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A new perylene bisimide bola amphiphile: synthesis, characterization, fluorescent properties and applications as a potential probe†

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Fluorescently-tagged lipids are a powerful tool for investigating the dynamics of lipids in cell biological studies and in biophysical applications. Herein we report the synthesis and the characterization of a new bola amphiphile, **PC12**, based on a bisimidic perylene moiety: a central perylene unit is symmetrically linked to two aliphatic chains both ending with a quaternary ammonium group. Absorption and fluorescent properties of the newly synthesized compound were investigated in DMSO and in water as a function of concentration and temperature. Further, the entrapment efficiency, the fluorescence behavior in dimyristoyl-*sn*-glycero-phosphocholine (DMPC) liposomes, and cell uptake on human and murine glioblastoma cell lines were evaluated. When loaded in liposomes, **PC12** follows the same destiny of liposomes themselves in the cell cultures: this is an interesting result because **PC12** could be used both as an aspecific dye (free form) and as an organelle-specific lipid probe.

Introduction

Fluorescently-tagged lipids are a powerful tool for investigating the dynamics of lipids in cell biological studies and in biophysical applications.[1] Several fluorescent lipids have been proposed and exploited since the early 1980s, some of which resemble their natural counterpart (*e.g.* fluorescently labeled phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylethanolamine, and fluorescently labeled fatty acid derivatives),[2] whereas others simply feature an amphiphilic molecular structure with no resemblance of natural lipids (*e.g.* 3,3′-diacylindocarbocyanine iodides[3] laurdan, 6-dodecanoyldimethylaminonaphthalene,[4] umbelliferone, 4-heptadecyl-7-hydroxycoumarin[5]). Depending on the lipid aspect to be investigated, the fluorescent dye can be covalently coupled either to the hydrophilic or to the hydrophobic region of the amphiphilic molecules. Perylene bisimides are known as optimal fluorescent dyes because they feature excellent chemical, thermal and photochemical stability and high fluorescence quantum yields.[6,7] In fact, they have been applied in different fields such as laser dyes,[8] photovoltaic cells,[9] and sensors.[10] Moreover, the extended aromatic system of the perylene dye has been exploited in the formation of supramolecular systems with photophysical properties peculiar with respect to single molecules.[11] However, the study of supramolecular aggregates of perylene bisimide derivatives has been restricted for many years to organic solvents; only recently, some water soluble perylene bisimide amphiphiles have been developed, belonging both to the class of conventional surfactants (where the hydrophobic perylene moiety is located at one side of the molecule) and to that of bola amphiphiles (where the perylene unit is the core of the molecule, linked at both sides to hydrophilic residues). A recent review of Würthner and coworkers provides an overview on this topic.[12] Amphiphiles with a perylene unit may be useful for many applications in an aqueous environment such as (i) dispersion of poorly soluble functional carbon materials (SWCNTs and graphenes), (ii) study of biomolecules (DNA and RNA) thanks to the fluorescence and electron-acceptor properties of perylene dye;[12b] (iii) development of anticancer drugs because of the ability of the perylene unit to stabilize the G-quadruplex inhibiting telomerase activity;[12c–g] (iv) development of fluorescent lipid probes for cell

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biology and biophysical studies. A very recent paper reports[13] the design and obtainment of fluorescent amphiphilic probes where the bisimidic perylene residue and dendritic polyethylene glycol (PEG) constitute the hydrophobic and hydrophilic regions, respectively, arranged in two different main architectures, the head–tail and the core–shell dyes. The first one is the architecture of a "conventional amphiphile" with a hydrophilic head group (the dendritic PEG) and a hydrophobic region (the perylene moiety), whereas in the core–shell architecture the bisimidic perylene unit is linked in opposite directions to symmetric dendritic PEGs, thus resulting in a *bola* type (or bipolar) amphiphile. The architecture of the fluorescent amphiphiles has been proved to influence the cellular uptake of the probes, in particular, the bola amphiphile was internalized more efficiently by cells.

Herein we report the synthesis of bola amphiphile **PC12 (1)** based on a bisimidic perylene moiety. A central perylene unit is symmetrically linked to two dodecyl chains both ending with a quaternary ammonium group. The absorption and emission features of the fluorescent bola lipid **PC12** were investigated in DMSO and in water as a function of concentration and temperature. Further, the entrapment efficiency in liposomes and cell uptake on glioblastoma cell lines were evaluated.

Results and discussion

Design rationale and synthesis of bola lipid PC12

Our objective was to synthesize a fluorescent amphiphilic probe characterized by two hydrophobic tails each linked on one side with a polar hydrophilic head and on the other with the fluorescent hydrophobic core.

Perylene anhydride is an excellent candidate for creating the desired fluorescent probe, since the insertion of side chains on the major axis of perylene is a well studied procedure.[14] Moreover perylene bisimide dyes are known for their high molar absorption coefficients of 30 000–90 000 cm^{-1} M^{-1},[15] fluorescence quantum yields in some cases close to unity[16] and excellent photostability.[17] The first challenge of this approach is represented by the low solubility of perylene in common organic solvents. Based on our previous experience,[18] we bypassed this main problem using perylene derivatives modified on the bay-area by bromination (compound **2**). In order to obtain a compound with the desired properties described above, we chose to insert as side chains on the dibromoperylene core a diamine with a long aliphatic chain: 1,12-diaminododecane. This compound has the right length to fit inside phospholipid bilayers and presents two amino groups which are suitable for the reaction with the anhydride function, leaving a polar group at the opposite end of the chain.

On the other hand, the simultaneous presence of two equivalent groups on the same molecule represents the second main challenge of the proposed synthetic route.

Thus our synthetic strategy (Scheme 1) begins with the protection with boc-anhydride of one of the two amino groups of the aliphatic chain to be inserted.

The addition of classical amino protecting group BOC led to an asymmetrical compound where the two amino groups present a different reactivity. The reaction was conducted using a BOC reactant at a 0.3 : 1 ratio with respect to the amine,[19] in dichloromethane at room temperature for 3 days, to obtain the desired compound **3**. The mono-protected product **3** was efficiently purified by column chromatography. Even though due to the use of a small amount of boc-anhydride a big percentage of the unreacted amine remained in the reaction mixture, it was possible to recover by the same column chromatography the purified starting compound, which could be recycled.

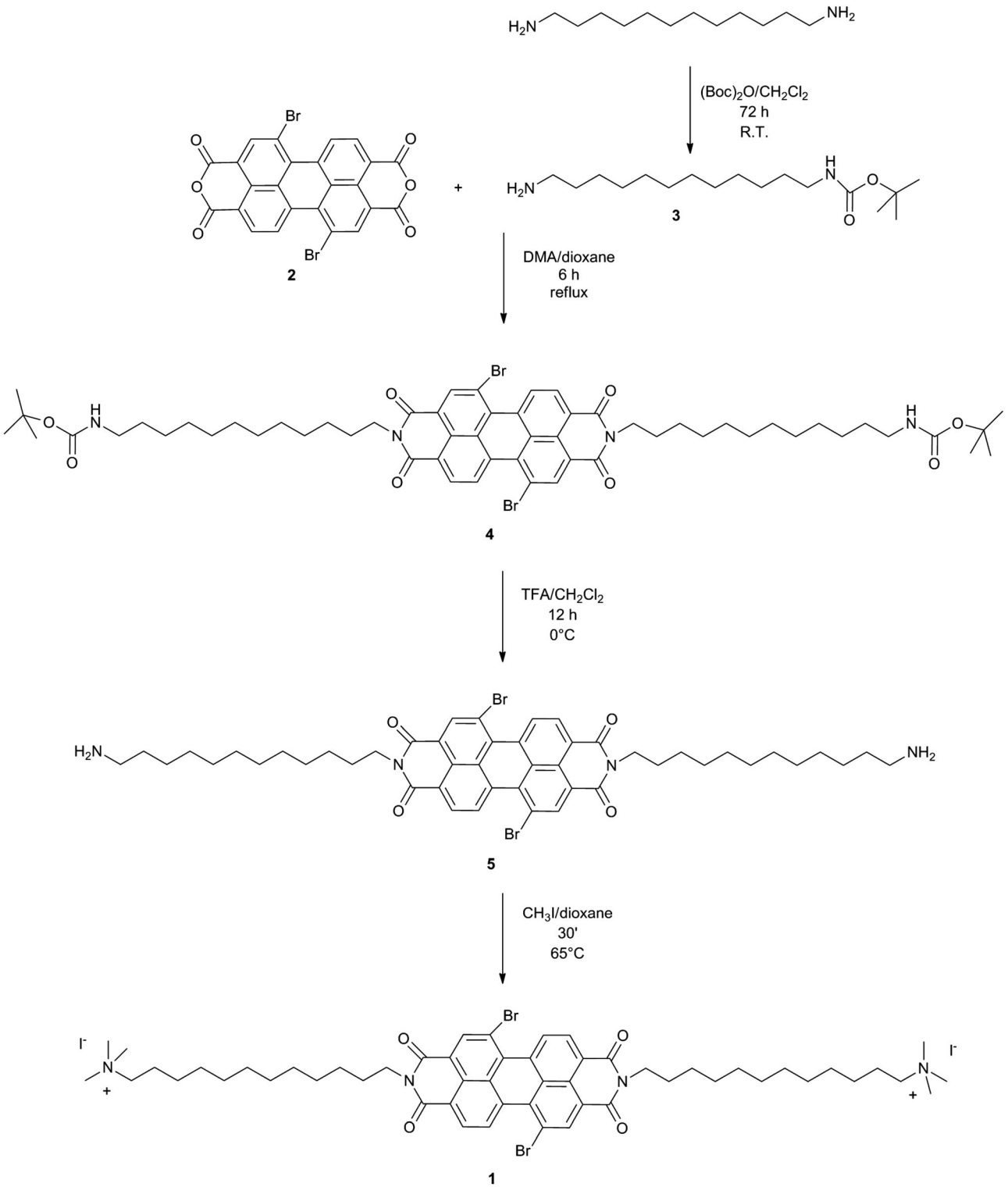
Once the asymmetrical compound **3** was synthesized, perylene diimide **4** was easily obtained by treatment of the brominated perylene anhydride **2** with an almost stoichiometric amount of **3**, in DMA/dioxane under argon. No more than 10% excess of reactant was used to avoid side reactions at the brominated positions, which can occur at higher ratios. It is worth noting that, in principle, substitution of bromine atoms by the primary amine can actually occur even at a stoichiometric ratio. In our experience, this can be avoided by strictly controlling the temperature and the time of reaction, TLC was used to follow the appearance of any blue-green spot, due to the formation of a new chromophore where a nitrogen atom from the primary amine is conjugated to the aromatic system.[17] If such a spot appears, the reaction must be stopped and the products can be purified by column chromatography.

The deprotection of pure compound **4** was conducted in dichloromethane adding TFA dropwise to obtain pure compound **5**. Finally, to obtain a *bola* type molecule, the polar hydrophilic heads were subjected to exhaustive methylation in order to obtain positively charged head groups. In particular, **PC12** was obtained in quantitative yield by adding CH_3I to perylene bisimide previously dissolved in dioxane.

Photophysical characterization of PC12

UV-visible absorption spectra. The absorption spectrum of 1×10^{-5} M **PC12** in DMSO is reported in Fig. 1 (long dash). The spectrum shows two main maxima in the UV/visible range, one of high intensity at 528 nm and the other at 494 nm. Moreover, a shoulder at higher energy (458 nm) can be detected. The observed pattern[20] is due to the vibronic fine structure of the S0–S1 transition of the perylene moiety; in particular, the lower energy band, corresponding to the 0–0 transition, is more intense with respect to the higher energy band, corresponding to the 0–1 transition, and the shoulder can be assigned to the 0–2 transition. As expected, the vibronic peaks are not well resolved as in other perylene derivatives without substituents in the "bay" positions. Qualitatively, this broadening originates from the loss of planarity of the perylene ring due to the presence of a bromine atom in the bay area.[21] However, little changes in maxima absorption wavelengths are observed with respect to pure perylene, as expected in the presence of electron-withdrawing substituents in the "bay" positions. Moreover, the maxima absorption wavelengths of the bands do not shift with concentration and the ratio of vibronic bands is constant with concentration (see Fig. S1, ESI†). Thus, in DMSO, the overall features of the spectra suggest the absence of aggregation involving a π–π interaction between the perylene rings.

In aqueous solution otherwise, **PC12** shows the typical absorption spectrum of aggregated perylene dyes (Fig. 1, solid line); in fact, the absorption intensities of all bands decrease,



Scheme 1 Preparation of bola amphiphile **PC12 (1)**.

and the 0–1 transition becomes more intense than the 0–0 one. The intensity reversal observed for the 0–0 and 0–1 bands indicates that in water the Franck–Condon factors favour the higher (0–1) excited state,[22] suggesting the formation of H-type π–π stacking aggregates. By heating the sample (1×10^{-5} M **PC12**) up to 55 °C the relative intensity of the perylene absorption peaks does not change (see Fig. 2b, ESI†).

Fluorescence spectra. The fluorescence emission spectrum of 6×10^{-6} M **PC12** in DMSO is reported in Fig. 2a. It shows a broad band with a maximum at 555 nm and a shoulder at

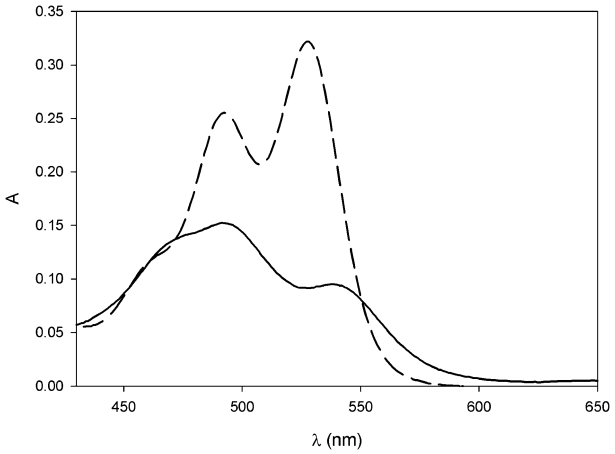


Fig. 1 Absorption spectra of 1×10^{-5} M **PC12** in DMSO (long dash) and in water (solid line).

595 nm, that is the mirror image of the absorption spectrum, as previously observed for other perylene derivatives.[23] Emission quantum yield for **PC12** was evaluated in air equilibrated DMSO medium with respect to $Ru(bpy)_3Cl_2$ (Φ = 0.028 in H_2O).[24] Moreover, in order to have as a touchstone for quantum yield a molecule belonging to the same class of **PC12**, we used also another standard, not commercially available but described in the literature, perylene-3,4,10-tetracarboxylic acid tetrapotassium salt. The fluorescence of this standard is independent of oxygen and displays a very high quantum yield (Φ = 1.00 in 0.1 M K_2CO_3, with respect to fluorescein).[25] Relative emission quantum yield was found to be 0.072 with respect to $Ru(bpy)_3Cl_2$ and 0.039 with respect to perylene-3,4,10-tetracarboxylic acid. In the field of perylene derivatives, the quantum yield of **PC12** is quite low. The reason for this low quantum yield can be ascribed mainly to the nature of the substituent at the imide group.[14a] The alkyl chains on the nitrogen are not fixed in an orthogonal conformation and are characterized by a high conformational freedom, the vibronic motions ('loose bolt effect')[26] are thus responsible for a drop in the quantum yield of perylene. Another cause could be the nature of the counterion, *i.e.* iodide, which is a well known fluorescence quencher. The bromine atoms in the bay area, otherwise, cannot be indicated as the main cause of the low quantum yield; in fact, for different perylene bisimide derivatives, with the same substituents on the nitrogen atoms, the presence of one[27] or two bromine atoms[28] in the bay area decreases the quantum yield by only 7% and 24%, respectively.

The fluorescence emission spectrum of 6×10^{-6} M **PC12** in water as a function of temperature (25–60 °C) is reported in Fig. 2b. The emission spectrum shows, as in DMSO, a broad band with a maximum at 562 nm and 595 nm. However, the aggregation behaviour of **PC12** in water is responsible for the decrease in the fluorescence quantum yield with respect to DMSO. In fact, with increasing temperature, the intensity of the fluorescence emission increases, suggesting a temperature driven disaggregation process. Note that the fluorescence emission of **PC12** as a function of temperature in DMSO decreases with increasing temperature, as expected for a fluorophore in the absence of aggregation (Fig. 2a).

Entrapment efficiency and fluorescence spectra of PC12 in DMPC liposomes

As a general rule, a solute unentrapped in liposomes remains in solution after the extrusion and can be removed from the liposome dispersion by filtration on an exclusion chromatography column. In some cases the unentrapped solute can self-assemble in solution to give large aggregates; in this case, aggregates will be removed in the extrusion procedure by the polycarbonate membrane. Therefore, the amount of unentrapped **PC12** excluded by extrusion and/or gel filtration is mainly dependent on its aggregation state and water solubility. The amount in molar percentages of **PC12** in DMPC liposome dispersions at each stage of the preparation has been calculated: 20% of **PC12** is lost upon extrusion while the amount of **PC12** remains constant upon gel filtration, suggesting that all unentrapped **PC12** is filtered off by the polycarbonate membrane and it is probably present in the buffer assembled in large aggregates.

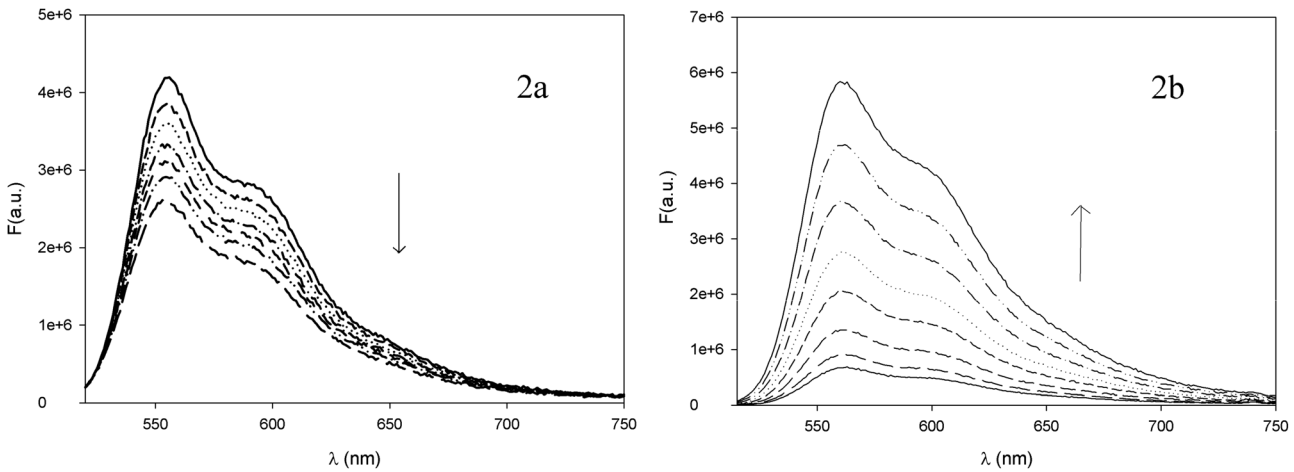


Fig. 2 Fluorescence emission spectra (λ_{exc} = 505 nm) at increasing temperature (25–60 °C) of 6×10^{-6} M **PC12** (a) in DMSO; (b) in an aqueous solution.

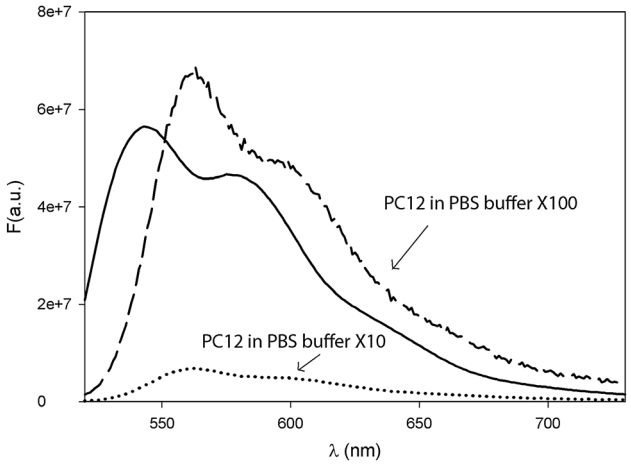


Fig. 3 Fluorescence emission spectra (λ_{exc} = 505 nm) of 6 × 10^{-6} M **PC12** (a) entrapped in DMPC liposomes (solid line) and in PBS buffer, magnified (b) 10 times (dotted line) and (c) 100 times (dashed line).

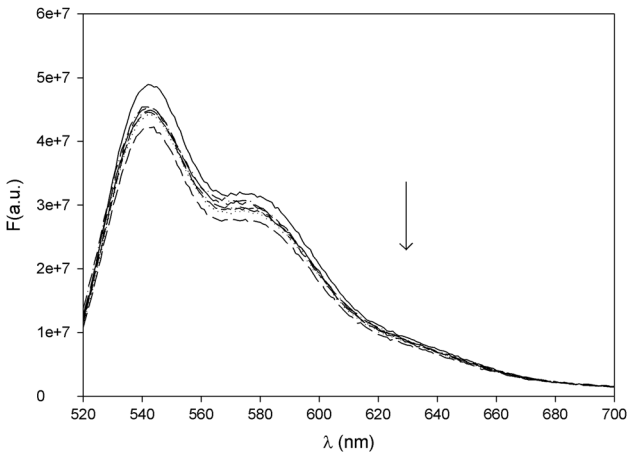


Fig. 4 Fluorescence emission spectra (λ_{exc} = 505 nm) of 6 × 10^{-6} M **PC12** entrapped in DMPC liposomes at increasing temperature.

The fluorescence emission spectrum of **PC12** entrapped in liposomes after gel filtration is shown in Fig. 3 (solid line). The fluorescence intensity is very high with respect to **PC12** at the same concentration (6 × 10^{-6} M) in the buffer (Fig. 3, dotted and dashed lines), suggesting that the perylene dye molecules are included in the lipid bilayer in the monomeric form. Moreover, the whole fluorescence spectrum is blue shifted, with the maximum at 543 nm (Fig. 3, solid line). Because the maximum is 562 nm in water and 555 nm in DMSO, respectively, this blue shift suggests a location of the perylene moiety in a less polar environment with respect to water. The hypothesis of the inclusion of **PC12** in the lipid bilayer in the monomeric form is strengthened by the fact that heating the **PC12** liposome solution up to 60 °C (Fig. 4) does not induce an increase in the intensity of fluorescence emission, rather, a slight decrease of fluorescence emission is detected.

Intracellular distribution of PC12

Finally, the interactions of **PC12** with cellular structures were studied in order to evaluate the potential of the newly synthesized

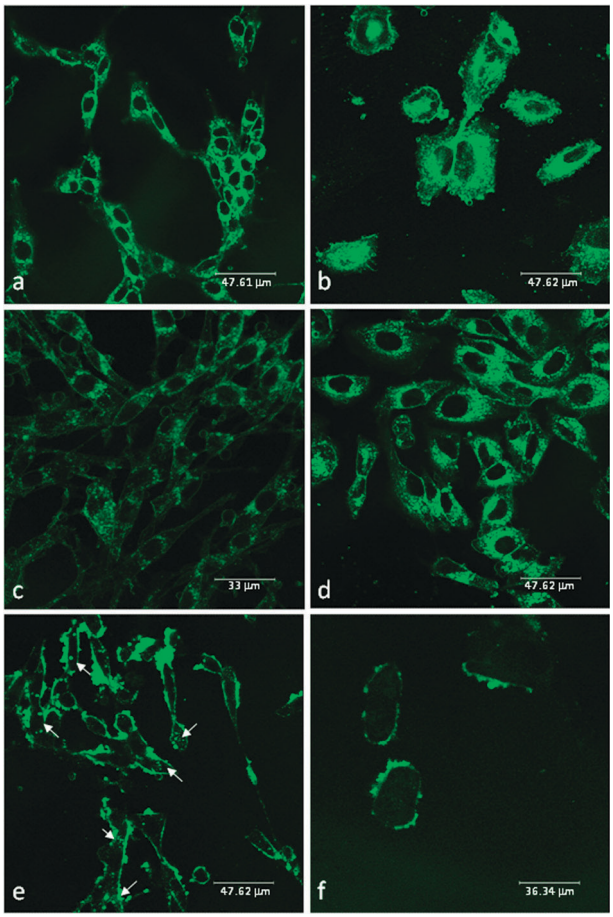


Fig. 5 Intracellular localization of **PC12** analyzed using laser scanning confocal microscopy. (a, c, and e) Rat C6 and (b, d, and f) human LN229 glioblastoma cells treated with **PC12** in DMSO for 5 (a and b) and 27 h (c and d). (e) C6 and (f) LN229 cells treated with **PC12** entrapped in liposomes for 27 h.

compound as a fluorescent lipid probe. In particular, intracellular localization of **PC12** administered either in DMSO or entrapped in liposomes was analyzed using laser scanning confocal microscopy (LSCM) in both human (LN229) and murine (C6) glioblastoma cells. As visualized in Fig. 5, after 5 (Fig. 5a and b) and 27 h (Fig. 5c and d) of treatment with a DMSO solution of the probe, **PC12** stained preferentially cellular membranes of both C6 and LN229 cells. Plasma membranes and intracytoplasmic organelles appeared strongly fluorescent, while no significant signal was revealed inside the nuclei. The cellular distribution of **PC12** completely changed when it was entrapped in DMPC liposomes. After 5 h of incubation it completely failed to stain C6 and LN229 cells, as demonstrated by the lack of the fluorescent signal in treated glioblastoma cultures (data not shown). In contrast, after 27 h of incubation, strongly fluorescent liposomes clusterized preferentially on the plasma membranes were revealed on both C6 and LN229 cells, and a low intracytoplasmic signal could be observed (Fig. 5e and f). Fluorescent clusters of liposomes that seem to be localized in the cytoplasm of some cells (Fig. 5e, arrows) are, actually, liposomes localized on the plasma membrane and visualized by the optical sectioning tangent to the cell surface. After the treatment with **PC12**

entrapped within liposomes, LN229 cells undergo morphological changes, most likely due to the interaction of vesicles with the membrane rather than to the entrapped probe.

Conclusion

A new dibromoperylene derivative, representing a fluorescently labelled lipid analogue, was synthesized. The synthetic procedure involved the bromination of the perylene bay-area to obtain a more soluble compound that could be handled more easily, and the insertion of a suitable alkyl chain to obtain molecules with the proper size and the amphiphilic character required for the interaction with phospholipid bilayers. The absorption and the fluorescence features of the new compound were investigated in DMSO, in water and upon its inclusion in DMPC liposomes. UV spectra of the perylene derivative showed the classical opposite behaviour in organic solvent and in water due to its monomeric form in DMSO and to the presence of aggregates in water. The emission spectra as a function of temperature (25–60 °C) confirmed this picture. Finally, the data collected for **PC12** in liposomes suggest that **PC12** is embedded in the liposome bilayer where the dye molecules are most probably in the monomeric form.

In the treatment of both C6 and LN229 cells with **PC12** (in the free form), no apparent signs of cell suffering or death were observed, suggesting that this new amphiphilic probe is not toxic and could be used as "vital stain" in all biological experiments where cells are not fixed. The different subcellular localization of free and liposome-entrapped **PC12** could provide the basis for its different uses as a fluorescent lipid probe. In fact, free **PC12** was detectable both in the plasma membrane and intracytoplasmic organelles, whereas **PC12** loaded in liposomes accumulates only on the plasma membrane of both C6 and LN229 cells. This difference suggests that **PC12** when loaded in liposomes follows the same destiny of liposomes themselves in the cell cultures. Therefore, in the free form it could represent an aspecific stain for lipids of cell membranes (plasma and organelle membranes), moreover, since no significant signal was revealed inside the nuclei, it can be particularly useful in multiple fluorescence experiments employing other dyes for nucleus stains. In the liposome-entrapped form, since it follows the same destiny of liposome itself, it could be used to label specific cell subcompartments using liposomes specifically formulated for targeting defined compartments.

Experimental section

Materials and methods

All commercial reagents, exclusion gel Sephadex G-50, phosphate buffer PBS tablets, RPE and HPLC grade solvents were purchased from Carlo Erba Reagenti, Fluka and Sigma-Aldrich and used without further purification. Dimyristoyl-*sn*-glycerophosphocholine (DMPC) was purchased from Avanti Polar Lipids (Alabaster, AL). TLCs were run on Merck silica gel 60 F254 plates. Silica gel chromatography was performed by using Merck silica gel 60 (0.063–0.200 mm). Perylene-3,4,10-tetracarboxylic acid tetrapotassium salt was prepared and purified as previously reported.[25]

^{1}H and ^{13}C NMR spectra were recorded using Bruker 300 and Varian Mercury 300 instruments. *J* values are given in Hz.

ESI-MS spectra were recorded on a Micromass Q-TOF MICRO spectrometer.

Spectrophotometric experiments were carried out on a Varian Cary 300 Bio using a cell of 1 cm path length.

Steady-state fluorescence emission spectra were carried out on a HORIBA Jobin-Yvon Fluoromax 4 spectrofluorimeter, all the proper corrections for spectral sensitivity in the near infrared region were applied in order to obtain true emission spectra.

The fluorescence quantum yield (Φ) was estimated using eqn (1) in DMSO by using as reference the integrated emission intensity of $Ru(bpy)_3Cl_2$ (Φ = 0.028 in H_2O at RT) and of perylene-3,4,10-tetracarboxylic acid tetrapotassium salt (Φ = 1.00 in 0.1 M K_2CO_3, with respect to fluorescein):

$$\Phi_f = \frac{I_{\text{sample}} A_{\text{std}} \eta_{\text{sample}}^2}{I_{\text{std}} A_{\text{sample}} \eta_{\text{std}}^2} \Phi'_f$$

where Φ'_f is the absolute quantum yield for the standard; I_{sample} and I_{std} are the integrated emission intensities of the sample and of the standard, respectively; A_{sample} and A_{std} are the absorbances of the sample and of the standard that are equal at the used excitation wavelength (484 nm for $Ru(bpy)_3Cl_2$ and 438 nm for perylene-3,4,10-tetracarboxylic acid tetrapotassium salt), and η_{sample} and η_{std} are the respective refractive indices of the solvents (1.479 for DMSO, 1.33 for 0.1 M K_2CO_3).

Laser Scanning Confocal Microscopy observations were performed by using a Leica TCS SP2 laser scanning confocal microscopy (Leica Microsystems, Mannheim, Germany).

Synthesis

Starting compound **2** (1,7-dibromoperylene-3,4:9,10-tetracarboxylic dianhydride) was prepared by reaction of 3,4,9,10-perylentetracarboxylic dianhydride with commercially available bromine, as previously described.[14b]

Together with compound **2**, we obtained also the corresponding 1,6-derivative (about 10%). This minor isomer is not separable from the 1,7-isomer at this stage; in the synthetic schemes only the main 1,7-isomer is reported.

12-(Amino-dodecyl)-carbamic acid *tert*-butyl ester (3). To a solution of 505 mg (2.52 mmol) of 1,12-diaminododecane in 5 ml of CH_2Cl_2 and 5 ml of chloroform at 0 °C 168 mg (0.77 mmol) of boc-anhydride in dichloromethane was added dropwise. The reaction was stirred at room temperature for a week. The residue was taken up with ethyl acetate, washed with brine (10 ml, 3×), dried over Na_2SO_4 and evaporated to obtain an oil. The residual oil was subjected to column chromatography using $CHCl_3$/MeOH (90/10) to obtain 153 mg (66%) of 12-(amino-dodecyl)-carbamic acid *tert*-butyl ester as a colourless oil. δ_H (300 MHz; $CDCl_3$) 4.53 (1H, br s, N*H*-BOC), 3.08 (2H, m, N_{BOC}-CH_2), 2.67 (2H, t, *J* 7, $NH_{2AMMINIC}$-CH_2), 1.43 (13H, br, CH_2- β $N_{AMMINIC}$, CH_2- β NH_{BOC}, (CH_3)$_3$-C), 1.31–1.18 (16H, br, -CH_2- alkyl chain); δ_C (300 MHz, $CDCl_3$) 142.5 (C═O), δ 42.5, 48.9, 34.0, 30.3, 29.8, 29.7, 29.5, 28.6, 27.1 (aliphatic C).

***N,N′*-Bis[(amino-dodecyl)-12-carbamic acid *tert*-butyl ester]-1,7-dibromoperylene-3,4:9,10-tetracarboxylic diimide (4).** To 257.6 mg of compound **2** (0.47 mmol) and 309 mg of compound **3** (1.03 mmol) anhydrous dioxane (2.5 ml) and anhydrous DMA (2.5 ml) were added. The reaction mixture was then refluxed, stirred under argon for 6 h. Successively, ice and brine were added and the solution was stored at 4 °C for a night. Then the solution was filtered on Hirsh imbute, washed with water. After drying the precipitate in an oven, 450 mg of compound **4** was obtained (85% yield). δ_H (300 MHz; CF_3COOD) 9.81 (2H, d, *J* 8, ar.), 9.16 (2H, s, ar.), 8.94 (2H, d, *J* 8, ar.), 4.44 (4H, t, *J* 8, N_{imidic}-CH_2), 3.35 (4H, t, J 8, NH_{BOC}-CH_2), 1.93 (4H, br, NH_{BOC}-CH_2-CH_2), 1.72 (18H, br, (CH_3)$_3$-C), 1.60–1.37 (40H, br m, aliphatic C); δ_C (300 MHz, CF_3COOD): 165.7 (C═O), 165.7 (C═O), 139.6 (ar.), 134.5 (ar.), 134.3 (ar.), 131.5 (ar.), 129.3 (ar.), 129.1 (ar.), 127.0 (ar.), 122.3 (ar.), 121.8 (ar.), 121.5 (ar.), 100.3 ((CH$_3$)$_3$-*C*), 42.1, 41.7, 29.3, 29.2, 29.0, 28.6, 27.7, 27.1, 26.8, 25.8 (aliphatic C). MS (ESI) *m*/*z*: 1137 $(M + Na)^+$.

***N,N′*-Bis(12-aminododecane) 1,7-dibromoperylene-3,4:9,10-tetracarboxylic diimide (5).** 372.1 mg of compound **4** was dissolved in dichloromethane (12 ml) and TFA (0.5 ml) was added dropwise. The reaction mixture was stirred for 12 hours, at 0 °C. Then, the reaction mixture was poured into ice and neutralized carefully with NH_3 (solution at 10%) until pH 8. The residue was extracted with dichloromethane or chloroform, washed with brine (5 ml, 3×), dried over Na_2SO_4 and evaporated to obtain 171.8 mg of the desired product **5** (57.0% yield). δ_H (300 MHz; CF_3COOD): 9.84 (2H, d, *J* 8, ar.), 9.18 (2H, s, ar.), 8.96 (2H, d, *J* 8, ar.), 4.47 (4H, t, br, N_{imidic}-CH_2), 3.37 (4H, br, $NH_{2AMMINIC}$-CH_2), 1.95 (8H, br, $NH_{2AMMINIC}$-CH-CH_2 and N_{imidic}-CH-CH_2), 1.60–1.30 (32H, br, aliphatic C); δ_C (300 MHz, CF_3COOD): 165.3 (C═O), 139.6 (ar.), 137.4 (ar.), 134.6 (ar.), 134.4 (ar.), 131.6 (ar.), 129.3 (ar.), 129.1 (ar.), 127.1 (ar.), 122.4 (ar.), 121.9 (ar.), 47.9, 29.3, 29.2, 29.1, 28.7, 27.7, 27.2, 26.8, 25.9 (aliphatic C). MS (ESI) *m*/*z*: 915 (M^+); 937 $(M + Na)^+$.

***N,N′*-Bis(12-trimethylammonium-dodecane) 1,7-dibromoperylene-3,4:9,10-tetracarboxylic diimide iodide (1).** To 84.7 mg of compound **5** (0.09 mmol) 3 ml of anhydrous 1,4-dioxane was added. The reaction mixture was stirred at 120 °C to favour the complete dissolution of compound **5**. Then the reaction mixture was cooled down, CH_3I (1 ml) was added and the reaction was heated to 65 °C for 30 minutes. The solid was separated by filtration to obtain 113.9 mg of the desired compound (yield 98%). δ_H (300 MHz; DMSO-d_6): 9.35 (2H, d, *J* 8, ar.), 8.60 (2H, s, ar.), 8.56 (2H, d, *J* 8, ar.), 4.03 (4H, t, *J* 7, N_{imidic}-CH_2), 3.27 (4H, br, $NH_{2AMMINIC}$-CH_2), 3.03 (18H, s, $NH_{2AMMINIC}$-CH_3), 1.66 (8H, br, $NH_{2AMMINIC}$-CH-CH_2 and N_{imidic}-CH-CH_2), 1.40–1.20 (32H, br, aliphatic C); δ_C (300 MHz, DMSO-d_6): 163.0 (C═O), 162.4 (C═O), 137.3 (ar.), 132.7 (ar.), 132.4 (ar.), 130.3 (ar.), 129.2 (ar.), 129.0 (ar.), 127.0 (ar.), 123.6 (ar.), 123.3 (ar.), 120.9 (ar.), 66.1, 52.9, 39.5, 22.7–22.6 (aliphatic C). MS (ESI) *m*/*z*: 500 (M^{2+}).

Liposome preparation

The aqueous dispersion of DMPC/**PC12** liposomes was prepared according to the procedure described by Hope *et al.*[29] Briefly, a film of lipid was prepared on the inside wall of a round-bottom flask by evaporation of $CHCl_3$ solution containing the proper amount of DMPC and **PC12** to obtain the desired percentage mixture (DMPC/**PC12** 500/1). To the obtained film stored in a desiccator overnight under reduced pressure 10 ml of PBS buffer solution was added (Aldrich, 10^{-2} M pH 7.4) in order to obtain a final concentration of 2.5×10^{-2} M in DMPC and 5×10^{-5} M in **PC12**. The dispersion was vortex-mixed and then freeze-thawed six times from liquid nitrogen to 313 K. Dispersion was then extruded (10 times) through a 100 nm polycarbonate membrane (Whatman Nuclepore). The extrusion procedure was carried out at 307 K, well above the transition temperature of DMPC (297.2 K), using a 10 ml extruder (Lipex Biomembranes, Vancouver, Canada).

Determination of entrapment efficiency

The unentrapped **PC12** in extruded DMPC liposomes was separated from the liposomes on a Sephadex G-50® gel column, equilibrated in PBS buffer solution. **PC12** concentration in liposome preparations before and after extrusion and before and after Sephadex filtration was determined by measuring the absorbance maximum (at 521 nm) of 3 ml of **PC12**/liposomes before extrusion, after extrusion, and after filtration; liposomes in the samples were disrupted by complete evaporation of water and dissolution of the residue in absolute ethanol. The percentage of entrapped drug was calculated using the equation:[30]

$$\%\mathbf{PC12} = 100 \times (M^{a}_{\mathbf{PC12}}\, M^{b}_{lip})/(M^{b}_{\mathbf{PC12}}\, M^{a}_{lip})$$

where $M^{a}_{\mathbf{PC12}}$ and $M^{b}_{\mathbf{PC12}}$ are the perylene derivative concentration in liposome dispersion, respectively, before and after extrusion and before and after gel filtration and M^{a}_{lip} and M^{b}_{lip} are the total lipid concentration before and after extrusion and before and after gel filtration, respectively.

Cell culture

Human (LN229) and murine glioblastoma (C6) lines (kindly provided by Dr S. Ciafre', University of Rome ''Tor Vergata'', Italy) were grown as a monolayer in DMEM medium supplemented with 1% non-essential amino acids, 1% L-glutamine, 100 IU ml^{-1} penicillin, 100 IU ml^{-1} streptomycin, and 10% fetal bovine serum at 37 °C in a 5% CO_2 humidified atmosphere in air.

Laser scanning confocal microscopy

Glioblastoma cells were analyzed using laser scanning confocal microscopy (LSCM) in order to investigate the intracellular distribution of **1** administered to cells in DMSO or delivered by liposomes. Cells, grown on 12 mm glass coverslips, were inoculated with **1** in DMSO or in liposomes, the final concentration of the dye was, in both cases, 8 μM. After 5 and 27 h of incubation at 37 °C, cells were fixed in 3.7% paraformaldehyde in PBS, for 10 min at room temperature.

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