ORIGINAL CONTRIBUTION

Polymerized mixed aggregates containing gadolinium complex and CCK8 peptide

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Abstract Two novel amphiphilic unimers containing an aliphatic hydrophobic chain (PDA) with two C≡C triple bonds and hydrophilic heads presenting the chelating agent DTPAGlu and the CCK8 bioactive peptide, respectively, have been prepared by solid phase synthesis. Aggregates obtained by mixing together PDA-DTPAGlu, or its Gd(III) complex, and PDA-L2-CCK8 in 70/30 molar ratio before and after a polymerization process carried out by UV irradiation have been structurally characterized by means of small angle neutron scattering. The relaxivity properties of aggregates containing Gadolinium complexes have also been investigated. Elongated mixed micelles have been observed, in which the relaxivity value r_{1p} for each Gadolinium complex, measured at 20 MHz and 298 K, is around 12 mM^{-1} s^{-1}.

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Introduction

Magnetic resonance imaging (MRI) is a very appealing medical diagnostic technique for its ability to give well-resolved in vivo images of many organs and districts [1, 2]. Due to its very low sensitivity, especially if compared to the Nuclear Medicine diagnostic techniques (single photon emission computed tomography and positron emission tomography), it needs to accumulate high concentration of contrast agents [3]. There is an increasing interest in developing new contrast agents for MRI with enhanced properties. One important approach is to address the contrast agent towards cell receptors overexpressed in well-defined pathologies. This approach has the obvious advantage of having easy accessibility to the molecular target, thus, giving well-resolved images of the area of interest and low concentration of the potentially toxic contrast agent in non target organs and districts [4]. However, in order to optimize the target to background ratio, a specific target MRI contrast agent has to be designed to have the following characteristics: (a) capacity to carry a high number of reporter compound having high relaxivity such as paramagnetic Gd(III) complexes because of the low receptor density on the cells; (b) sufficiently long intravascular half-time to allow the agents to interact and accumulate on the target site; (c) ability to be easily

modified with different targeting ligands and contrast agents; and (d) to be nontoxic and well tolerated [4].

Our aim concerns the development of a new class of supramolecular aggregates containing a large number of Gadolinium complexes and several units of a bioactive peptide well exposed on the aggregate surface, thus, combining in the same contrast agent the high relaxivity due to the large number of paramagnetic complexes and the target specificity of the bioactive peptide. These supramolecular aggregates are obtained by assembling two amphiphilic unimers: one containing the bioactive peptide and one containing the Gadolinium complex [5–8]. Many factors can influence the structural properties and the stability of the supramolecular aggregates [9]. In fact, it is well known that pH, temperature, and ionic strength could influence drastically colloidal aggregate structures [9].

Furthermore, the design of the unimers is critical in ruling the shape and size of the aggregates. The formation of spherical or rod-like micelles, open bilayers, and vesicles or liposomes can be determined by architectural parameters such as: (1) the length of the hydrocarbon chains of the unimer; (2) the ratio between the two unimers in the supramolecular aggregates; (3) the presence of spacers between the hydrophobic moiety and the bioactive peptide; and (4) the procedure of aggregate preparation, i.e., spontaneous coaggregation or the use of extrusion procedures [9]. It is worthy to note that in general, micelles respond rapidly to changes in their environment, while bilayers are much more sluggish.

This topic results of crucial importance for the use of this class of target selective contrast agents from the clinical point of view. In fact, in order to preserve the high relaxivity and the target selectivity, the supramolecular aggregates should remain in a compact-defined structure during the imaging analysis. The aggregates should not collapse upon of 1,000÷10,000-fold dilution once that stock solution of the contrast agent is injected in the human body.

Concerning this, the use of micelles in some cases could be unappropriated.

In order to overcome such deficiency, we have made an attempt to form polymerized structures formed of covalent bonds that in principle should not disaggregate even at high dilution.

Here, we report on the synthesis of two novel amphiphilic unimers in which the hydrophobic moiety has two C≡C triple bonds able to give, upon photochemical polymerization, stable aggregates covalently bound. The two unimers, PDA-DTPAGlu and PDA-L2-CCK8, are schematized in Fig. 1. In the abbreviation we used, PDA stays for the hydrophobic moiety 10,12-pentacosadiynoic acid [10], DTPAGlu (*N,N*-bis[2-[bis(carboxyethyl)amino]ethyl]-L-glutamic acid) is a DTPA-like chelating agent, L2 represents the two 8-amino-3,6-dioxaoctanoic acid used as spacers, and CCK8 is the cholecystokinin bioactive peptide used to target the aggregates on cholecystokinin receptors [11].
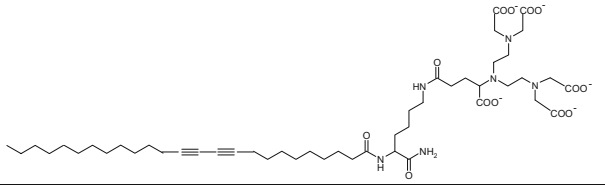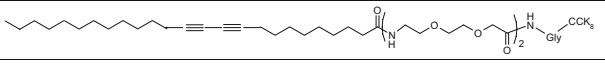
Aggregates obtained by mixing PDA-DTPAGlu, or its Gd(III) complex, PDA-DTPAGlu(Gd), and PDA-L2-CCK8 in 70/30 molar ratio, before and after polymerization are structurally characterized by using small angle neutron scattering (SANS) techniques. The relaxivity properties of the polymerized aggregates, containing Gadolinium complexes, are also investigated.

Experimentals

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 pentacosadynoic acid (PDA) was purchased by Lancaster Chemicals (CA). The DTPAGlu pentaester, *N,N*-Bis[2-[bis[2-(1,1-dimethyletoxy)-2-oxoethyl]-amino]ethyl]-L-glutamic acid 1-(1,1-Dimethylethyl) ester was prepared according to the experimental procedure reported in literature [12]. All other chemicals were commercially available by Sigma–Aldrich–Fluka (Bucks, Switzerland) or LabScan (Stillorgan, Dublin, Ireland) and were used as received unless otherwise

Fig. 1 Chemical structures and relevant parameters used in structural investigation by SANS of PDA-DTPAGlu and PDA-L2-CCK8

	Molecular volume (Å³)	Scattering length density (Å⁻²)	
		Hydrophobic core	Hydrophilic shell
	$1.6 \cdot 10^3$	$9.93 \cdot 10^{-8}$	$1.29 \cdot 10^{-6}$
	$3.6 \cdot 10^3$	$9.93 \cdot 10^{-8}$	$1.15 \cdot 10^{-6}$

stated. All solutions were prepared by weight using doubly distilled water. The pH of all solutions was kept constant at 7.4. Solid-phase peptide synthesis was performed by automatic solid-phase synthesis using a 433A Applied Biosystems automatic synthesizer according to the Fmoc methodology. HPLC columns were from Phenomenex (Torrance, CA, USA). The LC-MS system equipped with ESI sources was from ThermoElectron (Milan, Italy). UV-Vis spectra were carried out by using an UV-Vis Jasco (Easton, MD) model 440 spectrophotometer with a path length of 1.0 cm.

Synthesis of PDA-L2-CCK8

Peptide synthesis was carried out in solid-phase under standard conditions using Fmoc strategy [13]. Rink-amide MBHA resin (0.78 mmol/g, 0.5 mmol scale, 0.640 g) was used. The elongation of the CCK8-G peptide was achieved by sequential addition of Fmoc-AA-OH with HOBt/PyBop/DIPEA (1-hydroxybenzotriazole/benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium/N,N diisopropylethylamine) (1:1:2) as coupling reagents in N,N-dimethylformamide (DMF) in pre-activation mode. The mixture was stirred for 1 h and after filtration; the corresponding colorimetric test (Kaiser test) indicated the completion of the coupling. All couplings were performed twice for 1 h by using an excess of four equivalents for the single amino acid derivative. Fmoc deprotections were obtained by 30% solution of piperidine in DMF. When the peptide synthesis was complete, the Fmoc N-terminal protecting group was removed and the two residues of Fmoc-AdOO-OH were condensed by using an excess of two equivalents and a single coupling for each residue. Then, the resin was washed, the terminal Fmoc protection removed, and 0.756 g (2.0 mmol) of pentacosadiynoic acid in DMF were condensed. Coupling was repeated twice under nitrogen flow for 1 h. Peptide was cleaved from the resin with trifluoroacetic acid (TFA; 5 ml) containing 2.5% (v/v) water and 2.0% (v/v) tri-isopropylsylane as a scavenger at room temperature for 2 h. Free peptide was precipitated in cold ethyl ether (Et$_2$O) and lyophilized from a 50% H$_2$O/CH$_3$CN solution. Crude peptide was purified by reversed phase HPLC on a LC8 Shimadzu HPLC system (Shimadzu Corporation, Milan, Italy) equipped with a UV lambda-Max Model 481 detector using a Phenomenex C4 (300 Å, 250×21.20 mm, 5 μ), eluted with H$_2$O/0.1% TFA (A) and CH$_3$CN/0.1% TFA (B) from 20% a 95% over 25 min at 20 ml/min flow rate. Fractions were characterized by LC–MS analysis (0.2 μg/fraction) to assess purity and molecular weight. Pure fractions were pooled and lyophilized.

PDA-L2-CCK8; Rt=15.5 min; (MW=1767) [M + H]$^+$= 1768 u.m.a.

Synthesis of PDA-DTPAGlu

Fmoc-Lys(Mtt)-OH (624.79 mg (1.00 mmol)) activated by one equivalent of PyBop and HOBt and two equivalents of DIPEA in DMF were coupled to Rink-amide MBHA resin (0.78 mmol/g, 0.250 mmol scale, 0.320 g) stirring the slurry suspension for 1 h. The solution was filtered, and the resin was washed with three portions of DMF and three portions of dichloromethane (DCM). The 4-methyltrityl-protecting group (Mtt) was removed by treatment with 2.0 ml of DCM/triisopropylsylane (TIS)/TFA (94:5:1) mixture. The treatment was repeated several times until the solution became colorless. The resin was washed by DMF 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 equivalents of DTPAGlu-pentaester and O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium, and four equivalents 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 Kaiser test. After removal of the Fmoc group by 20% piperidine in DMF, the coupling of 10,12-pentacosadiynoic acid was performed in DMF 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 ESI spectrometry.

^{1}H-NMR (chemical shifts in δ, TMS as internal standard)= 4.30 (m, 1H, C*H*Glu α), 4.18 (m, 1H, C*H* Lys α), 3.40 (s, 8H, C*H*$_2$COOH) 3.2 (m, 2H, N-C*H*$_2$), 3.1 (m, 2H, N-C*H*$_2$), 3.95 (t, 2H, C*H*$_2$ Lys ε) 2.9 (m, 4H, N-C*H*$_2$), 2.25 (m, 2H, C*H*$_2$CO),1.95 (m, 2H, RC*H*$_2$C*H*$_2$CO), 1.5 (m, 1H, C*H*$_2$ Lys β), 1.32 (m, 2H, C*H*$_2$ Lys β,δ), 1.22 (m, 2H, C*H*$_2$ Lys δ,γ), 1.1 (m, 1H, C*H*$_2$ Lys γ), 1.1 (overlapped, 2H, RC*H*$_2$CH$_2$CO), 1.04 (m, 38H, C*H*$_2$ aliphatic), 0.60 (t, 3H, C*H*$_3$).

ESI: (MW=949) [M+H]$^+$=950 u.m.a.

Preparation of Gadolinium complex

The complexation has been carried out by adding light excess of the GdCl$_3$ to the aqueous solution of the PDA-DTPAGlu ligand at neutral pH and room temperature. The formation of Gd complex 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.

Solution preparation

Stock solutions were prepared in 0.1 M phosphate buffer at pH 7.4, and filtered through a 0.45 μm filter. Concentrations of solutions containing CCK8 peptide were determined by absorbance on a UV-vis Jasco (Easton, MD, USA) Model 440 spectrophotometer with a path length of 1.0 cm using a molar absorptivity (ε_{280}) of 6,845 M^{-1} cm^{-1} for CCK8. This value was calculated according to the Edelhoch method [14], taking into account contributions from tyrosine and tryptophan present in the primary structure, which amount to 1,215 and 5,630 M^{-1} cm^{-1}, respectively [15]. The solutions were stirred at room temperature until complete dissolution and then used without further treatment.

One milliliter of the binary system (PDA-DTPAGlu/H_2O), at unimers concentration higher than *cmc*, was poured in quartz cuvette and cooled at 0 °C in ice bath. The solution was irradiated by UV lamp at 254 nm for 1÷4 h keeping the temperature at 0 °C. The polymerization process was monitored by UV-vis spectroscopy following the progressive red shift of the electron transitions band. The polymerizations of lipophilic tails were carried out in the same way for PDA-DTPAGlu(Gd)/H_2O and PDA-L2-CCK8/H_2O binary systems and for the two ternary systems containing the two unimers in 70/30 molar ratio.

Fluorescence studies

Critical micellar concentration (*cmc*) values of micellar aggregates were obtained by Fluorescence spectroscopy using 1.0 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 experiments, with a recording speed of 125 nm/min and automatic selection of the time constant. The *cmc* were measured by using 8-anilinonaphthalene-1-sulfonate (ANS) as fluorescent probe. Small aliquots of aggregate solution ($c_1=1.0\cdot10^{-3}$ M; $c_2=1.0\cdot10^{-4}$ M $c_3=1.0\cdot10^{-5}$ M), were added to a fixed volume of $1.0\cdot10^{-5}$ M ANS dissolved in the same buffer. The fluorescence spectra were monitored at 480 nm, upon excitation at 350 nm as previously reported [16, 17].

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 (micropolarity). ANS is an anionic probe which is essentially nonfluorescent in water but highly fluorescent in nonpolar environments or micelles [18].

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, two scans). A typical 90° pulse width was 4 μs, and the reproducibility of the T_1 data was ±0.5%. The temperature was maintained at 298 K 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 0.00024 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%. Datapoints 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.

^{17}O measurements

Variable temperature ^{17}O NMR measurements were recorded at 2.1 T on the JEOL EX-90 spectrometer, equipped with a 5 mm probe by using a D_2O external lock. Experimental settings were: spectral width 10,000 Hz, 90° pulse (7 μs), acquisition time 10 ms, 1,000 scans and without sample spinning. Aqueous solutions containing 2.6% of ^{17}O isotope (Yeda, Israel) were used. The observed transverse relaxation rates (R_{2obs}^O) were calculated from the signal width at half-height $\left(\Delta\nu_{1/2} : R_{2obs}^O = \pi\Delta\nu_{1/2}\right)$.

Small angle neutron scattering

Small angle neutron scattering measurements were performed at the KWS2 instrument located at the Forschungsneutronenquelle Heinz Maier Leibnitz of Garching bei München, Germany. Neutrons with an average wavelength λ of 6.3 Å and a wavelength spread $\Delta\lambda/\lambda \leq 0.2$ were used. A two-dimensional array detector at two different sample-to-detector distances, 2 and 8 m, detected neutrons scattered from the samples. These configurations allowed collecting the scattering cross section in a range of the transferred momentum q between 0.006 and 0.16 $Å^{-1}$; q represents the modulus of the scattering vector $\vec{q}$ and for elastic, phenomena is related to the neutron wavelength λ and to the scattering angle θ through the equation

$$q = \frac{4\pi}{\lambda} \sin\frac{\theta}{2} \tag{1}$$

For the experiments samples were contained in 1 mm path length HELLMA 404QX quartz cells and measurements times ranged between 15 min to 1 h. D_2O was used as solvent for the preparation of all the solutions in order to minimize the incoherent scattering contribution to the total cross section.

Raw data were corrected for electronic background and empty cell scattering. Detector sensitivity corrections and

transformation to absolute scattering cross sections $d\Sigma/d\Omega$ were made with a secondary Plexiglass standard according to the standard procedure [19, 20].

Results and discussion

Unimers synthesis and aggregates preparation

The amphiphilic unimers were synthesized by solid-phase methods using Rink-amide MBHA resin as polymeric support and the Fmoc/tBu chemistry, unimer purified by RP-HPLC and characterized by LC-MS or MALDI-tof for their identity and purity. The Gadolinium complex of PDA-DTPAGlu was prepared mixing stoichiometric amounts of $GdCl_3$ and the ligand at neutral pH and room temperature. The excess of uncomplexed Gd(III) ions was removed by filtration with a 0.2-μm syringe filter of the solution brought to pH 10 and the absence of free Gd(III) ions was checked by orange xylenol UV measurements. The presence of free Gd(III) ions in solution, in fact, could yield a variation of the observed relaxation rate. The assembly of pure and mixed aggregates was achieved by simple dissolving unimers in 0.1 M phosphate buffer pH 7.4. In the ternary systems, the molar ratio between unimers, PDA-DTPAGlu(Gd) and PDA-L2-CCK8, was 70/30.

The polymerized aggregates were obtained by UV irradiation at 254 nm of the aqueous solutions containing the unimers. The polymerization process has been performed on the binary systems (PDA-DTPAGlu/H2O; PDA-DTPAGlu(Gd)/H2O and PDA-L2-CCK8) and on the two ternary systems containing the two unimers in 70/30 molar ratio. The polymerization process, schematically reported in Scheme 1, was monitored by UV-VIS spectroscopy. Red shift was observed in the visible region of the spectrum (data not shown), thus, indicating the formation of conjugated bonds in the polymerized micelles.

Fluorescence measurements

The fluorescence measurements allowed evaluating the critical micellar concentration for the systems not subjected to the polymerization process. In particular, the *cmc* has been evaluated at the intersection of the two lines fitted to the experimental data in the premicellar and postmicellar regions, respectively, as shown for one of the systems analyzed in Figs. 2 and 3. All the evaluated *cmc*s are reported in Table 1 [21].

Relaxometric characterization

The measured relaxivity value (r_{1p}) is defined, according to Eq. 2, as the paramagnetic contribution to the measured proton longitudinal relaxation rate (R_{1obs}) of a solution containing 1.0 mM concentration of the paramagnetic solute [22]
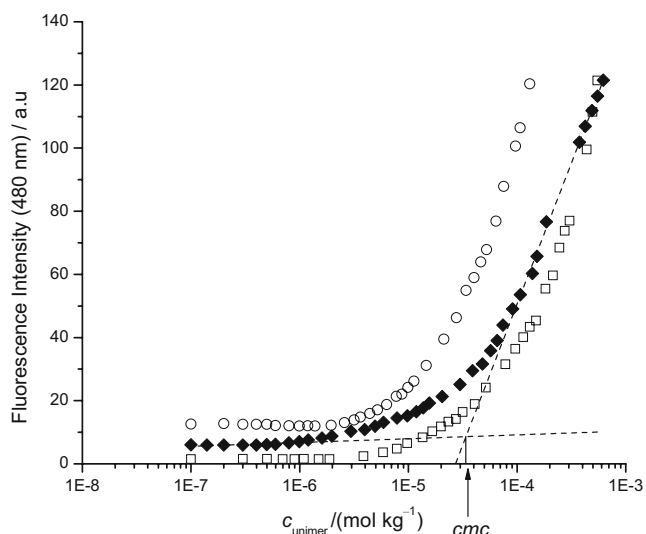
$$R_{1obs} = [Gd]r_{1p} + R_{1W} \qquad (2)$$

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

Relaxivity values at 20 MHz and 298 K (Table 2) were determined mineralizing a given quantity of sample with HCl 37% at 120 °C overnight in order to determine the exact concentration of Gd(III) present in solution: from the

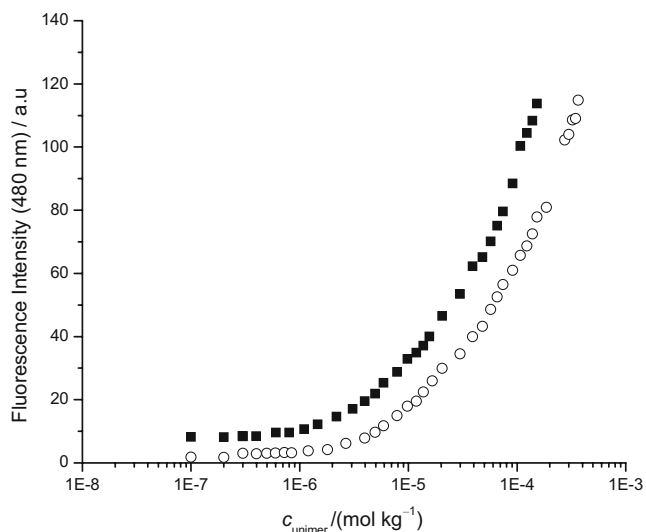

Scheme 1 Scheme of the formation of the polymer formation
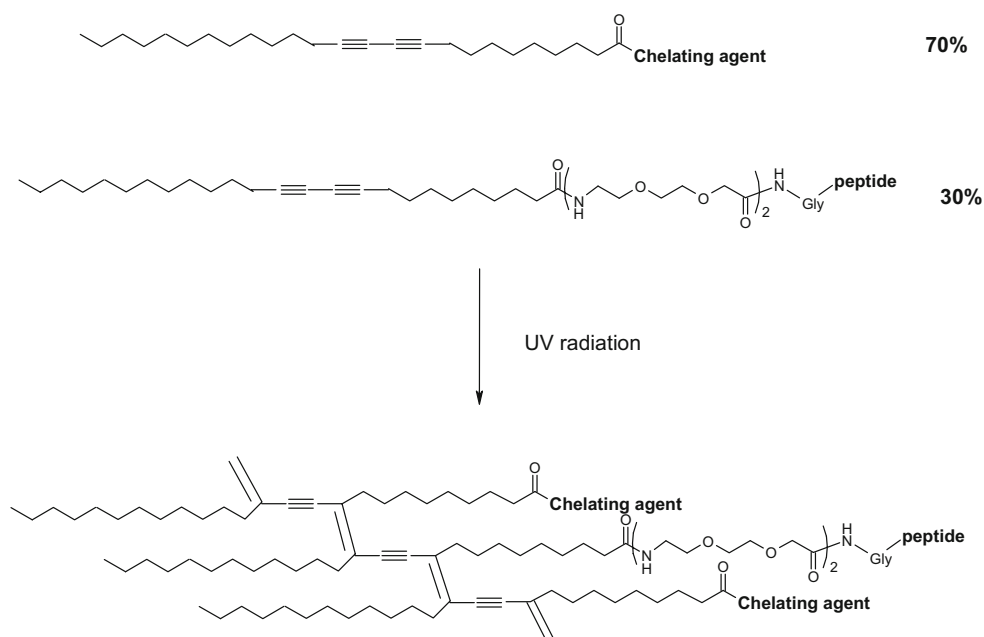
Fig. 2 Fluorescence intensity of ANS fluorophore at 480 nm versus amphiphiles concentration: PDA-DTPAGlu (*filled diamond*) and PDA-L2-CCK8 (*unfilled circle*) binary systems and PDA-DTPAGlu/PDA-L2-CCK8 (*unfilled square*) ternary system. The determination of *cmc* has been exemplified for PDA-DTPAGlu system and reported

measure of the observed relaxation rate (R_{1obs}) of the acidic solution and knowing the relaxivity (r_{1p}) of Gd(III) aquaion in acidic conditions (13.5 mM^{-1} s^{-1} at 20 MHz and 298 K—value determined by using standard GdCl$_3$ solutions whose concentrations were measured by ICP-MAS, accuracy 0.1%) and the diamagnetic contribution (R_{1W}) in acidic conditions (0.5 mM^{-1} s^{-1}), the exact Gd(III) concentration was calculated by using Eq. 2. Then, knowing [GdL] and measuring R_{1obs} of the aggregate containing solutions, the relaxivity values of the four systems considered were calculated using again Eq. 2.

Fig. 3 Fluorescence intensity of ANS fluorophore at 480 nm versus amphiphiles concentration: PDA-DTPAGlu(Gd) (*unfilled circle*) and PDA-DTPAGlu(Gd)/PDA-L2-CCK8 (*filled square*) ternary system

Table 1 *cmc* Values of supramolecular aggregates

System	$\frac{cmc}{mol\,kg^{-1}}$
PDA-L2-CCK8-H$_2$O	$\cong 2 \cdot 10^{-5}$
PDA-DTPAGlu-H$_2$O	$\cong 3 \cdot 10^{-5}$
PDA-DTPAGlu(Gd) -H$_2$O	$\cong 3 \cdot 10^{-5}$
PDA-DTPAGlu/PDA-L2-CCK8/H$_2$O	$\cong 8 \cdot 10^{-5}$
PDA-DTPAGlu(Gd)/PDA-L2-CCK8/ H$_2$O	$\cong 2 \cdot 10^{-5}$

In order to reach high relaxivity values, a very important parameter to account for is the exchange lifetime of the coordinated water molecule (τ_M) of a Gd(III) complex. The analysis of the temperature dependence of transverse relaxation rate of the metal bound ^{17}O water resonance may be considered the method of choice for the determination of τ_M values. The τ_M values determined for the PDA-DTPAGlu(Gd)–H$_2$O binary system, in polymerized and nonpolymerized form are 42 and 95 ns, respectively (see Table 2). The last value is in agreement with values found for other pure and mixed nonpolymerized aggregates already reported.

From the quantitative analysis of nuclear magnetic relaxation dispersion (NMRD) 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) [3] that is strictly related to the molecular dimensions of the investigated systems. In Figs. 4 and 5, the NMRD profiles relative to all systems complexed with gadolinium are reported. The analysis of experimental data has been made according to the Solomon–Bloembergen–Morgan model, modified according to the Lipari–Szabo approach [23, 24] for which it can be distinguished between a local faster motion (governed by τ_l) and a global slower motion (governed by τ_g). The extent of local to global contribution to the overall motion is determined by an order parameter (S^2) that can change from 0 to 1. For aggregated lipophilic systems such as micelles, this kind of approach is frequently utilized [25, 26] as it is likely that Gd(III) complexes on the surface of the micellar system are endowed with a faster individual motion but at the same time resent of the global slower motion of the supramolecolar system. All NMRD profiles have been fitted considering one water molecule in the inner coordination sphere for each Gd(III) complex ($n_w =$ 1), Gd-H distance of 3.1 Å, and fixing the exchange lifetime (τ_M) to the value obtained from ^{17}O-NMR studies, as reported elsewhere [27]. The τ_R values determined from the quantitative analysis of the NMRD profiles are collected in Table 2. The τ_R and τ_M values found for polymerized and nonpolymerized aggregates are roughly the same for the ternary systems, as expected, whereas some differences are observed for the binary systems. Since in all the systems we are in the presence of micelles with dimensions unaffected by the polymerization process, similar values of the

Table 2 Relaxometric parameters measured at pH 7.4, T=298 K calculated from the fitting of NMRD and ^{17}O-NMR (τ_M) data

System	r_{1p} (20 MHz,25 °C)	$\frac{\tau_1}{ps}$	$\frac{\tau_g}{ps}$	S^2	$\frac{\tau_M}{ns}$
PDA-DTPA(Gd) unpolymerized	14.8±2.1	224±14	1820±94	0.42±0.01	95±20
PDA-DTPA(Gd) polymerized	12.2±2.3	141±24	1472±181	0.41±0.01	40±10
PDA-DTPA(Gd)/PDA-L2-CCK8 unpolymerized	12.3±2.0	90.9±6.5	1650±82	0.44±0.01	95±20
PDA-DTPA(Gd)/PDA-L2-CCK8 polymerized	12.1±2.1	92.8±8.1	1597±112	0.42±0.01	40±10

Solution containing 1.0 mM concentration of the paramagnetic component are used. The errors associated to relaxivity values r_{1p} have been evaluated considering twice the standard deviation of the relaxation times. The meaning of the symbols used is reported in the text

relaxation times were predictable for both systems. On these basis, the relaxivity values measured for micelles present in the binary system DTPAGlu(Gd)–H$_2$O were unforeseen. It is likely that for all the systems the relaxivity value is around 13 mM^{-1} s^{-1} if we consider that the uncertainness associated to τ_1 and τ_g is twice the standard deviation reported in Table 2. The values reported in this table are those obtained from statistical analysis of experimental data that is underestimated. In fact, when we consider twice the standard deviation associated to the relaxation times, all the relaxivities are ranged within the uncertainness.

Structural characterization

Figures 6 and 7 show the scattering cross sections collected for the binary systems PDA-DTPAGlu/D$_2$O, PDA-DTPA Glu(Gd)/D$_2$O and for the corresponding ternary systems containing PDA-L2-CCK8. All the samples were analyzed before and after the polymerization process carried out by UV treatment. The structures of the unimers are reported in Fig. 1 together with their own volumes and scattering lengths. To clarify the obtained results, cross sections in Figs. 6 and 7 have been multiplied for a convenient scale factor as there indicated.

Inspection of the figures reveals the typical form factor of small aggregates, i.e., spherical or ellipsoidal micelles. The absence of a correlation peak suggests the absence of significant intermicellar interactions that, since the charged nature of the PDA-DTPAGlu and PDA-DTPAGlu(Gd) molecules, can be ascribed either to the presence of the 0.1 M buffer that further damp the structure factor or to the high dilution of the aggregates present in the system. This latter observation is also confirmed by the low absolute scattering cross sections detected for all the systems.

In order to extract structural information from the scattering cross sections, appropriate equations have been fitted to the experimental data by using a suitable model to describe the aggregates. Generally speaking, the scattering cross section $d\Sigma/d\Omega$ of a collection of monodisperse bodies is described by the equation [28]

$$\frac{d\Sigma}{d\Omega} = k_s\, n_p\, P(q)\, S(q) + \left(\frac{d\Sigma}{d\Omega}\right)_{incoh} \qquad (3)$$

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
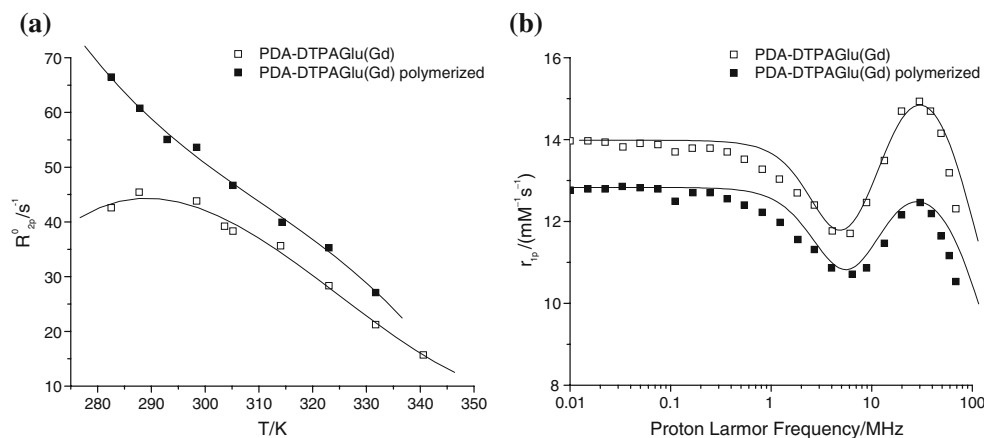
Fig. 4 a Temperature dependence of the paramagnetic contribution to the water ^{17}O NMR transverse relaxation rate (R^o_{2p}) for a solution of PDA-DTPAGlu(Gd) polymerized and non polymerized (10 mM, pH 7) at 90 MHz. The solid curve through the datapoints was calculated with the parameters reported in Table 2 and fixing the distance between Gd(III) ion and the oxygen of the coordinated water molecule to 2.5 Å and the Gd-^{17}O scalar coupling constant to $-3.8\cdot10^6$ rad/s; **b** nuclear magnetic resonance dispersion profiles of PDA-DTPAGlu(Gd) polymerized and nonpolymerized
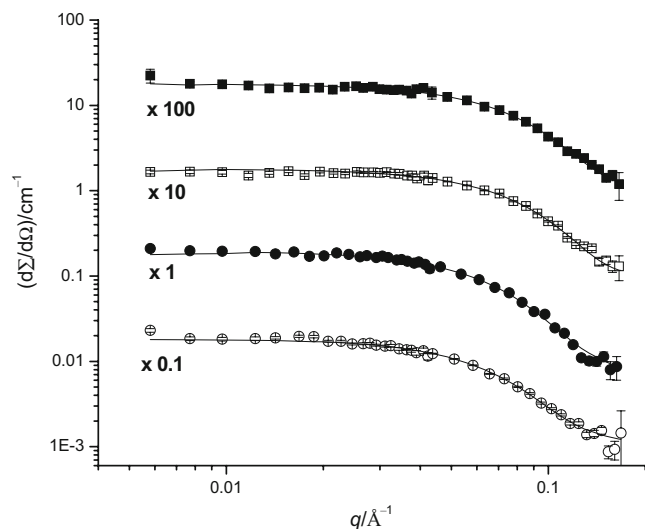
Fig. 5 Nuclear magnetic resonance dispersion profiles of: **a** PDA-DTPAGlu(Gd) (*unfilled square*) and PDA-DTPAGlu (Gd)/PDA-L2-CCK8 (*filled square*); **b** PDA-DTPAGlu(Gd) (*unfilled square*) and PDA-DTPAGlu(Gd)/PDA-L2-CCK8 (*filled square*) after polymerization process. NMRD are recovered at pH=7.4 and 25 °C, normalized to 1 mM concentration of Gd(III) ion. The *solid curves* through the datapoints were calculated with the parameters reported in Table 2
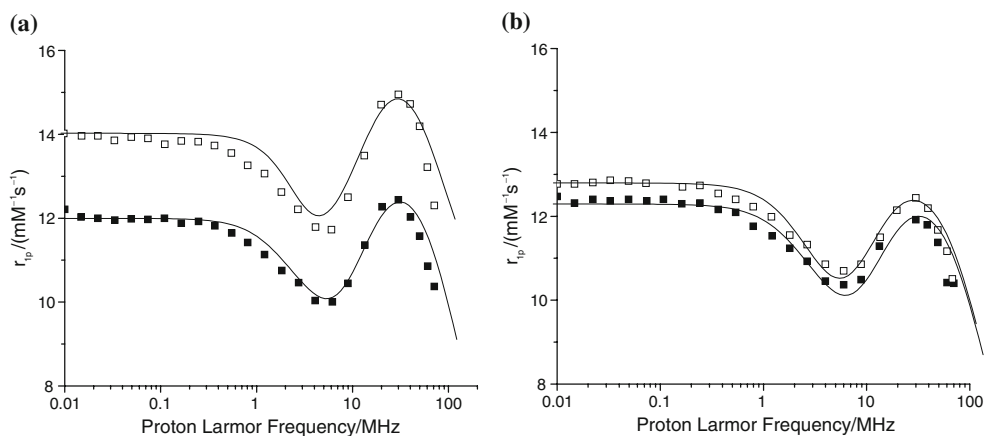
bodies, while $(d\Sigma/d\Omega)_{incoh}$ is the incoherent scattering cross section. The form factor $P(q)$ contains information on the shape of the scattering objects, while the structure factor $S(q)$ accounts for interparticle correlations and is normally important for concentrated or charged systems. The k_s is a scale factor that from a theoretical point of view should be unitary and the experimental value allows evaluating the goodness of the fitting as explained below.

According to the previous observations the structure factor can be approximated to the unity, and the scattering cross section is reduced to the equation

$$\frac{d\Sigma}{d\Omega} = k_s\, n_p\, P(q) + \left(\frac{d\Sigma}{d\Omega}\right)_{incoh} \tag{4}$$

Structural parameters of micelles have been established modeling the aggregates as ellipsoids made by a hydrophobic core with semiaxes a, $b=c$ and a hydrophilic part with a thickness d. The form factor $P(q)$ can be written as

$$P(q) = \int_0^1 |F(q,\mu)|^2 d\mu \tag{5}$$

where $F(q, \mu)$ is the angle-dependent form factor for ellipsoidal two-shell micelles

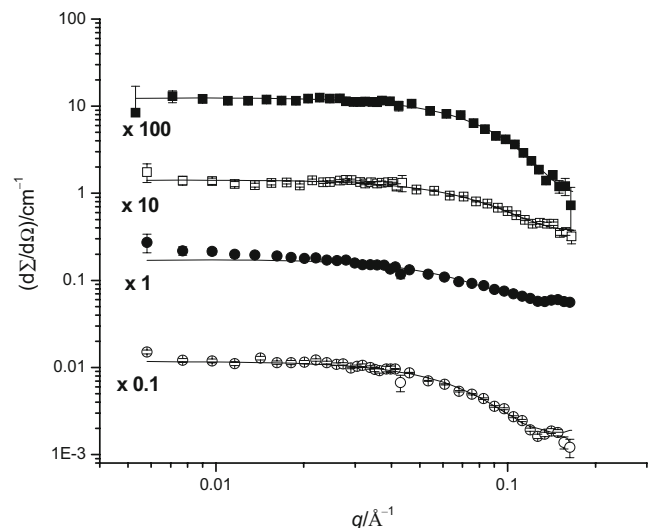$$F(q,\mu) = f\frac{3j_1(u_1)}{u_1} + (1-f)\frac{3j_1(u_2)}{u_2} \tag{6}$$

Fig. 6 Scattering cross sections obtained at 25°C for the following systems: (○) PDA-DTPAGlu(Gd) 1.06 mmol/kg polymerized – D$_2$O; (●) PDA-DTPAGlu(Gd) 1.10 mmol/kg unpolymerized – D$_2$O, (□) PDA-DTPAGlu 1.04 mmol/kg polymerized – D$_2$O, (■) PDA-DTPAGlu 0.96 mmol/kg unpolymerized – D$_2$O. For a better visualization, cross sections have been multiplied for a suitable scale factor. Furthermore, curves obtained from the fitting of the model described in the text are also reported

Fig. 7 Scattering cross sections obtained at 25°C for the following systems: (○) PDA-DTPAGlu(Gd) 0.67 mmol/kg – PDA-L2-CCK8 0.39 mmol/kg polymerized – D$_2$O, (●) PDA-DTPAGlu(Gd) 0.71 mmol/kg – PDA-L2-CCK8 0.28 mmol/kg unpolymerized – D$_2$O, (□) PDA-DTPAGlu 0.60 mmol/kg – PDA-L2-CCK8 0.31 mmol/kg polymerized – D$_2$O, (■) PDA-DTPAGlu 0.70 mmol/kg – PDA-L2-CCK8 0.34 mmol/kg unpolymerized – D$_2$O. For a better visualization, cross sections have been multiplied for a suitable scale factor. Furthermore, curves obtained from the fitting of the model described in the text are also reported

Table 3 Aggregation numbers and micellar spherical radii obtained at 25 °C for the systems inspected by means of SANS measurements

System	$\frac{10^{-18}n_p}{dm^{-3}}$	N_{agg}	$\frac{a}{A}$	$\frac{b}{A}$	$\frac{d}{A}$
PDA-DTPAGlu(Gd) polymerized/D$_2$O	7.1±0.7	87±5	33±2	12±2	16±2
PDA-DTPAGlu(Gd) unpolymerized/D$_2$O	0.15±0.02	77±5	33±2	11±2	16±2
PDA-DTPAGlu polymerized/D$_2$O	11±1.2	56±3	25±2	14±2	16±2
PDA-DTPAGlu unpolymerized/D$_2$O	10±0.9	54±3	26±2	12±2	15±2
PDA-DTPAGlu(Gd)/PDA-L2-CCK8 polymerized/D$_2$O	1.1±0.1	49±5	27±2	10±2	20±2
PDA-DTPAGlu(Gd)/PDA-L2-CCK8 unpolymerized/D$_2$O	9.4±0.9	62±5	30±2	10±2	20±2
PDA-DTPAGlu/PDA-L2-CCK8 polymerized/D$_2$O	17±1.5	29±3	21±2	9±2	16±2
PDA-DTPAGlu/PDA-L2-CCK8 unpolymerized/D$_2$O	17±1.4	30±3	19±2	9±2	17±2

The values reported have been obtained through a fitting of the model described in the text. The meaning of the symbols used is reported in the text

with

$$u_1 = q\sqrt{\mu^2 a^2 + (1-\mu^2)b^2} \qquad (7)$$

$$u_2 = q\sqrt{\mu^2(a+d)^2 + (1-\mu^2)(b+d)^2} \qquad (8)$$

$$f = \frac{\frac{4}{3}\pi ab^2(\rho_1 - \rho_2)}{\sum_i b_i - \frac{4}{3}\pi(a+d)(b+d)^2\rho_s} \qquad (9)$$

where j_1 is the spherical Bessel function of first order, V_{core} is the micelle core volume, ρ_1 and ρ_2 are the scattering length densities of the core and shell. In Eq. 9 the summation is carried out over all the nucleus composing the micelles.

For binary systems, ρ_2 has been taken equal to that of the solute hydrophilic part, whereas for ternary systems, a weighted average between those of the two solutes has been used. On the other hand, the molecules ρ_1 results are identical.

By using Eqs. 3–9, structural information on the aggregates present in the system have been obtained and reported in Table 3, together with the aggregation number N_{agg}. To verify the reliability of the fit, it is needed that not only that the shape of the scattering is reproduced but also that the absolute values of the scattering cross sections are in agreement. This has been checked through the value of the scale factor k_s that has been ranged within 10% in all fits.

Inspection of the table reveals that micellar aggregates have quite elongated shape (with an axes ratio ranged between 2 and 3), and dimensions of semiaxis ranged between ~10 and ~30 Å. However, depending on the system, the characteristics of the micelles are quite different. In fact, systems that differ only for the presence of the Gd^{3+} ion, like PDA-DTPAGlu and PDA-DTPAGlu (Gd), show a quite different aggregation number; in particular, the presence of the ions promote the formation of larger micelles. This can be ascribed to the smaller charge present on the PDA-DTPAGlu(Gd) heads if com-pared with the charge present on the PDA-DTPAGlu molecules. A smaller charge results in a smaller charge density and in a weaker intramicellar repulsions existing among the heads. As a final result, systems containing Gd^{3+} have a tendency to form larger aggregates.

On the other hand, comparing systems that differ for the presence of PDA-L2-CCK8, it is possible to note that ternary systems show a smaller aggregation number. This aspect can be explained by assuming that PDA-L2-CCK8 tends to repress the aggregation process, probably destabilizing the packing. Finally, inspection of the systems undergone to the UV process does not seem to show any marked difference with analogous systems where the UV process has not been performed. This behavior could suggest the hypothesis that the polymerization process, performed on water solutions containing the unimers at concentration higher than *cmc*, stabilizes already formed micellar structures.

Conclusions

Aggregates obtained by mixing together PDA-DTPAGlu, or its Gd(III) complex, PDA-DTPAGlu(Gd), and PDA-L2-CCK8 have been prepared and structurally characterized before and after polymerization. SANS measurements showed that in both cases, elongated micelles having similar structural parameters were formed. Relaxivity values obtained for polymerized systems agree with values reported for classical micellar systems [5]. Polymerization process should allow, through the formation of covalent bonds, forming micellar aggregates that should not break even after injection in the human body. This aspect makes suitable our systems as in vivo target selective contrast agents in MRI for imaging of cells and districts over-expressing the cholecystokinin receptors.

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