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PAPER

Polymeric micelles using pseudo-amphiphilic block copolymers and their cellular uptake†

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A PS-*b*-PMA block copolymer bearing a terminal carboxylic group has been synthesized using 3-(benzylsulfanyl thiocarbonylsulfanyl) propionic acid as RAFT agent. When dispersed in methanol, this block copolymer displays pseudo-amphiphilic behaviour providing micelles which can be suspended in water by osmosis. The carboxylic terminations are fundamental during the self-assembling process contributing to the formation of nanosized monodispersed particles. Furthermore, the anionic corona confers the to micelles stability in aqueous media. The core of the micelles was loaded with Nile Red to achieve the intracellular delivery of a lipophilic substance; this was demonstrated by Confocal Laser Scanning Microscopy. The particles, when loaded with doxorubicin, are able to overcome the intrinsic resistance of LoVo-MDR cancer cells. Further stabilization is given to the micelles by covalent shell crosslinking using triethylene glycol diacrylate. Because of the increased intracellular stability of the crosslinked micelles, they stain, when loaded with Nile Red, the aqueous cytosol instead of the lipophilic compartments. The unimers constituting the micelles were covalently labeled with fluorescein to follow their fate once incorporated into the cells.

1 Introduction

Nanotechnology sciences have recently benefited from "controlled/living radical polymerization",[1] an extremely powerful tool that has allowed new unexploited results to be reached in polymer chemistry. Such techniques made it possible to combine the versatility of radical polymerization, which is suitable for a vast variety of differently functionalized monomers, together with a precise control of the molecular weight and its distribution.[2–5] In particular, Atom Transfer Radical Polymerization (ATRP) and Radical Addition-Fragmentation chain Transfer (RAFT) have emerged as the choice procedures for the design and convenient synthesis of as-yet unexplored macromolecular architectures. These procedures allow for numerous applications in the field of nanostructured functional materials such as star polymers, bottle brush copolymers and amphiphilic block copolymers which are capable of self-assembling mainly into micelles or vesicles.[6,7] ATRP and RAFT are also successfully exploited in the synthesis of end-functional polymeric chains[8] and heterotelechelic polymers.[9,10] An increasing number of scientists have focused their attention on the RAFT technique because it can be simply applied to a conventional radical polymerization. This provides a very robust metal-free system capable of producing well defined polymeric architectures in a controlled way.

One of the most promising applications of the RAFT technique is its use in the preparation of functional polymeric micelles[11] that can be employed for the delivery of therapeutic agents. This makes it possible to administer lipophilic drugs while protecting unstable molecules from metabolic deactivation.[12,13] Such characteristics, together with a reduced clearance, increase the efficacy of drugs that are usually toxic, thus reducing their effective dose.[14] Another key feature of polymeric micelles is their specific passive targeting to tumor tissues through the enhanced permeability and retention (EPR) effect.[15,16] Active targeting is obtainable through linkages with monoclonal antibodies,[17] aptamers[18] or small molecules like biotin[8] and folic acid.[19]

Once its role as RAFT mediator has ended, the thiocarbonylthio function, carried by the polymer chains, is easily convertible through reduction reactions or nucleophilic substitution (by amines) to a thiol group.[20,21] Since the thiol functionality is easily oxidizable by atmospheric oxygen causing the formation of the polymeric disulfane dimer, the nucleophilic displacement must be carried out in an inert atmosphere. A recent work reported the nucleophilic displacement of the RAFT

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function by hydrazine achieving a fast reaction and, at the same time, avoiding the oxidation of the thiol.[22] The obtained –SH is reactive towards a large number of functionalities and can be exploited in bioconjugation processes *via* disulfane bridge formation or for linking with functional molecules bearing moieties that can undergo mercaptan addition.[23,24]

Polymeric micelles are characterized by a low critical micelle concentration (CMC), which makes them stable under diluted conditions. Nevertheless, their high stability may not be sufficient for the *in vivo* physiological extreme dilution. The polymeric micelles can be further stabilized by crosslinking, a process that can involve either the core or the shell. Core crosslinking (CCL) is a hyperbranching process that, being confined to the core of the micelles, does not bring complications; on the other hand, it has the disadvantage of reducing the loading capacity of the micelles. Shell crosslinked (SCL) micelles have a higher loading capacity but the hyperbranching process is more delicate due to the possibility of inter-micellar crosslinking. To prevent such an inconvenience, triblock copolymers have been used;[25] here the third block is usually poly(ethylene glycol) that, being highly hydrated, prevents micelle aggregation and more importantly provides steric protection against inter-micellar crosslinking. SCL micelles composed of a diblock copolymer have also been made.[26] The second block consists of a statistical block copolymer where the reactive monomeric units, which interacts with the crosslinker, resides in the inner shell of the assembled micelle, preventing the inter-micellar crosslinking.

As described by Maysinger,[27] the internalization of micelle-incorporated fluorescent probes into cells has been successfully followed using Confocal Laser Scanning Microscopy (CLSM), a technique that is also suitable for studying the subcellular localization of single molecules.[28] Functionalized polymeric micelles obtained by other techniques have been already used for the delivery of doxorubicin into cells.[29,30]

This paper describes the formation of stable micelles starting from a carboxyl terminated PS-*b*-PMA block copolymer that shows pseudo-amphiphilic properties when dispersed in methanol. Once assembled the micelles can be suspended in water by osmosis. The particles were loaded with Nile Red or doxorubicin. Cell internalization of the carried fluorescent compounds was followed with CLSM. The micelles were stabilized by SCL using triethylene glycol diacrylate as crosslinker. The unimers constituting the micelles were also labeled with fluorescein to follow their fate once incorporated into the cells.

Experimental

Materials

3-Mercapto propionic acid, benzyl bromide, 2,2′-azobis(cyclohexanecarbonitrile), styrene, 2,2′-azobis(2-methylpropionitrile), methyl acrylate, Nile Red, doxorubicin, hydroxyethyl acrylate, 2,2′-azobis(2-methylpropionamidine) dihydrochloride, fluorescein diacetate 5-maleimide and morpholine were purchased from Aldrich. Cell lines: colon cancer cell lines (LoVo) were purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 (Sigma, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, N.Y., USA), 2 mM glutamine, 50 U/mL penicillin, 50 μg mL^{-1} streptomycin and grown at 37 °C in a humidified atmosphere with 5% CO_2. The LoVo cell line was derived from a metastatic human colon carcinoma and LoVo-MDR was the derivative MDR clone. THF was distilled from $LiAlH_4$, methanol was distilled after reaction with sodium metal. Dialysis tube (cutoff 12000–14000 Dalton) was purchased from Medicell International Ltd.

Instrumentation

^{1}H-NMR spectra were recorded at 400 MHz on a Varian Mercury 400. Polymer molecular weights and their distribution were determined using a GPC instrument (MSI Concept PU III and refractive index detector Shodex RI-71) equipped with 2 × PL ResiPore 300 × 7.5mm (200–400 000 Daltons), the columns were calibrated using polystyrene standards. DLS and Z potential measurements were taken with a Malvern Instruments - Zetasizer 3000 HAS. Atomic Force Microscopy measurements were taken with a Veeco - Multimode IIIA microscope. The confocal imaging was performed on a Laica TCS SP2 confocal microscope equipped with an argon/krypton ion laser. Scanning Electron Microscopy micrographs were taken using a Philips Model XL 30 scanning electron microscope.

Synthesis of 3-(benzylsulfanyl thiocarbonylsulfanyl) propionic acid[19,31,32]

3-Mercapto propionic acid (10 mmol, 0.87 mL) was added to a solution of sodium hydroxide (20 mmol, 0.8 g) in methanol (20 mL), then carbon disulfide (50 mmol, 3.08 mL) was added. The color of the solution immediately turned deep yellow/orange. After 30 min benzyl bromide (11 mmol, 1.31 mL) was added and the mixture was stirred for two hours, the solvent was then removed under vacuum in a rotating evaporator. The solid residue was suspended in cyclohexane and filtered off. The filtrate was dissolved in water and neutralized with 1 M HCl (≈10 mL) then extracted with dichloromethane, dehydrated with Na_2SO_4 and, after solvent removal, purified by flash chromatography using toluene/acetic acid (95/5) as eluent. A crystalline yellow solid (2.49 g, 91.4%) was obtained. ^{1}H-NMR ($CDCl_3$, δ): 7.3 (m, 5H; Ar H), 4.6 (s, 2 H; CH_2), 3.63 (t, $J = 6.8$ Hz, 2 H; CH_2), 2.85 (t, $J = 6.8$ Hz, 2 H; CH_2).

Synthesis of polystyrene macro-RAFT

A stock solution composed of 3-(benzylsulfanyl thiocarbonylsulfanyl) propionic acid (100 mg, 3.7×10^{-4} mol), 2,2′-azobis(cyclohexanecarbonitrile) (24.4 mg, 1×10^{-4} mol) and styrene to a volume of 10 mL (9.7 mL, 8.4×10^{-2} mol) was prepared in a volumetric flask then divided in two ampoules which were degassed with four freeze–thaw cycles. The ampoules were placed in a thermostatic bath set at 90 °C and left respectively for 8 (**a**) and 16 (**b**) hours. Unreacted styrene was removed under reduced pressure in a rotating evaporator; then the polymeric material was dissolved with toluene and evaporated, this procedure was repeated three times or until complete styrene depletion. Sample **a** yielded 2.5 g (55.5%) of polystyrene Mn = 6800, Mw/Mn = 1.20. Sample **b** yielded 4 g (86.9%) of polystyrene Mn = 11000, Mw/Mn = 1.08.

Synthesis of PS-*b*-PMA block copolymer

A solution composed by the polystyrene macro-RAFT **b** (1.2 g, 1.1×10^{-4} mol), AIBN (0.82 mg, 5×10^{-6} mol), methyl acrylate (7 mL, 7.8×10^{-2} mol) and benzene to a volume of 10 mL was placed in an ampoule and degassed with four freeze–thaw cycles, then placed in a thermostatic bath set at 70 °C for 5.5 h. Unreacted methyl acrylate was removed under reduced pressure in a rotating evaporator; then the polymeric material was dissolved with chloroform and evaporated, this procedure was repeated three times or until complete methyl acrylate depletion. The polymerization yielded 2.8 g (41.2%) of PS-*b*-PMA block copolymer $^{GPC}Mn = 35000$, $^{NMR}Mn = 34000$, $Mw/Mn = 1.09$. NMR molecular weight was determined by comparing the integrals of the areas between 3.2–4.0 ppm (PMA) and 6.2–7.6 ppm (PS) in a ^{1}H-NMR experiment.

Micelle formation

PS-*b*-PMA block copolymer (30 mg) was dissolved in 0.5 mL of THF/NEt_3 solution (dry THF 5 mL, NEt_3 1.2 μL), then dry methanol (25 mL) was quickly added with a syringe. A clear suspension formed. Methanol was replaced by water using a dialysis tube (cutoff 12000–14000 Dalton). Nile Red (0.2 mg) or neutralized doxorubicin (0.2 mg) were dissolved together with the block copolymer when preparing the loaded micelles.

Shell crosslinked micelles (SCL-M)

PS-*b*-PMA block copolymer (30 mg) was dissolved in 0.5 mL of THF/NEt_3 (dry THF 5 mL, $NEt_3$1.2 μL) solution, then dry methanol (25 mL) was quickly added with a syringe (0.2 mg of Nile Red was dissolved together with the block copolymer when preparing loaded SCL micelles). A clear suspension formed. Triethylene glycol diacrylate[33] (SCL-M1 1.04 μL, 4.5×10^{-6} mol; SCL-M2 10.4 μL, 4.5×10^{-5} mol; SCL-M3 104 μL, 4.5×10^{-4} mol), 2-hydroxyethyl acrylate (SCL-M1 0.5 μL, 4.5×10^{-6} mol; SCL-M2 5 μL, 4.5×10^{-5} mol; SCL-M3 50 μL, 4.5×10^{-4} mol) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (1×10^{-7} mol) were added to the methanolic suspension. The mixture was degassed by bubbling argon for 20 min, then the sealed vials were kept at 50 °C for 24 h in an oil bath. An aliquot of 4.5 mL was taken, methanol was removed and 1 mL of THF was added to the residue; this solution was used for GPC measurements. The methanolic suspension was put in a dialysis tube (cutoff 12000–14000 Dalton) and left in deionized water until complete replacement of methanol with water.

Labeling of the micelles with fluorescein

To the methanolic suspension of micelles, obtained as described above, fluorescein diacetate 5-maleimide (0.5 mg, 9.8×10^{-7} mol) and morpholine (0.24 μL, 2.7×10^{-6} mol) were added. The resulting suspension was degassed by bubbling argon for 20 min. The sealed ampoule was heated at 50 °C for 16 h. Then, methanol was replaced by water using a dialysis tube (cutoff 12000–14000 Dalton). The deionized water of the dialysis bath was renewed until the fluorescence release ceased (about one week). Fluorescence assays details are provided with the electronic supplementary information.†

Atomic force microscopy measurements

The methanolic suspension resulting from the crosslinking process was diluted four times with methanol, then spin-coated (3000 RPM) on Si (100) substrates covered by native oxide. Prior to deposition, the substrates were cleaned by SC1 treatment (NH_4OH 25% : H_2O_2 30% : H_2O 1 : 1 : 5 at 70 °C) and rinsed with water. AFM topographical images were recorded in tapping mode.

Scanning electron microscopy measurements

A methanolic suspension of micelles (SCL-M3) was deposited on a silica plate which, after solvent evaporation, was plasma gold-plated.

Apoptosis assay

Colon cancer cell (10.000/cmq) lines were grown on coverslips and after 24 h of treatment cells were fixed with 4% paraformaldehyde and, thereafter, permeabilized with Triton X-100 (0.1% in PBS). Cells were stained with 2.5 μg mL^{-1} DNA dye Hoechst 33258 in PBS for 30 min at 20 °C and analyzed by fluorescence microscopy. At 20-fold magnification cells with condensed or fragmented nuclei were counted on adjacent four fields of each coverslip for a total of 160–180 cells. The percentage of cell death was determined through two coverslips for each condition and compared with controls.

Cell viability assay

The effect of doxorubicin on cell viability was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, based on the reduction of the number of metabolically active cells, and the results were expressed as percentage of the controls. 3×10^{-3} cells/well were seeded into a 96-well plate and treated with several concentrations of doxorubicin, for 24 h and 72 h, after which 0.5 mg ml^{-1} of MTT (Sigma, USA) was added to each well and incubated for 4 h at 37 °C. Following the incubation, a solution containing 10% Sodium Dodecyl Sulfate (SDS) and 0.01M HCl was added. After at least 18 h at 37 °C, the absorbance of each well was measured in a microplate reader at 570 nm. The results were expressed as percentage of the controls.

Confocal microscopy and image processing analysis

Cells (10.000/cmq) were treated with labeled micelles, added to the culture medium at dilution higher than 1 : 100, and grown on coverslips for 0.5–12 h. Then, cells were washed five times with PBS and fixed with 4% paraformaldehyde. After washing, cells were mounted in glycerol–PBS medium containing 50 mg ml^{-1} DABCO. The confocal imaging was performed on a confocal microscope equipped with an argon/krypton ion laser. A green laser line was used to visualize Nile Red and doxorubicin fluorescence at respectively 535 nm and 545 nm. A blue laser line at 488 nm was used to visualize fluorescein fluorescence. Optical sections were obtained at increments of 0.25 μm in the *Z*-axis and were digitized with a scanning mode format of 1024 × 1024 pixels. The image processing and the volume rendering were performed using the Leica TCS software. Negative controls consisted of samples not incubated with labeled micelles or

incubated with unlabeled micelles. To visualise fluorescein-labeled unimers, cells were treated with labeled micelles for 1 h, then were washed three times with fresh medium, and grown on coverslips for an additional 2–11 h. Four coverslips were analysed by CLSM for each condition tested.

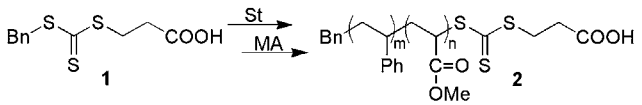
Results and discussion

Synthesis of the block copolymer

The RAFT agent 3-(benzylsulfanyl thiocarbonylsulfanyl) propionic acid (**1**, Scheme 1) was synthesized modifying an already reported procedure.[30] This compound was used to control the radical polymerization of styrene. The process, conducted at 90 °C, is initiated by 2-2′-azobis(cyclohexane-carbonitrile). Carboxyl terminated polystyrene was obtained after a reaction time of 8 and 16 h. The molecular weight and polydispersity were respectively Mn = 6800 g mol^{-1}, Mw/Mn = 1.20 (**a**) and Mn = 11000 g mol^{-1}, Mw/Mn = 1.08 (**b**). The macro-RAFT **b** was used to synthesize the polystyrene-*b*-poly(methyl acrylate) block copolymer **2** (Scheme 1), after accurate removal of the residual styrene. Traces of styrene inhibited the subsequent polymerization of methyl acrylate that requires milder conditions than styrene (lower temperature and higher dilution). Similar behaviour has been observed in the synthesis of PS-*b*-PMA.[34] The polymerization of methyl acrylate, initiated by AIBN, proceeded at 70 °C for 5.5 h. The molecular weight and polydispersity of the resulting block copolymer were Mn = 35000 g mol^{-1}, Mw/Mn = 1.09 from GPC analysis and Mn = 34000 g mol^{-1} from NMR analysis.

Micelle formation

Self-assembly in methanol. We casually observed, working on a different project, the formation of rather stable particles when diluting with methanol a THF solution of PS_{100}-*b*-PMA_{134} block copolymer. This material was obtained while optimizing the synthesis of PS-*b*-PMA-*b*-PVA triblock copolymer. The switchable RAFT agent *S*-(methyl propion-2-yl) *N*-methyl *N*′-(pyridin-4-yl) carbamodithioate *p*-toluene sulfonic acid salt[34,35] used for this synthesis provides polymeric chains bearing a positively charged head. The DLS characterization of the particles, which were obtained in not dry solvents, gave 280 nm as hydrodynamic diameter with low polydispersity and +10 mV as Z potential. The observation of this phenomenon prompted us to use the easily synthesizable RAFT agent 3-(benzylsulfanyl thiocarbonylsulfanyl) propionic acid[19,31,32] in order to obtain ionizable carboxyl ended polymeric species. The RAFT polymerization of styrene gave a carboxyl ended PS-macro-RAFT that, upon reaction with methyl acrylate, provided the species PS_{106}-*b*-PMA_{267}-$SC(S)S(CH_2)_2COOH$ (**2**, Scheme 1). The particles obtained when a THF solution of carboxyl terminated block copolymer is diluted with methanol confirmed what was previously observed. This phenomenon, which is characteristic of amphiphilic block copolymers, is observed despite the fact that both polystyrene and poly(methyl acrylate) are insoluble in methanol. We suppose that the driving force for the self-assembling process of PS-*b*-PMA is due to a different grade of (poor) solubility in methanol of the polystyrene block with respect to the poly(methyl acrylate) one, and it is helped also by the solvated charged heads. Hence, the particles formed are composed of a PS core and a partially solvated PMA shell. Such behaviour is consistent with the self-assembly of the polymeric chains (unimers) and results in the formation of core–shell particles (micelles). Beside the charged (or not charged) head, the presence of water in the solvents also influences the process. In fact, the particles formed in not anhydrous solvents (commercial grade) by non-deprotonated carboxyl ended unimers are thermodynamically unstable and tend to increase their dimensions from 350 nm to 1.5 μm in 1.5 h. The particles formed by unimers with deprotonated carboxyl functions in not anhydrous solvents are thermodynamically more stable, their hydrodynamic diameter is 120 nm and their Z potential is −15 mV. Micelles formed by diluting with dry methanol a solution of copolymer **2** in dry THF containing one equivalent (with respect to the carboxylic function) of triethylamine are characterized by a much smaller hydrodynamic diameter, *i.e.* 20–30 nm. In brief, when the solvents employed contain traces of water, larger particles are formed; this is due to a less thermodynamically stable sol–gel interface. Thus, well defined monodispersed spherical micelles are obtained only when the acidic groups are deprotonated and by using dry solvents.



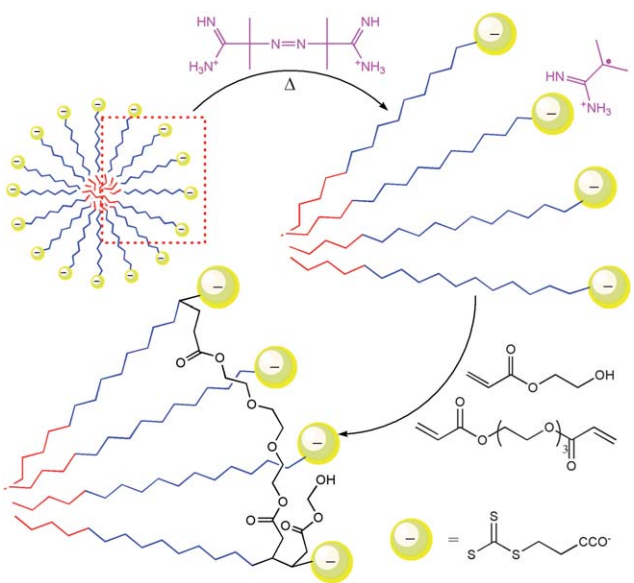
Scheme 1 Synthesis of PS-*b*-PMA block copolymer.

Stability of the micelles in water. Once small micelles are obtained by self-assembling of anionic unimers in dry methanol, they are easily dispersible in water. The negatively charged corona on the micelles, enhancing the thermodynamic stabilization, causes the formed particles to remain stable even when the dispersant medium (methanol) is replaced with water by osmosis. After suspension in deionized water, the micelles were dispersed in a 10 mM solution of $NaHCO_3$ in the same dialysis tube. The hydrodynamic diameter of the micelles was found to be 25 nm and the Z potential was −54 mV. To verify the stability of the neutralized micelles in water, solutions of acetic acid at different concentration were added. The addition of 2 mL of acetic acid 20 mM to 2 mL of the micellar suspension in 10 mM $NaHCO_3$ causes rapid particle coalescence that makes it impossible to determine the hydrodynamic diameter by DLS analysis. The acidic medium shifts to neutrality the acid–base equilibrium of the carboxylated corona which loses the negative charge. Therefore, the lost thermodynamic stabilization causes the rapid particle coagulation. The addition of 2 mL of 10 mM acetic acid to 2 mL of the micellar suspension in 10 mM $NaHCO_3$ causes slow particle coalescence. Indeed, three hours after neutralization, their dimension was found to be 160 nm. In this case, a neutral environment makes the micelle surface partially ionized due to the weak acidity of the carboxylic groups. In neutral medium the particles are characterized by a partial thermodynamic stabilization that slows down the coagulation process.

Shell-crosslinking

Another key feature of the carboxylic acid terminated unimers that affords micelles with an anionic corona is the ability to direct elongation processes that involve the RAFT function only at the particle interface. This is particularly useful for starting on the surface of the micelles (and only there) a crosslinking polymerization process that involves the bifunctional monomer triethylene glycol diacrylate, the intercalating monomer 2-hydroxyethyl acrylate and the radical initiator 2,2′-azobis(2-methylpropionamidine) dihydrochloride. Because of electrostatic interactions this initiator settles, in its cationic form, only in proximity to the micelle surface (Scheme 2) initiating there the crosslinking polymerization process. To prove the importance of the electrostatic interactions between the micelles and the initiator, the azo compound 4,4′-azobis(4-cyanopentanoic acid) was instead used to initiate the crosslinking process, and a dramatically different behaviour was observed. In this case, due to the opposing interactions between the azo compound and the particles, the initiation stage starts in the sol phase forming amorphous micro-gel aggregates which, eventually, incorporate and desegregate the micelles as evidenced by AFM analysis (Fig. 1a). The charged corona of the micelles also has the function of hampering inter-micellar crosslinking because of the electrostatic repulsion of the particles. Fig. 1 shows AFM topography images of an amorphous microgel incorporating a few micelles (**a**) and the well separated monodispersed SCL micelles (**b**). The double function of the anionic corona that constitutes the outer shell of the micelles must be emphasised: one function is to direct the hyperbranching process only at the surface of the particles, the other one is to prevent micelle aggregation by electrostatic repulsion.

The crosslinking degree is governed by the crosslinker amount and three different crosslinker/polymer molar ratios have been tested (5/1 SCL-M1; 50/1 SCL-M2; 500/1 SCL-M3). Because of the dilution of the reagents (macro-RAFT 3.6×10^{-5} M) the rate of the polymerization of the crosslinker is low and an appreciable



Scheme 2 Micelle shell crosslinking.

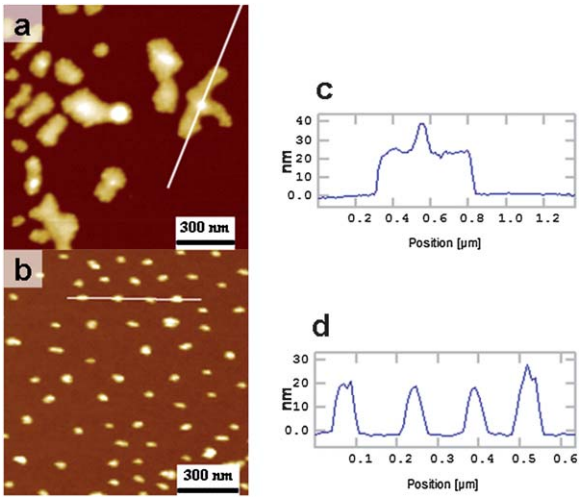


Fig. 1 AFM topography images of amorphous microgel (**a**, z-range 53 nm) and SCL-M1 micelles (**b**, z-range 31 nm); in **c** and **d** are shown the corresponding height profiles, taken along the white lines.

crosslinking degree is observed with a reaction time of 24 h at 50 °C. Thus, as evidenced by GPC analysis (Fig. 2), for SCL-M1 around 15% of unimers are dimerized, for SCL-M2 around 25% of chains are dimerized (also a small amount of higher hyperbranching grade is observed), and for SCL-M3 around 50% of chains are hyperbranched (of which 50% is still in the dimeric form). The diameters of the particles in methanol were determined as 25 nm (SCL-M1), 30 nm (SCL-M2) and 50 nm (SCL-M3) through DLS measurements. Fig. 3 shows a SEM micrograph of SCL-M3.

SCL micelles can be suspended in an aqueous phase, as easily as the uncrosslinked ones, by osmosis. The hydrodynamic diameter of SCL-M3 micelles in 10 mM $NaHCO_3$ was 50 nm and the Z potential was −52 mV. The increased stability of the shell crosslinked micelles SCL-M3 was tested by adding solutions of acetic acid at different concentrations to the micelles suspended in 10 mM $NaHCO_3$. The acidic environment, resulting from the addition of 2 mL of 20 mM acetic acid to 2 mL of the micellar suspension in 10 mM $NaHCO_3$, causes particle coalescence but the dispersion is not destabilized. Indeed, after 16 h the dimension of the particles was found to be 130 nm. The neutral

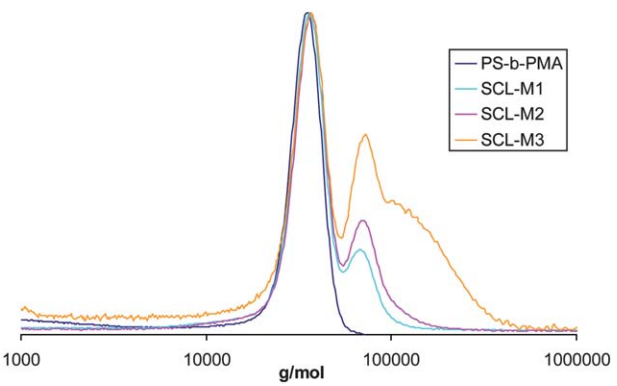


Fig. 2 GPC traces of the starting block copolymer (PS-*b*-PMA) and of the crosslinked block copolymer (SCL micelles).

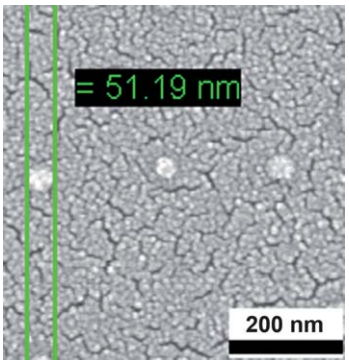


Fig. 3 SEM micrograph of SCL-M3.

environment, resulting from the addition of 2 mL of 10 mM acetic acid to 2 mL of the micellar suspension in 10 mM $NaHCO_3$, causes a little coalescence of the particles that were found to be 70 nm after three hours. This behaviour shows the increased stability of the SCL-M3 micelles compared to the uncrosslinked ones. This increased stability in neutral or acidic media is not attributable to thermodynamic stabilization but to the covalent hyperbranching between the unimers.

Micelle loadability

The micelles obtained with this method can be easily loaded with lipophilic compounds like fluorescent markers or therapeutic agents. Nile Red or neutralized doxorubicin were dissolved together with the block copolymer in the THF solution before adding methanol. During the self-assembling process, the lipophilic environment of the forming micellar core segregates the lipophilic substances that are not compatible with the dispersant media. The Nile Red loaded micelles dispersed in methanol were also crosslinked. The loaded particles, suspended in water by osmosis, confined their lipophilic contents in the core.

Cellular uptake

Upon methanol replacement with water the micelles were ready for dilution in cell medium and administration to cultured cells. The micelles (uncrosslinked and SCL-M1) loaded with Nile Red were incubated with Human Umbilical Venin Endothelial (HUVE) cells, with GN11 neurons or with colorectal carcinoma LoVo cells for times between 30 min and 12 h. After fixation with paraformaldehyde, the cells were observed with CLSM at 535 nm excitation. The obtained images of cells (Fig. 4) that have internalized the micelles indicate the staining of different cellular compartments according to the kind of micelles used. Cells treated with uncrosslinked micelles are stained in their lipophilic domains (*e.g.* membrane and intracellular organelle membranes), Nile Red being highly lipophilic. Conversely, the treatment with SCL micelles leaves Nile Red dispersed in the aqueous cytoplasm (Fig. 4c and d). Although the time course of micelle internalization shows that the particles undergo a rapid and massive internalization in all cell types tested, in HUVE and LoVo cells the maximum fluorescence intensity is reached after 30 min of incubation, while in GN11 neurons it is reached after 60 min, indicating different internalization rates in different cell types.

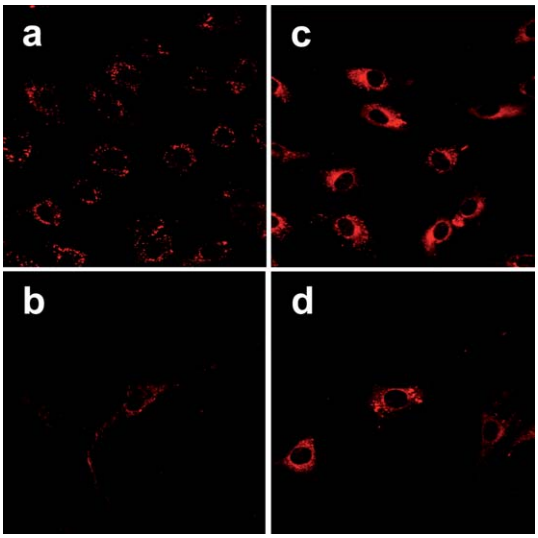


Fig. 4 Confocal laser scanning microscopy analysis of human umbilical venin endothelial (HUVE) cells (**a**, **c**) and GN11 mouse neurons (**b**, **d**) treated with Nile Red loaded free micelles (**a**, **b**) or with Nile Red loaded SCL-M1 micelles (**c**, **d**).

SCL micelles, even at the lower degree of shell crosslinking (SCL-M1), showed intracellular resistance and did not desegregate within 12 h keeping the dye confined in their core. This confinement also prevents dye depletion by the laser energy as shown by brighter images provided by cells treated with SCL micelles (Fig. 4c and d).

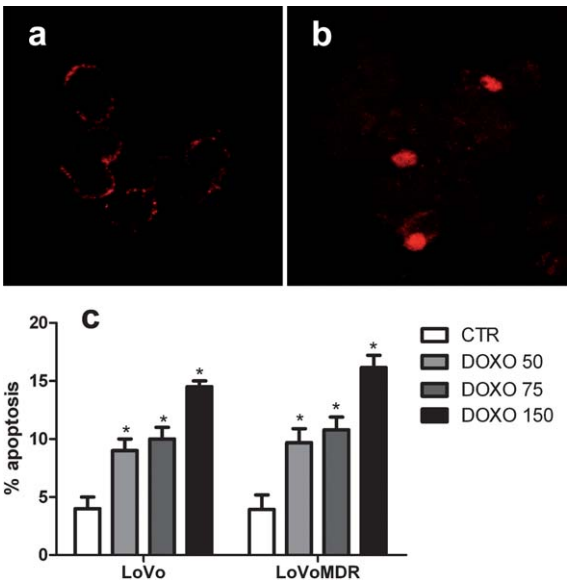


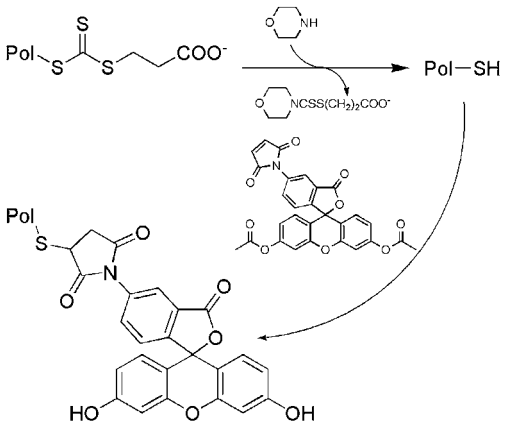
Fig. 5 Confocal laser scanning microscopy analysis of LoVo-MDR cells treated with free doxorubicin (**a**) or doxorubicin loaded micelles (**b**). Doxorubicin loaded into micelles is capable of inducing similar apoptosis levels in LoVo and LoVo-MDR cells (**c**). Apoptosis was evaluated after 24 h exposure to different concentrations of doxorubicin loaded micelles. Bars represents the mean (±SEM) of three independent experiments, performed in duplicate. * = $p < 0.05$ with respect to controls.

Doxorubicin delivery

Uncrosslinked micelles were loaded with doxorubicin and administered to doxorubicin resistant cells (LoVo-MDR). The comparison of the images of cells treated with free doxorubicin (Fig. 5a) and with doxorubicin loaded micelles (Fig. 5b), obtained by CLSM at 545 nm excitation, shows that in the former case the fluorescent drug remains confined in the area around the cell membrane whereas in the latter the drug is allocated within the nucleus because of the DNA intercalating properties of doxorubicin. This behaviour reveals that uncrosslinked micelles are incorporated into cells, overcoming their intrinsic resistance to doxorubicin, probably by endocytosis. The apoptosis induction of doxorubicin loaded micelles in LoVo and LoVo-MDR cells (Fig. 5c) is almost identical, confirming that micelles containing doxorubicin bypass the drug efflux due to MDR phenotype. Moreover, in LoVo cells the LD50 of doxorubicin is 10 times lower when using doxorubicin loaded in uncrosslinked micelles than free doxorubicin (respectively 3 μM and 300 nM) directly added to the culture medium, as evaluated by cell viability assay.

Unimer labeling

To trace the unimer fate after the internalization of micelles into the cells we labeled the block copolymer chains exploiting the electrophilic reactivity of the RAFT function. So the trithiocarbonate group located in the corona of the crosslinked micelles (SCL-M3) suspended in degassed methanol was displaced by morpholine. This leaves a free thiol group which reacts with fluorescein diacetate 5-maleimide resulting in unimers covalently bound with the fluorescent dye (Scheme 3). It should be noticed that the not fluorescent moiety (fluorescein diacetate) is cleaved by a transesterification process that proceed in methanol and it is catalyzed by morpholine; this turned the fluorescein moiety fluorescent. This kind of micelle was administered to LoVo, HUVE and GN11 cells that, after fixation, were observed with CLSM at 488 nm excitation. The fluorescein labeled micelles were promptly incorporated into the cells within one hour (Fig. 6a) and localized everywhere in the cytosol and in the nucleus. After 12 h most of the marked unimers were eliminated by the cells as shown in Fig. 6b. The excretion of fluorescein labeled unimers will be traced in future *in vivo* experiments.



Scheme 3 Trithiocarbonate displacement by morpholine followed by mercaptan addition to fluorescein diacetate 5-maleimide.

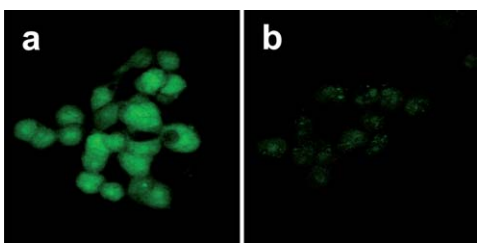


Fig. 6 LoVo cells treated with micelles assembled by fluorescein covalently bound unimers 1 h (**a**) or 12 h (**b**) after micelle administration.

Conclusions

With this work, we exploited many of the RAFT technique peculiarities ranging from controlled polymerization for synthesizing functional polymers to post-polymerization conjugation reactions that involve the RAFT function. The synthesized functional polymers conjugate self-assembling properties with the needed post-micellization specific functions. So we showed that micelles composed of PS-*b*-PMA block copolymer can be easily suspended in water after their self-assembly in methanol. The process is favoured and the nanoparticles are stabilized by the anionic carboxylate function terminating each unimer. The anionic corona also has the fundamental function to direct the process of shell crosslinking only at the interface between the micelles and the free solution. This prevents the formation of an amorphous micro-gel and, at the same time, inter-micellar crosslinking. Free and SCL micelles rapidly cross the cellular plasma membrane of many cell types, if not all, and thus can be very efficient vehicles for intracellular delivery of lipophilic fluorescent probes and drugs. Indeed, free micelles loaded with doxorubicin act as very efficient drug shuttles that overcome the intrinsic resistance of LoVo-MDR cells. The SCL process is capable of stabilizing the micelles in the intracellular environment, as evidenced by the different cellular compartments stained, and of also increasing the sensitivity of cellular labeling. The stability conferred to the SCL micelles by covalent crosslinking has been confirmed by changing the pH of the dispersant medium. The micelles can also be labeled in their constituent unimers which were covalently bound with fluorescein in order to trace the unimer fate *in vitro*, and possibly *in vivo*.

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