Containing CCK Peptides and Gd Complexes Designed as Tumor Specific Contrast Agents in MRI†

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Abstract: New amphiphilic molecules containing a bioactive peptide or a claw moiety have been prepared in order to obtain mixed micelles as target-specific contrast agents in magnetic resonance imaging. The first molecule, $C_{18}H_{37}CONH(AdOO)_2$-G-CCK8 (C18CCK8), contains a C18 hydrophobic moiety bound to the C-terminal cholecystokinin octapeptide amide (CCK 26−33 or CCK8). The second amphiphilic compound, $C_{18}H_{37}CONHLys(DTPAGlu)CONH_2$ (C18DTPAGlu) or its gadolinium complex, (C18DTPAGlu-(Gd)), contains the same C18 hydrophobic moiety bound, through a lysine residue, to the DTPAGlu chelating agent. The mixed aggregates as well as the pure C18DTPAGlu aggregate, in the presence and absence of Gd, have been fully characterized by surface tension measurements, FT-PGSE-NMR, fluorescence quenching, and small-angle neutron scattering measurements. The structural characterization of the mixed aggregates C18DTPAGlu(Gd)−C18CCK8 indicates a spherical arrangement of the micelles with an external shell of ∼21 Å and an inner core of ∼20 Å. Both the DTPAGlu(Gd) complexes and the CCK8 peptides point toward the external surface. The measured values for relaxivity in saline medium at 20 MHz proton Larmor frequency and 25 °C are 18.7 mM^{-1} s^{-1}. These values show a large enhancement in comparison with the isolated DTPAGlu(Gd) complex.

Introduction

The more promising medical diagnostic imaging procedures currently in use are based on nuclear medicine (γ-scintigraphy and positron emission tomography, PET) and magnetic resonance imaging (MRI) techniques. The critical point for these techniques is that both require a different amount of reporter compounds (contrast agents) to be accumulated in the area of interest.[1,2] In fact, contrast agents help to discriminate between normal and pathological regions. The quantity of reporter compound to be accumulated in the area of interest strongly varies between these imaging techniques. While the very sensitive nuclear medicine techniques require very low tissue concentration (10^{-10} M) of radionuclide to give diagnostically significant, low resolved images, MRI gives very resolved images but, due to its very low sensitivity, needs higher concentration (10^{-4} M) of contrast agents such as paramagnetic Gd(III) complexes. To reach the required local concentration of the contrast agent, many carriers have been developed such as liposomes[3] and other microparticulates,[4] micelles,[5] dendrimers,[6] linear polymers,[7,8] proteins,[9] or peptides,[10] all of these derivatized with the metal complex of interest. Among these

† Abbreviations: AdOO, 8-amino-3,6-dioxaoctanoic acid; Boc, *tert*-butoxycarbonyl; tBu, *tert*-butyl; C12E5, polyoxyethylene-5-lauryl ether; CCK, cholecystokinin; CCK8, C-terminal octapeptide of cholecystokinin; CCK_A-R, CCK_B-R, cholecystokinin receptor types A and B; cmc, critical micellar concentration; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DpyCl, dodecylpyridinium chloride; DTPAGlu, *N*,*N*-bis[2-[bis(carboxyethyl)amino]-ethyl]-L-glutamic acid; EDT, ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; FT-PGSE-NMR, Fourier transform pulsed gradient spin-echo nuclear magnetic resonance; GPCR, G-protein coupled receptor; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium; HOBt, 1-hydroxybenzotriazole; ICP, inductively coupled plasma; MRI, magnetic resonance imaging; Mtt, 4-methyltrityl; NMRD, nuclear magnetic relaxation dispersion; PyBop, benzotriazol-1-yl-oxytris(pyrrolidino)phosphonium; R_t, retention time; SANS, small-angle neutron scattering; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TMS, tetramethylsilane.

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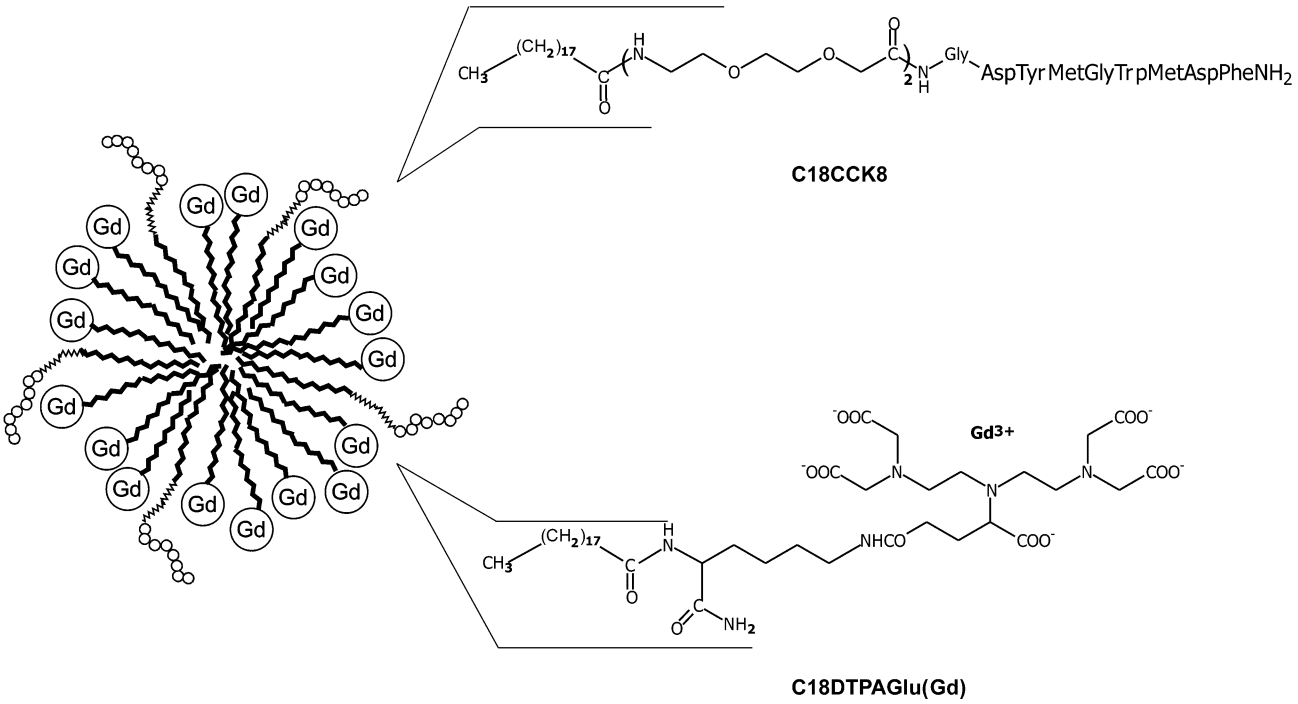

Figure 1. Schematic representation of mixed micellar aggregates.

carriers, micellar aggregates, formed by amphiphilic molecules and structurally constituted by a hydrophobic core and a hydrophilic shell, have recently drawn much attention owing to their easily controlled properties and good pharmacological characteristics.[11] For example, the self-assembling micellar organization of Gd(III)(DOTA) complex derivatized with a liphophilic tail allows obtaining a high relaxivity MRI contrast agent.[12]

There is an increasing interest in developing new contrast agents with enhanced properties. First, the new contrast agents should have high contrast activity of the metal complex. This is critical for MRI applications: high relaxivity of the paramagnetic Gd complexes allows reducing the very high concentration of the reporter compound to be realized in the area of interest. Second, the new contrast agents should be selective for a specific target: this property allows addressing the reporter compound only to the targets, such as membrane receptors overexpressed by cancer cells. Therefore, the total amount of the contrast agent to be injected for diagnostic analysis could be reduced: this result is very important in γ-scintigraphy in which radioactive isotopes are used.

This paper is concerned with the development of new contrast agents based on mixed micelles that fulfill both these properties. The basic idea behind the new contrast agents is that the two different monomers synthesized, one of which contains a bioactive molecule able to address the aggregates on the specific biological target and the other containing a chelating moiety able to form stable complexes with the metal of interest, and both presenting also a lipophilic tail, could self-assemble with each other in a mixed micelle. The hydrophobic chains allow the monomers to form mixed micelles, leaving the hydrophilic heads on the surface of the aggregates available for the appropriate task. In Figure 1 is reported a schematic representation of the mixed micellar structure formed in aqueous solution by the two monomers. The first monomer $C_{18}H_{37}CONH(AdOO)_2$-G-CCK8 (C18CCK8) contains a C18 hydrophobic moiety bound to the C-terminal cholecystokinin octapeptide amide (CCK 26−33 or CCK8).[13] Moreover, to ensure that the bioactive peptide remains on the external surface of the micelle when the aggregate is formed, two oxoethylene linkers and a glycine residue were introduced between the lipophilic tail and the CCK8 peptide. The choice of the CCK8 peptide is based on the knowledge that this peptide displays high affinity for both cholecystokinin receptors, CCK_A-R and CCK_B-R.[14] These receptors belong to the G-protein coupled receptors (GPCRs) superfamily and are localized in the cell membrane. Both CCK_A-R and CCK_B-R are very promising targets for specific contrast agents due to their overexpression in many tumors: CCK_A-R is overexpressed in pancreatic cancer and CCK_B-R is found in small cell lung cancer, colon, and gastric cancers, medullary thyroid carcinomas, astrocitomas, and stromal ovarian tumors.[15]

The second monomer, $C_{18}H_{37}CONHLys(DTPAGlu)CONH_2$ (C18DTPAGlu), or its gadolinium complex (C18DTPAGlu(Gd)), contains the same C18 hydrophobic moiety bound, through a lysine residue, to the DTPAGlu as chelating agent.[16] This monomer has been designed to keep the chelating agent on the external surface of the mixed micelle. The chelating moiety could complex radioactive metal isotopes such as ^{111}In(III) for application of the mixed micelles as reporter compounds in

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diagnostic γ-scintigraphy, or paramagnetic ions such as Gd(III) for application as contrast agent in MRI. A DTPAGlu−CCK8 conjugate has been recently used to give in vitro and in vivo very stable complexes with the radioactive isotope ^{111}In(III),[17] while Gd(III) complexes of DTPAGlu and of DTPAGlu−CCK8 conjugate have been completely characterized for both their stability and relaxometric properties.[18] The supramolecular aggregation of the C18DTPAGlu(Gd) complexes in the mixed micelle gives rise to a slow rotation and, consequently, increases the proton relaxivity with respect to that shown by monomeric Gd(III)−chelate complexes.[12] Finally, the presence in the micelles of two separate monomers gives the unique opportunity to tune the ratio between the two active components in order to find the right compromise between the number of bioactive peptides on the micellar surface to address the micelle on the target receptors and the number of the metal−chelate complexes able to give high contrast activity of the supramolecular adduct.

In this paper we present a complete physicochemical characterization of mixed micelles formed in aqueous solution by the two monomers C18CCK8 and C18DTPAGlu in the presence or in the absence of a nonionic surfactant (polyoxyethylene-5-lauryl ether, C12E5) used to stabilize the micelle formation. Moreover, the mixed micelles containing Gd(III) ions complexed by the chelating DTPAGlu agent have been studied for their relaxometric behavior in view of their use as CCK receptor specific contrast agents for MRI applications.

Materials and Methods

Materials. 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 DTPAGlu pentaester, *N*,*N*-bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxoethyl]amino]ethyl]-L-glutamic acid 1-(1,1-dimethylethyl) ester,[16] was provided by Bracco Imaging SpA (Milan, Italy) and was used without further purification. For its synthesis refer to published methods.[16] C12E5 (stated purity >99%) was purchased from Sigma-Aldrich (Milwaukee, WI), as reagent grade, and it was used without further purification. All other chemicals were commercially available by Sigma-Aldrich, Fluka (Bucks, Switzerland), or LabScan (Stillorgan, Dublin, Ireland) and were used as received unless otherwise stated. The molar mass of the C12E5 surfactant was 406.60 g mol^{-1}. All solutions were prepared by weight using doubly distilled water. Samples to be measured by FT-PGSE-NMR and SANS techniques were prepared using heavy water (Sigma-Aldrich, purity >99.8%). The pH of all solutions was kept constant at 7.4.

Chemical Synthesis. Solid phase peptide synthesis was performed on a Shimadzu (Kyoto, Japan) Model SPPS-8 fully automated peptide synthesizer. Analytical RP-HPLCs were carried out on a Shimadzu 10A-LC using a Phenomenex C18 column (Torrance, CA), 4.6 × 250 mm, eluted with an H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B). Two gradients from 60% to 80% B over 10 min at 1 mL/min flow rate and from 80% to 95% B over 15 min at 1 mL/min flow rate were used. Preparative RP-HPLCs were carried out on a Waters (Milford, MA) Model Delta Prep 4000 equipped with a UV lambda-Max Model 481 detector using a Vydac C18 column (Columbia, MD), 22 × 250 mm, eluted with an H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) linear gradient at 20 mL/min flow rate. UV−vis spectra were carried out by using an UV−vis Jasco (Easton, MD) Model 440 spectrophotometer with a path length of 1 cm. Mass spectra were carried out on a Maldi-tof Voyager-DE Perseptive Biosystem (Framingham, MA) apparatus using α-cyano-4-hydroxycinnamic acid as matrix and bovine insulin as internal reference. The monodimensional ^{1}H NMR in DMSO-d_6 spectrum was performed on a Varian (Palo Alto, CA) 400 MHz spectrometer.

$C_{18}H_{37}CONH(AdOO)_2$-G-CCK8 (C18CCK8). Peptide synthesis was carried out in the solid phase under standard conditions using Fmoc strategy. Rink amide MBHA resin (0.78 mmol/g, 0.1 mmol scale, 0.128 g) was used. The peptide chain was elongated by sequential coupling and Fmoc deprotection of 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, and two residues of Fmoc-AdOO-OH. All couplings were performed twice for 1 h, by using an excess of 4 equivalents (equiv) for the single amino acid derivative. The α-amino acids were activated in situ by the standard HOBt/PyBop/DIPEA procedure.[19] DMF was used as a solvent. Fmoc deprotections were obtained by 20% solution of piperidine in DMF. Only the two residues of Fmoc-AdOO-OH were added in a single coupling by using an excess of 2 equiv. When the peptide synthesis was complete, the resin was washed and the terminal Fmoc protection removed. To obtain the lipophilic monomer, 0.119 g (0.4 mmol) of nonaoctanoic acid were coupled on the linker peptide N-terminus by using 0.208 g (0.4 mmol) of PyBop, 0.0612 g (0.4 mmol) of HOBt, and 134 μL (0.8 mmol) of DIPEA in 2 mL of a mixture DMF/DCM = 1/1. Coupling time was 1 h under stirring at room temperature.

The yield for aliphatic acid coupling, monitored by the Kaiser test, was in the range 95−98%. For deprotection and cleavage, the fully protected resin was treated with TFA containing triisopropylsilane (2.0%), ethanedithiol (2.5%), and water (2.5%), and the free product precipitated at 0 °C by adding ethyl ether dropwise. Purification of the crude mixture was carried out by RP-HPLC, R_t = 21.9 min. Maldi-tof-MS confirms the product identity: $C_{18}H_{37}CONH(AdOO)_2$-G-CCK8 (MW = 1687), m/z = 1688.

$C_{18}H_{37}CONHLys(DTPAGlu)CONH_2$ (C18DTPAGlu). A sample of 624.79 mg (1.00 mmol) of Fmoc-Lys(Mtt)-OH activated by 1 equiv of PyBop and HOBt and 2 equiv of DIPEA in DMF was coupled to Rink amide MBHA resin (0.78 mmol/g, 0.250 mmol scale, 0.320 g), with stirring of the slurry suspension for 1 h. 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 with 2.0 mL of DCM/TIS/TFA (94:5:1) mixture.[19] The treatment was repeated several times until the solution became colorless. The resin was washed by DMF and then the DTPAGlu pentaester chelating agent was linked, through its free carboxyl function, to the α-NH_2 of the lysine residue. This coupling step was performed using 2.0 equiv of DTPAGlu pentaester and HATU, and 4 equiv of DIPEA in DMF as solvent. The coupling time, compared with the classical solid phase peptide synthesis protocol, was increased up to 2 h, and the reaction was tested for completion by the Kaiser test. After removal of the Fmoc group by 20% piperidine in DMF, the coupling of nonaoctanoic acid was performed in a mixture DCM/DMF (1:1) in the previously described condition. For deprotection and cleavage, the fully protected fragment was treated with TFA containing TIS (2.0%) and water (2.5%). The crude product was precipitated at 0 °C, washed several times with small portions of water, and recrystallized from methanol and water. The product was characterized by ^{1}H NMR spectroscopy and Maldi-tof mass spectrometry.

^{1}H NMR (chemical shifts in δ, TMS as internal standard) = 4.18 (m, 1H, C*H* Lys α), 3.80 (overlapped, 1H, C*H*Glu α), 3.40 (overlapped, 8H, CH_2COOH) 3.02 (t, 2H, CH_2 Lys ϵ) 2.81 (m, 8H, N−CH_2), 2.22

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(t, 2H, CH_2CO), 2.14 (m, 2H, RCH$_2$CH_2CO), 1.87 (m, 1H, CH_2 Lys β), 1.76 (m, 1H, CH_2 Lys β), 1.65 (m, 1H, CH_2 Lys δ), 1.51 (m, 3H, CH_2 Lys γ,δ), 1.42 (t, 2H, RCH_2CH$_2$CO), 1.25 (m, 30H, CH_2 aliphatic), 0.90 (t, 3H, CH_3). Maldi-tof-MS confirms the product identity: $C_{18}H_{37}$-CONHLys(DTPAGlu)CONH$_2$ (MW = 870), *m*/*z* 871.

Preparation of Gadolinium Complex, C18DTPAGlu(Gd). The complexation has been carried out by adding light excess of the $GdCl_3$ to the aqueous solution of the C18DTPAGlu ligand at neutral pH and room temperature. The formation of Gd complex, C18DTPAGlu(Gd), was followed by measuring the solvent proton relaxation rate ($1/T_1$). The excess of uncomplexed Gd(III) ions, which yields a variation of the observed relaxation rate, was removed by centrifugation of the solution brought to pH 10; further relaxation rate measurements were made to check the complete Gd(III) ions removal.

Preparation of Solutions. All solutions were prepared by weighting, buffering the samples at pH 7.4 with 0.10 M phosphate and 34 mM NaCl. pH measurements were made by using the pH meter MeterLab PHM 220. The pH meter was calibrated with standards at pH 7 and pH 10. In most cases the samples to be measured were prepared from stock solutions. All solutions were stirred at room temperature until complete dissolution and then used without further treatment.

In C18DTPAGlu−C18CCK8−water and C18DTPAGlu−C18CCK8−C12E5−water solutions the imposed ratio between the solutes was as such to have an average of three peptide derivatives per micelle. This has been verified through the SANS measurements, as discussed below.

In C18DTPAGlu−C12E5−water and C18CCK8−C12E5−water ternary systems the imposed ratio between stoichiometric solute concentrations was 1:3, and 1:9, respectively.

Surface tension, fluorescence quenching, and SANS measurements were also performed on mixtures where C18DTPAGlu was replaced with the Gd complex C18DTPAGlu(Gd).

Surface Tension Measurements. The surface tension, γ, was measured with an accuracy of 0.1 dyn cm^{-1}, by the Du Nouy ring method, using a KSV Sigma 70 digital tensiometer, equipped with an automatic device to select the rising speed of the platinum ring and to set the time lag between two measurements. The temperature was kept constant at 25.00 ± 0.01 °C. The tensiometer was calibrated with water and acetone.[20]

Self-Diffusion Measurements. The self-diffusion coefficients were measured by the FT-PGSE-NMR technique at 25 °C.[21] A spectrometer operating in the ^{1}H mode at 80 MHz and equipped with a pulsed magnetic field gradient unit, made by Stelar (Mede, Pavia, Italy), was employed. The temperature was controlled within 0.1 °C through a variable-temperature controller, Model VTC-87. The expression for individual spin−echo peak amplitude for a given line is

$$A = A_0 \exp[-\gamma_{mg}{}^2 g^2 D \delta^2 (\Delta - \delta/3)] \quad (1)$$

where A_0 is a constant for a specific set of experimental conditions, γ_{mg} is the gyromagnetic ratio of the proton, D is the self-diffusion coefficient of the species responsible for the NMR signal, g is the magnitude of the applied gradient, and Δ and δ are spacing and duration of the gradient pulses, respectively. Echo delays were kept constant so that the relaxation effect must not be accounted for. Equation 1 was fitted by nonlinear least-squares routine to the attenuation of the echo amplitude sampled as a function of g.

Fluorescence Quenching. To evaluate the mean aggregation number of surfactants into the micelles, fluorescence quenching measurements were performed by a Jasco FP-750 (Easton, MD) spectrofluorimeter at 25 °C. The fluorescence probe pyrene and the quencher dodecylpyridinium chloride (DpyCl) have been used in all systems. The excitation wavelength was set at 335 nm, and fluorescence intensity was detected at 383 nm. This latter wavelength corresponds to the third out of five peaks of vibronic fluorescence that the pyrene spectrum exhibits. The aggregation numbers were measured in the assumption that the intramicellar "static" quenching between the probe and the quencher, both following Poisson distribution, is dominant. The pyrene concentration was 2×10^{-6} mol dm^{-3}, and the fluorescence intensity at 383 nm was monitored at decreasing amount of the DpyCl quencher starting from a molar concentration ratio R = [quencher]/[micelle] = [q]/[M] ~ 1.[22−24]

The fluorescence intensity in the presence of the quencher is given by

$$I = I_o \exp(-[q]/[M]) \quad (2)$$

where I_o is the fluorescence intensity in the absence of inhibitor. In the approximation of the phase separation model the following equation holds:

$$[M] = (C - \text{cmc})/N_{agg} \quad (3)$$

where C is the concentration of the aggregating species. Hence, the aggregation number, N_{agg}, is obtained from the slope of $\ln(I/I_o)$ vs $[q]/(C - \text{cmc})$.[25,26] Due to the very low concentration of the systems studied, in eq 3 molarity has been replaced by molality.

Water Proton Relaxation Measurements. The longitudinal water proton relaxation rates were measured on a Stelar Spinmaster (Mede, Pavia, Italy) spectrometer operating at 20 MHz, by means of the standard inversion−recovery technique (16 experiments, 2 scans). A typical 90° pulse width was 4 μs and the reproducibility of the T_1 data was ±0.5%. The temperature was kept at 25 °C with a Stelar VTC-91 air-flow heater equipped with a copper−constantan thermocouple (uncertainty ±0.1 °C). The proton $1/T_1$ NMRD profiles were measured over a continuum of magnetic field strength from 0.000 24 to 0.28 T (corresponding to 0.01−12 MHz proton Larmor frequency) on a Stelar Fast Field-Cycling relaxometer. This relaxometer works under complete computer control with an absolute uncertainty in $1/T_1$ of ±1%. Data points at 20 and 90 MHz were added to the experimental NMRD profiles and were recorded on the Stelar Spinmaster (20 MHz) and on a JEOL EX-90 (90 MHz) (Tokyo, Japan) spectrometer, respectively.

^{1}H Water Relaxation Rate. The relaxivity of a Gd(III) complex results from contributions arising mainly from water molecules in the inner and outer coordination spheres:

$$r_1 = r_1{}^{His} + r_1{}^{Hos} \quad (4)$$

$r_1{}^{His}$ refers to the contribution from the exchange of the water protons in the first coordination sphere of the paramagnetic metal ion:

$$r_1{}^{His} = \frac{n_w[C]}{55.6(T_{1M} + \tau_M)} \quad (5)$$

where n_w is the hydration number, [C] is the molar concentration of the paramagnetic chelate, T_{1M} is the longitudinal relaxation time of the inner sphere water protons, and τ_M is their residence lifetime.

The outer sphere term, $r_1{}^{Hos}$, describes the contribution from water molecules which diffuse around the paramagnetic complex (bulk solvent).

The complex theory that governs the relaxation process in a paramagnetic chelate is well described by the models developed by Solomon−Bloembergen−Morgan for what concerns the inner sphere contribution and by Hwang and Freed for the outer sphere one.[27]

Small-Angle Neutron Scattering. SANS measurements were performed at 25 °C on the KWS2 located at the Forschungszentrum

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Julich, Germany. Neutrons with an average wavelength of 7 Å and wavelength spread $\Delta\lambda/\lambda < 10\%$ were used. A two-dimensional array detector at two different sample-to-detector distances, 2 and 8 m, detected neutrons scattered from the sample. These configurations allowed collecting the scattered intensity in a range of moment transfer Q between 0.003 and 0.12 $Å^{-1}$. Samples were contained in 1 mm path length quartz cells, and measurement times ranged between 20 min and 2 h. The data were then corrected for background, empty cell, and solvent contribution, and then reduced to scattering cross section (in absolute units per centimeter), following the standard procedure.[28]

Method of Data Analysis. The general scattering intensity, containing information about shape, size, and interactions of monodisperse scattering bodies, is given by[29]

$$I(Q) = N_b \left(\sum_i b_i - V_b\rho_s\right)^2 P(Q)\, S(Q) + I_{inc} \qquad (6)$$

where N_b is the number density of scattering bodies, V_b is the volume of each scattering particle, $\sum_i b_i$ is the sum of the scattering lengths over the atoms constituting the body, and ρ_s is the solvent scattering length density. $P(Q)$ and $S(Q)$ are the form factor and structure factor, respectively. I_{inc} is the incoherent scattering cross section.

The form factor contains information on the shape of the scattering objects, whereas the structure factor $S(Q)$ accounts for interparticle correlations and is normally important for concentrated or charged systems.

The structure of micelles present in the systems under study has been established by analyzing the scattering data through eq 6, imposing that the number density of scattering bodies and their volume are $N_b = (C - \text{cmc})L_a/N_{agg}$ and $V_b = N_{agg}V_m$, where C is the stoichiometric solute concentration, V_m is the volume of the free surfactant, N_{agg} is the aggregation number of the micelles, and L_a is Avogadro's constant.[29] The micelles were modeled as a spherical "core + shell", and hence the form factor $P(Q)$ is reduced to a sum of two Bessel first-order spherical functions:[29]

$$P(Q) = \left(f\frac{3j_1(u_1)}{u_1} + (1-f)\frac{3j_1(u_2)}{u_2}\right)^2 \qquad (7)$$

with

$$u_1 = Qa \qquad (8)$$

$$u_2 = Q(a+d) \qquad (9)$$

$$f = \frac{V_f(\rho_1 - \rho_2)}{\sum_i (b_i - V_m\rho_s)} \qquad (10)$$

$$j_1(x) = \frac{\sin x - x\cos x}{x^2} \qquad (11)$$

V_f is the micelle core volume; a and d are the radius of the core and the thickness of the hydrophilic shell, respectively. ρ_1 and ρ_2 are the densities of scattering length of the core and shell.

The structure factor, $S(Q)$, can be calculated through the Hayter and Penfold theory.[30,31]

Results

Synthesis of Monomers. The peptide synthesis was performed by the solid phase approach using Fmoc/tBu chemistry. The CCK8 peptide was synthesized as previously described.[32] At the Asp N-terminal residue of the CCK8 peptide a glycine residue and two units of 8-amino-3,6-dioxaoctanoic acid were added directly on the solid phase. As a final step, the nonaoctanoic acid was bound to the N-terminal position of the peptide derivative. The cleavage of resin and the deprotection of the side chain functions were performed in a TFA/TIS/EDT/water mixture. The peptide derivative was isolated in good yields and purified by RP-HPLC. The analitycal data (Maldi-tof mass spectrum and RP-HPLC) confirm compound identity and purity. The two units of the hydrophilic linker increase the affinity for the polar solvent of the peptide moiety.

The amphiphilic chelating moiety C18DTPAGlu was also synthesized in the solid phase using a resin support. Fmoc-Lys-(Mtt)-OH was anchored to the resin and the side chain was selectively deprotected. Then, DTPAGlu pentaester, the chelating agent fully protected by *tert*-butyl (tBu) groups on its carboxyl functions with the exception of the carboxyl group on the side chain of L-glutamic acid, was linked, by a single coupling step, to the ϵ-NH_2 amino function of the lysine residue. Successively, the Fmoc protecting group was removed by the α-NH_2 amino function of the lysine residue and the nonaoctanoic acid was bound. After cleavage from the resin the product was characterized by ^{1}H NMR spectroscopy and Maldi-tof mass spectrometry.

Surface Tension. The surface tension was measured on C12E5−water, C18DTPAGlu−water, and C18CCK8−water binary systems; on C18DTPAGlu−C12E5−water, C18CCK8−C12E5−water, and C18DTPAGlu−C18CCK8−water ternary systems; and on C18DTPAGlu−C18CCK8−C12E5−water quaternary system. The surfactant concentration was raised well above the expected critical micellar concentration, cmc. Surface tension measurements were also performed on all systems once C18DTPAGlu was replaced by C18DTPAGlu(Gd).

All the above systems show a concentration dependence of the surface tension, γ, typical of systems containing amphiphilic molecules that form micelles. Addition of solute to pure water ($\gamma_o = 72.0$ dyn cm^{-1}) has the effect of decreasing γ to the cmc value, at which the surface tension curve changes its slope and approaches a constant value. However, there are significant differences among the systems studied, as inspection of Table 1 suggests. For the C18CCK8−water system the typical change in the slope of the γ vs ln m curve, of micellar systems, has not been observed.

Binary Systems: C18DTPAGlu−Water, C18CCK8−Water, and C12E5−Water. In Figure 2 the surface tension diagram of the C18DTPAGlu−water and C12E5−water binary systems along with C18DTPAGlu(Gd)−water system are reported. The cmc values detected for the C18DTPAGlu−water and C12E5−water systems are about the same, 5×10^{-5} and 4×10^{-5} mol kg^{-1} respectively, although the limit value of the surface tension for the two systems is quite different. This is to be assigned to the higher hydrophobic contribution of C12E5 to the surface composition with respect to the other compounds studied in this paper.

The behavior of C18DTPAGlu(Gd)−water is substantially similar to that of the corresponding system in the absence of

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Table 1. Physicochemical Parameters of Pure and Mixed Aggregates

	cmc (mol kg^{-1})	N_{agg}[a]	$D \times 10^9$ (m^2 s^{-1})	R_{Hy} (Å)
C12E5−water	4×10^{-5}	140 ± 7	0.0083 ± 0.0004	240 ± 15
C18DTPAGlu−water	5×10^{-5}	35 ± 5	0.054 ± 0.003	37 ± 3
C18DTPAGlu(Gd)−water	7×10^{-5}	60 ± 5		
C18DTPAGlu−C12E5−water	9×10^{-5}	70 ± 5	0.045 ± 0.002	44 ± 3
C18DTPAGlu(Gd)−C12E5−water	2×10^{-4}	70 ± 5		
C18DTPAGlu−C18CCK8−water	2×10^{-5}	40 ± 4	0.054 ± 0.003	37 ± 4
C18DTPAGlu(Gd)−C18CCK8−water	4×10^{-5}	58 ± 4		
C18CCK8−C12E5−water	4×10^{-5}	80 ± 5	0.019 ± 0.001	120 ± 5
C18DTPAGlu−C18CCK8−C12E5−water	2×10^{-4}	60 ± 5	0.048 ± 0.003	41 ± 3
C18DTPAGlu(Gd)−C18CCK8−C12E5−water	2×10^{-4}	70 ± 5		

[a] Values determined by fluorescence quenching measurements.

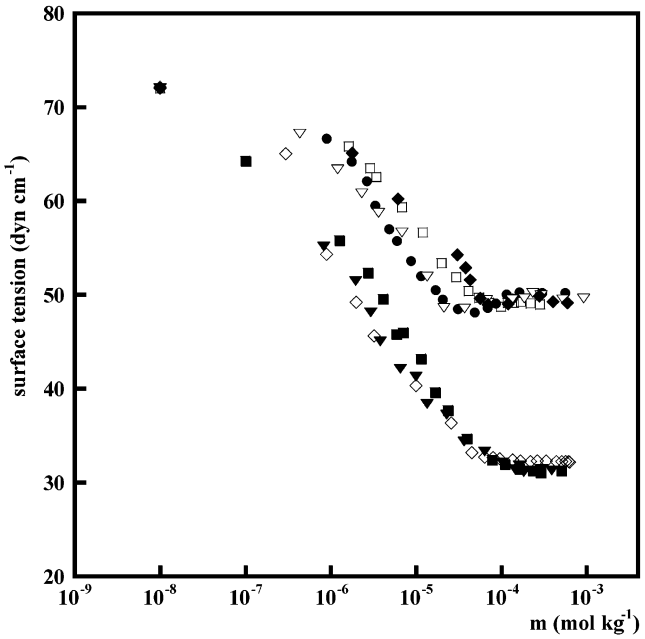

Figure 2. Surface tension of the following systems: C12E5−water (◇), C18DTPAGlu−C12E5−water (▼), C18CCK8−C12E5−water (■), C18DTPAGlu−water (▽), C18DTPAGlu(Gd)−water (□), C18DTPAGlu−C18CCK8−water (●), and C18DTPAGlu(Gd)−C18CCK8−water (◆).

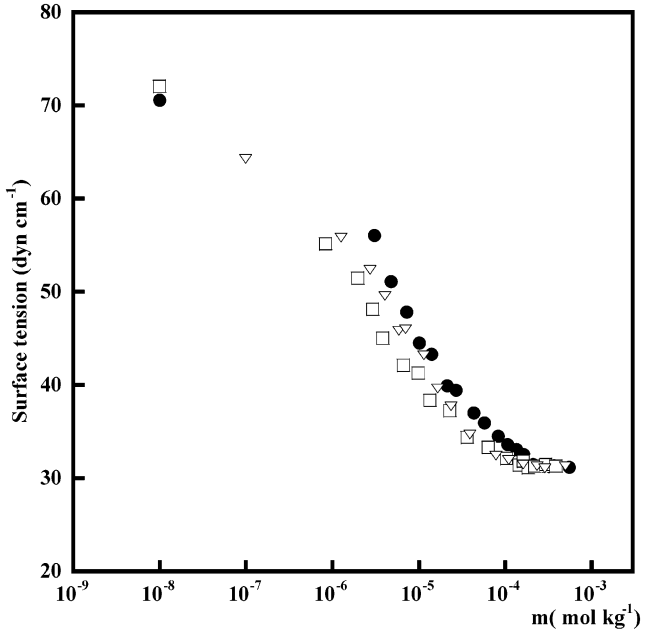

Figure 3. Concentration dependence of surface tension of the C18DTPAGlu−C18CCK8−C12E5−water (●) system and, for comparison, that of C18CCK8−C12E5−water (□) and that of C18DTPAGlu−C12E5−water (▽).

Gd, although for the former one a slightly higher value of the cmc has been observed, about 7×10^{-5} mol kg^{-1}.

For the C18CCK8−water system the typical change in the slope of the γ vs ln m curve has not been observed up to its solubility limit ($\sim 10^{-4}$ mol kg^{-1}). Indeed addition of C18CCK8 to pure water has only the effect to reduce the surface tension of the system (from $\gamma_o = 72.0$ to $\gamma = 55.0$ dyn cm^{-1}). This implies that C18CCK8 does not form micellar aggregates.

Ternary Systems: C18DTPAGlu−C18CCK8−Water, C18DTPAGlu−C12E5−Water, and C18CCK8−C12E5−Water. In Figure 2, the surface tension data, γ vs ln m, for C18DTPAGlu−C18CCK8−water, C18DTPAGlu−C12E5−water, and C18CCK8−C12E5−water systems are also shown. For the C18DTPAGlu−C18CCK8−water system the break point detected is at C18DTPAGlu concentration (2×10^{-5} mol kg^{-1}) very similar to that observed for the pure C18DTPAGlu−water system (5×10^{-5} mol kg^{-1}). As expected, from the evidence collected on the binary system, when C18DTPAGlu is replaced with the corresponding C18DTPAGlu(Gd), a slight increase of the cmc is observed.

Inspection of Figure 2 indicates that there are sensible differences between the micelles containing C12E5 and those without the surfactant. The surface tension is determined by C12E5, and as discussed in the above section, its higher contribution to the surface composition produces a marked decrease in the γ values approaching nearly equal values as measured for the C12E5−water binary system, as a result of the solution composition increase.

The measured values of the cmc for the C18DTPAGlu−C12E5−water and C18CCK8−C12E5−water systems are $\sim 9 \times 10^{-5}$ and $\sim 4 \times 10^{-5}$ mol kg^{-1}, respectively.

Interestingly, the measured cmc value for the C18DTPAGlu−C12E5−water ternary system is significantly higher than that of the corresponding binary ones, whereas for the C18CCK8−C12E5−water system the cmc is substantially similar to that of the pure C12E5−water solution.

Quaternary System: C18DTPAGlu−C18CCK8−C12E5−Water. In Figure 3 the surface tension concentration dependence of the C18DTPAGlu−C18CCK8−C12E5−water system and, for comparison, that of C18CCK8−C12E5−water and C18DTPAGlu−C12E5−water are reported.

Figure 3 shows that the presence of C18CCK8 in the C18DTPAGlu−C12E5 system does not change substantially the trend of the surface tension with respect to that observed in the absence of the amphiphilic peptide. In fact, as the solute concentration is raised, the γ values of the quaternary system

overlap those of the ternary ones, so that the only break point detectable corresponds to the cmc of the C18DTPAGlu−C18CCK8−C12E5−water system, $\sim 2.0 \times 10^{-4}$ mol kg^{-1}.

Self-Diffusion. The self-diffusion coefficients were measured for the C18DTPAGlu−water and C12E5−water binary systems; for the C18DTPAGlu−C18CCK8−water, C18DTPAGlu−C12E5−water, and C18CCK8−C12E5−water ternary systems; and for the C18DTPAGlu−C18CCK8−C12E5−water quaternary system. For each system, the total solute molality was 1000 times the cmc determined by surface tension measurements. Under these conditions, the contributions of monomeric species are negligible, and only micelles are responsible for measured self-diffusion coefficients. Because of the line broadening due to the paramagnetic ion, no self-diffusion measurements could be performed on the systems containing Gd complex monomer. In all self-diffusion measurements, two NMR lines were followed: the former (chemical shift 1.2 ppm) due to the methylene groups of the tail of all solutes; the latter (chemical shift ~3.5 ppm) due to both the ethoxylic group (for C18CCK8 and C12E5) and the CH_2COOH group (for C18DTPAGlu) of the solute heads. For both lines and for all measurements, a single-exponential decay was observed, leading to the same D value. The self-diffusion coefficients were computed as a weighed average of the values obtained following the two NMR lines.

In ternary and quaternary systems this experimental evidence indicates that the solutes present in solution form mixed aggregates and diffuse together.

The experimental self-diffusion coefficients, D, are collected in Table 1. As mentioned above, due to the relatively high solute molality (1000 × cmc), only the micellar aggregates are responsible for the measured D. Consequently, these data can be directly related to the hydrodynamic radii of the aggregates, R_{Hy}, through the Stokes−Einstein equation[33] (see Table 1):

$$R_{Hy} = \frac{k_B T}{6\pi\eta D} \tag{12}$$

where k_B is the Boltzmann constant, T is the absolute temperature, and η is the medium viscosity (for heavy water $\eta = 1.095$ cP). This relation holds for noninteracting hard spheres diffusing in a continuous medium. Inspection of Table 1 shows that in some cases R_{Hy} is clearly larger than what is reasonable for spherical aggregates. This means that some of the assumptions on which eq 12 is based are not valid, i.e., the presence of strong micelle−micelle interactions, or the micelle shape is far from spherical.

Binary Systems: C18DTPAGlu−Water and C12E5−Water. In aqueous solution, C18DTPAGlu forms spherical micelles with a relatively small hydrodynamic radius. In contrast, the high R_{Hy} value obtained for C12E5 is not compatible with the spherical shape. Jonströmer et al.,[33] by analyzing the dependence of the C12E5 self-diffusion coefficient as a function of the surfactant composition, proposed a rodlike shape for the aggregates.

Ternary Systems: C18DTPAGlu−C18CCK8−Water, C18DTPAGlu−C12E5−Water, and C18CCK8−C12E5−Water. C18DTPAGlu−C18CCK8 mixed aggregates are similar to C18DTPAGlu micelles. This suggests that the octapeptide chain (i) is not incorporated in the micellar core and (ii) is far from being completely extended in the aqueous medium. In fact, in both cases a significantly larger R_{Hy} value with respect to that calculated should be expected.

C18DTPAGlu−C12E5 mixed micelles (1:3 ratio) are similar to C18DTPAGlu micelles. The presence of C18DTPAGlu molecules, because of the steric obstruction of bulky heads, favors spherical rather than rodlike micelle formation.

The high R_{Hy} values obtained for C18CCK8−C12E5 mixed aggregates, similar to that evaluated for C12E5 aggregates, is not compatible with the spherical shape. This means that the presence of C18CCK8 molecules in the C12E5 aggregates does not modify their shape. Consequently, C18CCK8−C12E5 mixed aggregates appear to be rodlike micelles.

Quaternary System: C18DTPAGlu−C18CCK8−C12E5−Water. C18DTPAGlu−C18CCK8−C12E5 mixed aggregates are similar to C18DTPAGlu micelles and C18DTPAGlu−C18CCK8 mixed micelles. This suggests that in these ratios C18DTPAGlu molecules discriminate the shape of the aggregates. Because the R_{Hy} value is not very different by binary and ternary systems containing C18DTPAGlu, it is possible to say that also in C18DTPAGlu−C18CCK8−C12E5 mixed micelles the octapeptide chain is not included in the micellar core and, at the same time, is far from extended in the aqueous medium.

Fluorescence Quenching. Fluorescence quenching measurements were performed on all the systems in order to evaluate the aggregation number of the pure and mixed aggregates. The measurements were carried out on samples at concentration as high as 100 times the cmc values evaluated by surface tension. The aggregation numbers calculated by using eq 3 are reported in Table 1.

Binary Systems: C18DTPAGlu−Water and C12E5−Water. The aggregation number obtained for the pure C12E5 micelle is ~140. Instead, the aggregation number obtained for the pure C18DTPAGlu micelle is ~35. Last number is quite small if compared to that expected for a molecule with such a long hydrophobic tail (18 carbons). Thus, it is possible to state that the size as well as the charge of the hydrophilic head (5 negative charges) has an overriding effect in ruling the aggregation number and the size of the micelles.

Ternary Systems: C18DTPAGlu−C18CCK8−Water, C18DTPAGlu−C12E5−Water, and C18CCK8−C12E5−Water. Table 1 shows that the aggregation number of the micelles present in the C18DTPAGlu−C18CCK8−water system ($N_{agg} \sim 40$) is nearly equal to that of pure C18DTPAGlu aggregates ($N_{agg} \sim 35$). In contrast, the presence of C12E5 in most cases leads to a significant increase of the aggregation number of the micelles in comparison with the systems where it is absent. In particular, for the C18DTPAGlu−C12E5−water system the evaluated aggregation number is ~70, whereas for C18CCK8−C12E5−water it is ~80.

Quaternary System: C18DTPAGlu−C18CCK8−C12E5−Water. Fluorescence quenching measurements were also performed on the C18DTPAGlu−C18CCK8−C12E5−water system at $m_{tot} = 2.2 \times 10^{-2}$ mol kg^{-1}. The aggregation number in this system was measured to be ~60.

Finally, for all systems the presence of Gd in the micelles is reflected in an increase of the micelle aggregation number, with respect to those formed by C18DTPAGlu; see Table 1.

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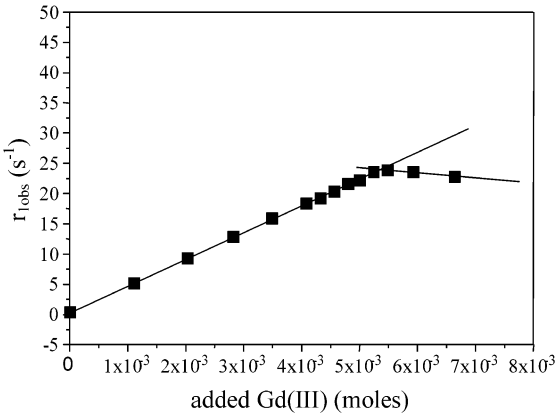

Figure 4. Water proton relaxation rate of a C18DTPAGlu solution as a function of addition of increasing amounts of $GdCl_3$ (20 MHz, 298K, pH 7). R_{1obs} increases linearly up to the saturation of free C18DTPAGlu ligand; further addition of Gd(III) is relaxometrically silent (R_{1obs} remains constant) as at neutral pH it precipitates as hydroxide.

Water Proton Relaxation Measurements. Upon addition of increasing amounts of a concentrated $GdCl_3$ solution to the C18DTPAGlu water solution, the chelating ligand DTPAGlu became progressively saturated, leading to the formation of the binary micellar system C18DTPAGlu(Gd)−water. The Gd(III) titration is conveniently followed by measuring ^{1}H relaxation rates (Figure 4).

Applying this methodology, the formation of a paramagnetic complex should yield a straight line (eq 13) whose slope corresponds to the relaxivity (r_{1p}) of the complex, defined as the paramagnetic contribution to the measured proton longitudinal relaxation rate (R_{1obs}) of a solution containing 1 mM concentration of the paramagnetic solute:[34]

$$R_{1obs} = [Gd]r_{1p} + R_{1w} \quad (13)$$

where R_{1w} is the diamagnetic contribution of pure water (0.38 s^{-1}).

As the Gd(III) concentration at which these measurements were performed (1.0 mM) is higher than the calculated cmc, the relaxivity determined from the fitting of the first tract of the straight line in Figure 4 for C18DTPAGlu(Gd) (r_{1p} = 17.5 mM^{-1} s^{-1} at 20 MHz and 25 °C) is certainly the one of the micellar form. This value (as well as the ones determined for ternary and quaternary systems) was then confirmed mineralizing, with 37% HCl at 120 °C overnight, a given quantity of sample solution to determine the exact concentration of Gd(III) present in the solution; from the measure of the observed relaxation rate (R_{1obs}) of the acidic solution, knowing the relaxivity (r_{1p}) of Gd(III) aquo ion in acidic conditions (13.5 mM^{-1} s^{-1}), it was possible to calculate the exact Gd(III) concentration (eq 13). (This method was calibrated using standard ICP solutions, and the accuracy was determined to be 1%.) At this point, knowing [GdL] and measuring R_{1obs} of the micellar mother solution, the same eq 13 was used to calculate the micellar relaxivity.

Relaxivity measurements on C18DTPAGlu(Gd)−C18CCK8−water ternary and C18DTPAGlu(Gd)−C18CCK8−C12E5−water quaternary systems were performed using mixed micelles prepared with C18DTPAGlu already complexed with Gd(III) ion. The measured relaxivity values for this species are 18.7 and 12.8 mM^{-1} s^{-1} respectively, at 20 MHz and 25 °C.

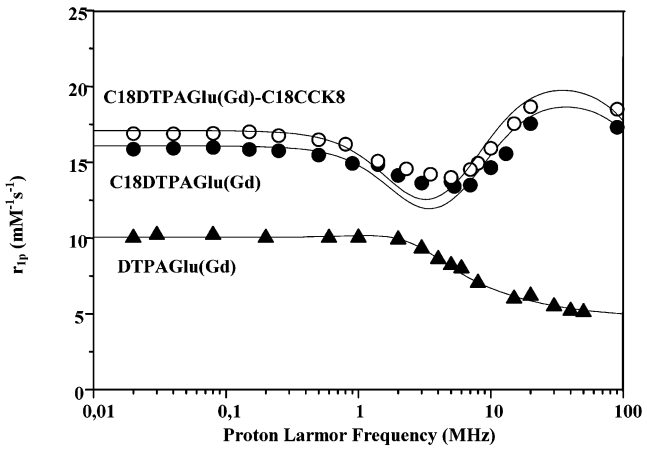

Figure 5. Nuclear magnetic resonance dispersion profiles of C18DTPAGlu(Gd)−C18CCK8−D_2O (○), C18DTPAGlu(Gd)−D_2O (●), and DTPAGlu(Gd)−D_2O (▲) at pH 7 and 298 K, normalized to 1 mM concentration of Gd(III) ion.

The slight difference in the relaxivity values determined for the binary and ternary species is due to the difference in their molecular dimensions; in fact, the N_{agg} value in Table 1 is higher for C18DTPAGlu(Gd)−C18CCK8 than for C18DTPAGlu(Gd). Surprisingly, the relaxivity of the quaternary system is lower than the one of the other two systems despite the fact that their aggregation numbers are quite similar. As the presence of C12E5 in the mixed micelle produces a substantial decrease of the water proton relaxation value, any further relaxometric characterization of this system was avoided.

With the field used for the relaxometric experiments (20 MHz), the relaxivity of Gd(III) complexes is given by

$$r_{1p} \propto \frac{1}{T_{1M}} = Kf(\tau_R) \quad (14)$$

where K is a constant related to the strength of the dipolar interaction between the protons and the electronic magnetic moment, and τ_R is the correlation time associated with the reorientation of the Gd−H magnetic vector.

From the quantitative analysis of NMRD (nuclear magnetic relaxation dispersion) profiles, in which relaxivity is a function of the applied field strength, it is possible to obtain an accurate determination of the reorientational correlation time (τ_R),[35] which is strictly related to the molecular dimensions of the investigated system. In Figure 5 the NMRD profiles of C18DTPAGlu(Gd)−C18CCK8 and C18DTPAGlu(Gd) are reported in comparison with the one of the monomeric precursor complex DTPAGlu(Gd). Data were analyzed using the Solomon−Bloembergen−Morgan model,[27] considering one water molecule in the inner coordination sphere for each Gd(III) complex (n_w = 1) and fixing the exchange lifetime (τ_M) at the value obtained from ^{17}O NMR studies[36,37] for DTPAGlu(Gd) complex.

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Table 2. Structural Parameters of Pure and Mixed Aggregates Determined by Small-Angle Neutron Scattering

	N_{agg}	a (Å)	d (Å)	z	n_w	$r_{1p}{}^a$ (mM^{-1} s^{-1})	$\tau_R{}^a$ (ps)
C18DTPAGlu−D_2O	42 ± 1	17 ± 1	18 ± 1	−28 ± 2	90 ± 5		
C18DTPAGlu(Gd)−D_2O	60 ± 2	20 ± 1	21 ± 2	−26 ± 8	78 ± 6	17.5 ± 0.1	650 ± 6.5
C18DTPAGlu−C18CCK8−D_2O	43 ± 1	17 ± 1	19 ± 1	−22 ± 1	95 ± 5		
C18DTPAGlu(Gd)−C18CCK8−D_2O	58 ± 1	20 ± 1	21 ± 1	−24 ± 2	80 ± 7	18.7 ± 0.1	700 ± 7

[a] Values determined by water proton relaxation measurements.

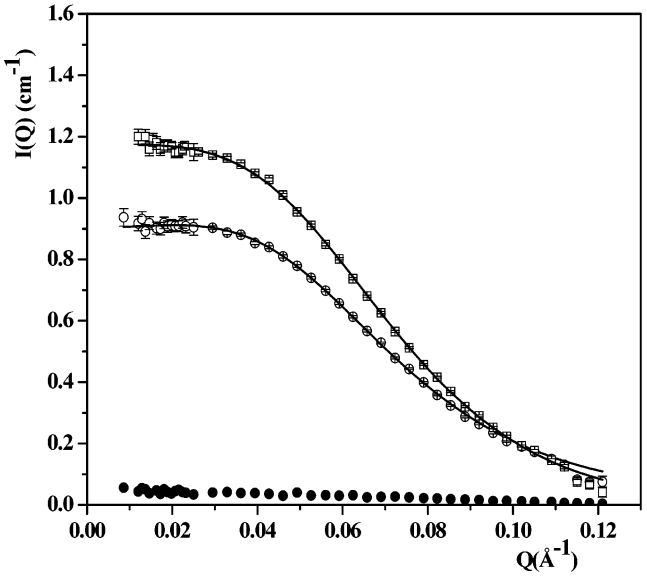

Figure 6. Scattering intensity profile for C18CCK8−D_2O (●), C18DTPAGlu−D_2O (○), and C18DTPAGlu−C18CCK8−D_2O (□).

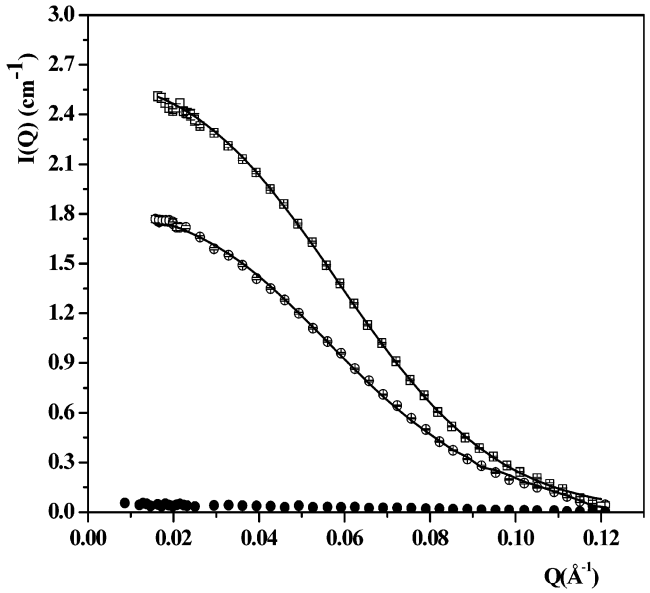

Figure 7. Scattering intensity profile for C18CCK8−D_2O (●), C18DTPAGlu(Gd)−D_2O (○), and C18DTPAGlu(Gd)−C18CCK8−D_2O (□).

The quantitative analysis of the NMRD profiles of the micellar forms gave τ_R values of 650 and 700 ps, respectively, for the species without and with C18CCK8 peptide.

Small-Angle Neutron Scattering. SANS measurements were performed only on the binary and ternary systems containing C18DTPAGlu (in the presence and absence of Gd ion) and C18CCK8 in order to gain structural information. As reported above, the presence of C12E5 in the mixed micelle does not produce a substantial improvement in the water proton relaxation value, and thus such an aggregate was considered of scarce interest with respect to the aim of the present research.

SANS experiments were performed on the C18DTPAGlu−D_2O (m = 0.0105 mol kg^{-1}), C18CCK8 (m = 0.0001 mol kg^{-1})−D_2O, and C18DTPAGlu (m = 0.0105 mol kg^{-1})−C18CCK8 (m = 0.0009 mol kg^{-1})−D_2O systems and on the systems where C18DTPAGlu was replaced by the corresponding C18DTPAGlu(Gd).

Binary Systems: C18DTPAGlu−D_2O and C18CCK8−D_2O. Figure 6 shows the scattering intensity distribution, $I(Q)$ vs Q, of the C18DTPAGlu−D_2O and C18CCK8−D_2O binary systems. The scattering profile of the sample containing C18CCK8 is quite flat, confirming that at this concentration solute aggregates are absent. In contrast, for C18DTPAGlu−D_2O the magnitude of $I(Q)$ is high at quite low Q values. This is characteristic of a slightly charged or uncharged micellar system, in which there are slight interparticle interactions among the micelles and the scattering is dominated by the form factor. Replacement of C18DTPAGlu chelating molecule by the corresponding Gd complex results in a substantial increase of $I(Q)$, in comparison with that collected in the absence of Gd (Figure 7). This difference is expected and reflects the additional contribution to the scattering length density, due to the metal ion in the hydrophilic shell of the micelles, in comparison to micelles formed by pure C18DTPAGlu. To gain structural information on the micelles present in the systems containing C18DTPAGlu, complexed or uncomplexed with Gd, eq 6 was fitted to the intensity scattering distributions taking into account eqs 7−10 with N_{agg}, d, and z as unknown parameters (z is the actual micelle charge). In Figures 6 and 7 the solid line shows good agreement between the experimental and calculated data. The parameters extracted are collected in Table 2. Inspection of the data reported in this table allows further considerations. All aggregates formed by C18DTPAGlu are substantially spherical micelles. The aggregation number of such micelles is ∼42, with a value for the total radius (core + shell) of ∼35 Å. Both values are in good agreement with those measured through fluorescence quenching (N_{agg} ∼ 35) and PGSE-NMR (R_{Hy} ∼ 37 Å) techniques. The actual micellar charge is ∼ −28.

The micelles formed by C18DTPAGlu(Gd) exhibit a larger value of the aggregation number (∼60) with respect to that of the chelating molecule in uncomplexed form (Table 2). This also produces an increase of the core radius (∼20 Å) and shell thickness (∼21 Å).

Ternary System: C18DTPAGlu−C18CCK8−D_2O. Addition of C18CCK8 to the C18DTPAGlu−D_2O system results in a marked change in the scattering of C18DTPAGlu. In particular, the magnitude of the $I(Q)$ at low Q values is much higher in the ternary system in comparison with that observed for the system where only C18DTPAGlu is present. On the basis that the pure C18CCK8 aqueous solution does not lead to an appreciable scattering intensity, the above observation suggests

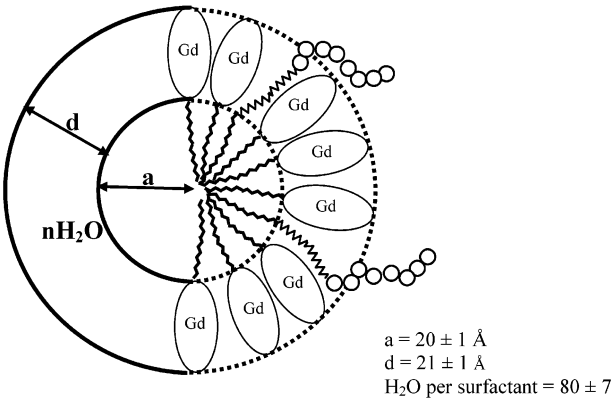

Figure 8. Structural data of the mixed micelles formed in aqueous solution by the two monomers C18DTPAGlu(Gd) and C18CCK8 at pH 7.4 and 25 °C.

that, at the imposed concentration, in the ternary system mixed micelles are present as revealed by the self-diffusion measurements. Structural information on the mixed C18DTPAGlu−C18CCK8 micelles has been obtained using the same procedure described in the previous section. However, in the present case, the micelle composition has been fixed taking into account that for each micelle an average of three C18CCK8 molecules were present for the C18DTPAGlu−water system, and the model was adjusted according to that composition. It is worth noting that several values of the peptide/micelle ratio were considered in our model. However, better agreement between the experimental data and the model was found when the imposed ratio was equal to 3.

The data extracted from the fitting curve (Figure 7) are nearly equal to those obtained for the pure C18DTPAGlu and C18DTPAGlu(Gd) micelles. A schematic representation of the structural parameters for the mixed C18DTPAGlu(Gd)−C18CCK8 micelle is reported in Figure 8.

Discussion

The two monomers used to form micelles have been successfully synthesized by using solid phase strategies. At the end of synthesis and purification procedures the two monomers were obtained with high purity grade, necessary for micelle formulation and physicochemical characterization.

The analysis of the physicochemical data collected allows several considerations. The surface tension measurements have clearly shown that the amphiphilic molecule C18DTPAGlu behaves as an anionic surfactant and at a concentration of $\sim 5 \times 10^{-5}$ mol kg^{-1}, namely at its cmc, forms aggregates. The corresponding molecules containing Gd(III) show the same behavior, although in this case the cmc has been detected at slightly higher solute concentration. From the structural point of view the C18DTPAGlu micelle is substantially spherical, with a hydrodynamic radius of ∼37 Å as measured by PGSE-NMR and SANS measurements. Its aggregation number evaluated through fluorescence quenching and SANS technique is nearly 40. Interestingly, a large amount of water is present in the micelle, about 90 molecules per C18DTPAGlu monomer. Such a large number suggests that there is a high penetration of water, probably, even in the hydrophobic micelle core. This should be the reason for the relatively small value of the length of the measured micellar core (∼17 Å), in comparison with that expected for a fully extended alkylic 18 carbon chain (∼24 Å).[38] In the aggregation, amphiphilic molecules release the hydrophobic tail contraction that they usually adopt to lower the surface exposed to water in order to minimize unfavorable interactions. Such an effect, known also as solvophobic contraction,[39] is eliminated when the chains experience a hydrophobic environment, such as that present in the micellar core. Thus, it is presumable that, due to the water penetration in the core of the micelle, the hydrophobic tail of the C18DTPAGlu cannot completely release its contraction. When the C18DTPAGlu molecule is replaced by the complex C18DTPAGlu(Gd), the micelle seems to be more compact and the aggregation number increases substantially as well as its size. This probably reflects the reduction of the charge borne by the DTPAGlu moiety (in the presence of Gd^{3+} the actual charge of the complex DTPAGlu(Gd) becomes −2) that in turn decreases the unfavorable electrostatic interaction between the headgroup of the chelating molecules placed in the shell of the micelle. As result, a larger size (∼41 Å) and a smaller amount of water (∼78 molecules) for surfactant of the C18DTPAGlu(Gd) micelle has been measured.

The measured proton relaxivity value, at 20 MHz and 25 °C, for C18DTPAGlu(Gd) is 17.5 mM^{-1} s^{-1}. This value is markedly larger than that measured for the simple complex DTPAGlu(Gd), 6.2 mM^{-1} s^{-1}.[18] According to eq 14, the slow rotation of the C18DTPAGlu(Gd) micelle decreases the longitudinal relaxation time of the water protons bound to the Gd and produces a substantial raise of the relaxivity. Indeed, from the quantitative analysis of NMRD profile, a τ_R value of 650 ps was determined. This value is much longer than the one obtained for the monomeric low molecular weight DTPAGlu(Gd) complex ($\tau_R \sim 100$ ps);[18] this elongation confirms the formation of aggregated species as there is an enhancement in the reorientational correlation time that reflects the increased dimensions of the object containing the Gd. In conclusion, the relaxometric behavior of the self-assembling C18DTPAGlu(Gd) micelles is similar to that exhibited by micelles obtained by Merbach and Maecke with lipophilic DOTA(Gd) monomers[12] and by mixed micelles reported by Anelli et al.[5]

With regard to C18CCK8 there is no evidence of self-aggregation. Indeed, its solubility in water is quite low (∼10^{-4} mol kg^{-1}). However, when C18CCK8 is added to the C18DTPAGlu (or C18DTPAGlu(Gd))−water system, its solubility increases as result of mixed micelle formation. This result is confirmed by PGSE-NMR and SANS measurements (Figures 6 and 7). In fact, in the self-diffusion measurements, the NMR signals due to both solutes never show a multiexponential decay, thus indicating that the two monomers co-micellize and, consequently, diffuse together. Accordingly, the magnitude of the scattering profile of the system containing C18CCK8, measured by SANS technique, is significantly increased. In particular for the ternary system, the surface tension data reveal that the cmc of the C18DTPAGlu−C18CCK8−D_2O system is substantially equal to that observed for the corresponding pure C18DTPAGlu−water system ($\sim 5 \times 10^{-5}$ mol kg^{-1}). The same result has been found when the complex C18DTPAGlu(Gd) (cmc $\sim 7 \times 10^{-5}$ mol kg^{-1}) is present in the system. The aggregation number of the mixed micelles is similar to that

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obtained in both the C18DTPAGlu−water (∼40) and C18DTPAGlu(Gd)−water (∼60) systems, as measured through the quenching fluorescence and SANS experiments (Tables 1 and 2). The same values have been obtained from SANS so that, in detail, the structure of the mixed C18DTPAGlu−C18CCK8 micelle, in the presence or absence of Gd, is substantially similar to that of the corresponding C18DTPAGlu pure aggregate. This is expected due to the large amount of the C18DTPAGlu monomers contained in the mixed micelle (the system was prepared imposing an average of three C18CCK8 molecules per micelle). Thus, as discussed above, also for the ternary system the presence of Gd, which reduces the actual charge on the DTPAGlu, increases the aggregation number of the mixed micelle. The SANS results are in agreement with PGSE-NMR and quenching data. In fact, R_{Hy} measured for the pure C18DTPAGlu micelle is equal to that measured for the C18CCK8−C18DTPAGlu mixed micelle ($R_{Hy} \sim 37$ Å). The structural parameters (Figure 8) also suggest that the bioactive CCK8 peptide is exposed on the external surface of the micelle: the length of the two oxyethylene linkers and the lysine residue, present in the shell, should be sufficient to allow a good exposition of the bioactive peptide of C18CCK8. However, the formation of a mixed C18DTPAGlu(Gd)−C18CCK8 micelle results in a slight increase of relaxivity value (18.7 mM^{-1} s^{-1}) with respect to that of the pure C18DTPAGlu(Gd) self-aggregate (17.5 $mM^{-1}s^{-1}$), reflecting the small difference in their molecular reorientation times ($\tau_R = 700$ and 650 ps for micelles with and without C18CCK8, respectively).

Finally, the addition of the C12E5 to the above systems does not produce a substantial improvement of the relaxivity value of the micelles containing Gd, although a sensible difference in the structure of the mixed micelles is expected. C12E5, in fact, forms rodlike micelles in water solution; as a consequence their aggregation number is quite large. In all the systems studied in this work, except those containing C18DTPAGlu, when present, C12E5 is in large amount and as a result the structural parameters of the mixed micelles are essentially dominated by it. In fact, as reported in Table 1, the mixed micelles containing C12E5 show a large aggregation number. In contrast, if C18DTPAGlu is also present in the system, due to the large size of its head (DTPAGlu), the mixed micelle exhibits an aggregation number similar to that of the pure C18DTPAGlu self-aggregate.

Conclusion

New amphiphilic molecules, constituted basically by a hydrophobic tail (18 carbon atoms) and the CCK8 peptide or the DTPAGlu moiety, have been prepared to obtain a mixed micelle to be used as a potential new contrast agent in magnetic resonance imaging technique. This is, to the best of our knowledge, the first attempt to form a supramolecular aggregate containing a bioactive molecule able to direct to a specific place, i.e., where tumor tissues are present, a large amount of paramagnetic gadolinium ions. Here we have reported a fully physicochemical characterization of mixed C18DTPAGlu−C18CCK8 micelles along with the corresponding pure C18DTPAGlu aggregate in the presence and absence of gadolinium. In all cases the micelles are substantially spherical with chelating agents and the CCK8 peptides pointing toward the external surface.

The present study, in particular the structural characterization and the relaxometric behavior, indicates that these kinds of mixed micelles are promising candidates as target-specific MRI contrast agents. In vitro and in vivo investigations are now in progress to confirm the efficiency of the high relaxing micellar aggregates in binding the colecystokinin receptors.

The constructs here described could be the first example of a new class of target-specific contrast agents. In fact, many parameters can be tuned in order to improve the biological properties (receptor binding, biodistribution behavior, toxicity) of these new diagnostics: the number of peptide-containing monomers to be included in the micelle; the number and nature of the spacers between the hydrophobic tail and the bioactive molecule; the nature of the bioactive molecule to address the contrast agents on relevant biological targets.

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