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A Dendrimer-Hydrophobic Interaction Synergy Improves the Stability of Polyion Complex Micelles

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Polyion complex (PIC) micelles incorporating PEG-dendritic copolymers display an unprecedented stability towards ionic strength that is amplified *via* hydrophobic interactions. The tridimensional orientation of peripheral hydrophobic linkers between charged groups and the globular/rigid dendritic scaffold maximizes this stabilization compared to PIC micelles from linear polymers. As a result, micelles stable at concentrations higher than 3 M NaCl are obtained, which represents the highest saline concentration attained with PIC micelles. Advantage of this stabilizing dendritic effect has been taken for the design of a robust, pH-sensitive micelle for the controlled intracellular release of the anticancer drug doxorubicin. This micelle displays a slightly higher toxicity, and distinctive mechanisms of cell uptake and intracellular trafficking relative to the free drug. The preparation of mixed PIC micelles by combining differently functionalized PEG-dendritic block copolymers has allowed to fine-tune their stability, paving the way towards the facile modulation of properties like biodegradability, drug loading, or the response to external stimuli.

Introduction

During the last decades drug delivery (DD) nanosystems have attracted a great deal of attention to improve the efficacy of medicines.[1] Among them, polyion complex (PIC) micelles, originally described by the groups of Kataoka and Kabanov,[2,3,4,5,6] have consolidated as carriers for a plethora of pharmaceuticals, including nucleic acids and proteins.[7] PIC micelles are prepared by electrostatic interaction from oppositely charged polymers or biopolymers in stoichiometric charge ratios, with at least one of the components carrying a neutral hydrophilic block, usually poly(ethylene glycol) (PEG). Their electrical neutrality, nanometric scale, narrow size distribution, and core-corona structure are well suited properties for DD to ensure an improved biocompatibility, long circulation times in the bloodstream, and passive accumulation into solid tumors [enhanced permeability and retention (EPR) effect].[8] However, *in vivo* applications of PIC micelles are strongly limited because their electrostatic nature renders them extremely salt-sensitive. Accordingly, covalent cross-linking[9,10,11] or more sophisticated charge-conversional approaches[12,13,14] have been proposed by Kataoka to prevent their premature dissociation.

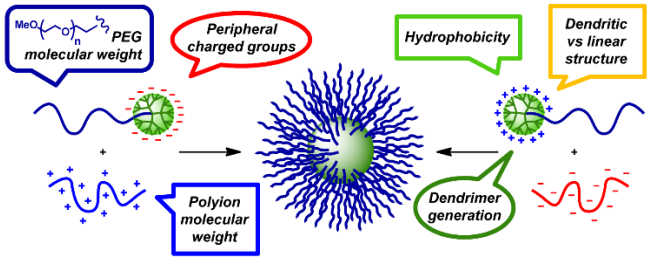

Figure 1. Structural elements controlling the stability of PIC micelles.

As an alternative way to secure stable PIC micelles, we have recently described with promising results the use of PEG-dendritic block copolymers of the GATG (gallic acid-triethylene glycol) family[15,16,17,18,19] carrying a rigid dendritic block (PEG_{5000}-[G3]-OSO_3^-, Figures 1, 2).[20,21] The rigidity of dendrimers has been pointed by Kataoka[22,23] and more recently by Kishimura[24] as responsible of enhanced stability in related systems, though no stability improvements with dendrimers have been observed by others.[25] In addition, a detailed comparison between dendritic and linear PIC micelles has remained elusive. Herein we report our efforts to dissect the existence of a stabilizing dendritic effect[26,27] in PIC micelles: a program to unveil the relative influence of the linear *vs* dendritic architecture as well as other structural elements on the stability of these systems [molecular weight (MW) of the polymers/blocks, dendrimer generation (G), nature of the charged groups, and hydrophobicity of linkers] (Figure 1). Our findings confirm the enhanced stability of dendritic PIC micelles and identify key structural elements in their design to achieve integrity under physiological conditions (150 mM NaCl, 37 °C). Remarkably, dendritic PIC micelles reveal an unprecedented leaning to increase their stability by incorporation of hydrophobic linkers

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between the dendritic scaffold and peripheral charged groups. This synergistic effect, not observed in PIC micelles derived from linear polymers, is presumably associated to the globular nature of the dendritic block. By combining these findings, we have succeeded in preparing an optimized pH-sensitive DD system (DDS) for the controlled intracellular release of the anticancer drug doxorubicin (DOX).

Experimental

Materials

Methoxy-poly(ethylene glycol)-block-poly(L-glutamic acid sodium salt) copolymers [PEG_{5000}-PGA_{25} (DP 23), PEG_{5000}-PGA_{50} (DP 48), PEG_{5000}-PGA_{100} (DP 92), by ^1H NMR] were purchased from Alamanda polymers. $CuSO_4{\cdot}5H_2O$ was obtained from Prolabo. Alexa Fluor-488 succinimidyl ester, LysoTracker Green and LysoTracker Red were obtained from Invitrogen-Molecular Probes. BocHN-PEG_{5000}-NH_2 (M_n 5018, M_w 5043 by MALDI-TOF) was purchased from Iris Biotech. MeO-PEG_{10000}-OH (M_n 9892, M_w 9918 by MALDI-TOF), poly-L-lysine hydrobromide [PLL, MW 13400 (DP64), 22600 (DP 108) and 32800 (DP 157), by viscosity], poly-L-glutamic acid sodium salt [PGA, MW 20500 (DP 136), by viscosity] and all others reagents were purchased from Sigma-Aldrich.

PEG_{5000}-[G2]-N_3,[28] PEG_{5000}-[G3]-N_3,[28] PEG_{5000}-[G4]-N_3,[29] PEG_{2000}-[G3]-N_3,[28] PEG_{5000}-[G3]-CO_2^-,[20] PEG_{5000}-[G3]-NH_3^+,[29] and GATG repeating unit[30] were prepared following procedures previously described by our group. PEG_{10000}-[G2]-$NH_2{\cdot}HCl$ was prepared from commercial MeO-PEG_{10000}-OH following procedures reported by us.[28] 4-ethynylbenzoic acid,[31] ammonium 4-ethynyl benzene sulfonate,[32] 4-ethynylbenzylamine hydrochloride[33] and 1-azido-3-aminopropane[34] were prepared following known procedures. The synthesis of PEG-dendritic block copolymers is described in detail in the Electronic Supplementary Information (ESI). A549 cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC).

General Methods

H_2O was purified with a Milli-Q purification system from Millipore. NMR spectra were recorded on 400, 500, or 750 MHz spectrometers. Chemical shifts are reported in ppm (δ units) downfield from the HOD solvent peak (D_2O) or internal 3-(trimethylsilyl)propionic acid-d_4 (D_2O). All spectra were analyzed with MestReNova software. IR spectra were obtained using a Bruker IFS-66v or Perkin-Elmer Spectrum Two spectrophotometers. MALDI-TOF MS experiments were carried out in an Applied Biosystems/MDS SCIEX 4800 MALDI-TOF/TOF spectrometer, using 2,5-dihydroxybenzoic acid (DHB) as matrix. Ultrafiltration was carried out with Amicon® stirred cells [YM1 and YM3 membranes, molecular weight cut-off (MWCO) 1 and 3 KDa respectively]. Dialysis were carried out with SpectrumLabs Spectra/Por®6 tubing (MWCO 1 KDa) and Thermo Scientific Slide-A-Lyzer® (MWCO 3.5 KDa)

CuAAC Reactions

General Procedure for CuAAC. Azide containing block copolymers and alkynes (200 mol % per terminal azide groups) were dissolved in DMF/H_2O or *t*-BuOH/H_2O mixtures. Then, freshly prepared aqueous solutions of $CuSO_4$ (5 mol % per azide, 0.1 M) and sodium ascorbate (25 mol % per azide, 0.5 M) were added (final concentration of terminal azides 0.1 M). After 24 h of stirring at rt protected from light, reaction mixtures were purified by ultrafiltration (YM3) washing with aq 0.1 M EDTA pH 6 (2 x 30 mL), sat $NaHCO_3$ (1 x 30 mL), and H_2O (5 x 30 mL) to afford functionalized block copolymers after freeze-drying.

PEG_{5000}-[G3]-$PhCO_2^-$. Starting from PEG_{5000}-[G3]-N_3 (50 mg, 3.84 µmol), 4-ethynyl benzoic acid (30.3 mg, 207 µmol), $NaHCO_3$ (34.8 mg, 415 µmol), $CuSO_4$ (51.8 µL, 5.19 µmol, 0.1 M) and sodium ascorbate (51.8 µL, 26.0 µmol, 0.5 M) dissolved in DMF (0.52 mL)/H_2O (0.41 mL) and following the general procedure for CuAAC reactions, PEG_{5000}-[G3]-$PhCO_2Na$ (66 mg, 98%) was obtained as a pale yellow foam. ^1H NMR (500 MHz, D_2O) δ: 8.14-7.95 (m, 27H), 7.93-7.67 (m, 54H), 7.51 (br s, 54H), 7.01-6.65 (m, 26H), 4.44 (br s, 54H), 4.25-3.28 (m, ~879H), 3.18 (br s, 2H). ^{13}C NMR (100 MHz, D_2O) δ: 174.1, 168.5, 151.2, 146.3, 139.1, 136.0, 131.4, 129.4, 128.7, 124.7, 122.3, 109.9, 105.7, 71.6, 70.9, 69.5, 69.1, 68.8, 68.4, 67.9, 60.3, 57.9, 49.9, 39.6. IR (KBr, cm^{-1}): 3422, 2872, 1589, 1541, 1421, 1115. MALDI-TOF MS (DHB, linear mode, *m/z*): M_p 16746, M_n 17157, M_w 17331. Calcd: M_p 16918 ($[M+H^+]$), M_n 16967.

PEG_{5000}-[G3]-$BnNH_3^+$. Starting from PEG_{5000}-[G3]-N_3 (23.3 mg, 1.79 µmol), 4-ethynylbenzylamine hydrochloride (16.2 mg, 96.6 µmol), $CuSO_4$ (24.2 µL, 2.4 µmol, 0.1 M) and sodium ascorbate (24.2 µL, 12.1 µmol, 0.5 M) dissolved in *t*-BuOH (0.24 mL)/H_2O (0.19 mL) and following the general procedure for CuAAC reactions, PEG_{5000}-[G3]-$BnNH_2{\cdot}HCl$ (29 mg, 93%) was obtained as a brown foam after purification by ultrafiltration [YM3; washing with 0.01 M HCl (3 x 30 mL) and H_2O (3 x 30 mL)] and freeze-drying. ^1H NMR (500 MHz, D_2O) δ: 8.04 (br s, 27H), 7.40 (br s, 54H), 7.19 (br s, 54H), 6.94-6.46 (m, 26H), 4.41 (br s, 54H), 4.08-3.89 (m, 54H), 3.88-3.18 (m, ~879H), 3.12 (br s, 2H). ^{13}C NMR (126 MHz, D_2O) δ: 168.6, 158.5, 151.8, 146.7, 139.8, 132.9, 130.3, 129.8, 126.1, 122.9, 106.3, 72.4, 70.1, 69.4, 69.0, 68.6, 60.9, 58.5, 50.5, 43.1, 40.1. IR (KBr, cm^{-1}): 3417, 2878, 1111. MALDI-TOF MS (DHB, linear mode, *m/z*): M_p 16049, M_n 15832, M_w 15892. Calcd: M_p 16513 ($[M+H^+]$), M_n 16564.

PIC Micelles

Preparation of PIC Micelles. PIC micelles were prepared from the following polyion solutions:

(i) (volume ratio 1:2) anionic PEG-dendritic or PEG-polypeptide copolymers dissolved in 10 mM Na_2HPO_4/0.1 M NaOH (15% v/v) and PLL·HBr (0.5 mg/mL) dissolved in 10 mM NaH_2PO_4,

(ii) (volume ratio 1:2) PEG_{5000}-[G3]-NH_3^+ or PEG_{5000}-[G3]-$BnNH_3^+$ dissolved in 10 mM NaH_2PO_4/0.1 M HCl (5% v/v) and PGA Na salt (0.25 mg/mL) dissolved in 10 mM Na_2HPO_4.

After mixing the solutions at stoichiometric charge ratios, PIC micelles were obtained (pH 7.2-7.5) and aged for at least 1-3 h before analysis or further treatment.

General Note on PIC Micelles. Filtering of PIC micelles before dynamic light scattering (DLS) measurements was avoided to

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prevent removal of large aggregates that could obscure the analysis of micelle formation and stability.

Preparation of Fluorescently Labeled (Alexa Fluor 488) PIC Micelles. Alexa Fluor 488 (AF488) labelled PIC micelles were prepared from PLL_{108} and a mixture of PEG_{5000}-[G3]-$PhCO_2^-$ and AF488-PEG_{5000}-[G3]-$PhCO_2^-$ (9:1 molar ratio) following the above procedure.

Efficiency of the Formation of PIC Micelles. The formation efficiency of PIC micelles was assessed by quantifying the amount of free PLL in a solution of micelles prepared from PEG_{5000}-[G3]-$PhCO_2^-$ (1.04 mg/mL PIC) and PLL_{108} (0.33 mg/mL PIC). To this end, we exploited the characteristic fluorescence of substituted isoindoles produced by reaction of primary amines with *o*-phthalaldehyde in the presence of thiols.[35,36] First, any free PLL_{108} in the solution of PIC micelles (4 mL in 10 mM PB 7.4) was separated by ultracentrifugation (50000 rpm, 15 °C, 4 h). A solution of PLL_{108} (4 mL in 10 mM PB 7.4, 0.5 mg/mL) was submitted to identical ultracentrifugation conditions as control to rule out precipitation of PLL during the process. Then, supernatants of PIC micelles and control PLL_{108} (50 μL each) were separately mixed in a 96-well plate in the dark with a solution containing *o*-phthalaldehyde (0.04% w/v), 2-mercaptoethanol (0.2% v/v) and diethyl ether (0.1% v/v) in 0.4 M borate buffer, pH 9.7 (50 μL). Afterwards, the concentrations of PLL_{108} in micelle and control solution were determined by measuring their relative fluorescence emission intensity (excitation wavelength 355 nm, emission wavelength 460 nm, Tecan Infinite F200 PRO plate reader) compared to a calibration line made with freshly prepared solutions of PLL_{108} of known concentrations. It was revealed that 95.0% (±2.5%) of PLL_{108} control remains in solution after ultracentrifugation, confirming the validity of the method. When applied to PIC micelle supernatant, only 5.8% (±2.2%) of free PLL_{108} was detected, which accounts for a formation efficiency of PIC micelle higher than 94%.

Measurement of z-Potentials. Z-potential values of PIC micelles were obtained by laser doppler anemometry (LDA), measuring the mean electrophoretic mobility (Malvern Zetasizer Nano ZS, Malvern Instruments). Measurements were performed in 10 mM PB pH 7.4. The z-potential of PEG_{5000}-[G3]-$PhCO_2^-$/PLL_{108} and PEG_{5000}-[G3]-$BnNH_3^+$/PGA_{136} micelles were 0.15 and -1.42 mV, respectively.

Dynamic Light Scattering (DLS) and Stability of PIC Micelles. DLS measurements were performed on a Malvern Nano ZS (Malvern Instruments, U.K.) operating at 633 nm with a 173 ° scattering angle, at 25 or 37 °C. DLS histograms and mean diameters were obtained from the volume particle size distribution provided by Malvern Zetasizer Software.

PIC micelles prepared following the general procedure shown above were left at rt for 3 h before DLS measurements (upon formation; PB 7.4, 25 °C). Then, to assess the stability of micelles towards ionic strength, increasing concentrations of NaCl were added (one experiment per concentration) and DLS measurements (at 25 °C) repeated after 1 h at rt. Finally, the stability of micelles towards simulated physiological conditions was analyzed by DLS (at 37 °C) after heating at 37 °C for 12 h in the presence of 150 mM NaCl. These results are summarized in Tables S1 and S2, and DLS histograms shown in Figures S3-S19 in the ESI.

Mixed PIC Micelles. PIC micelles from PLL_{108} and mixtures of PEG_{5000}-[G3]-CO_2^- and PEG_{5000}-[G3]-$PhCO_2^-$ (stoichiometric charge ratio) were prepared (10 mM PB, pH 7.4, supplemented with 150 mM NaCl) following the procedure shown above. After 1 h at rt, micelle formation was assessed by DLS (25 °C). Then, increasing concentrations of NaCl were added (one experiment per concentration) and DLS measurents (25 °C) repeated after 1 h at rt.

Doxorubicin (DOX) Loaded PIC Micelles

Preparation of DOX-loaded PIC micelles. PIC micelles from PEG_{5000}-[G3]-$PhCO_2^-$ and PLL_{108} were prepared as described above. Then, an aqueous solution of DOX (1/3 of total PIC micelle volume, 1 mg/mL in H_2O) was added to the solution of micelles and the resulting mixture was stirred at rt overnight. Unloaded DOX was removed by dialysis (Spectra/Por® 6, MWCO 1 KDa) against PBS pH 7.4 in the dark (12 h, 37 °C for encapsulation and release studies; 24 h for cellular uptake and viability experiments).

Determination of Encapsulation Efficiency and Drug Loading. Encapsulation efficiency (EE) describes the fraction of drug incorporated into a micelle compared to the total amount of drug used in its preparation (EE = weight of drug in micelle × 100 / weight of feeding drug). Drug loading (DL) refers to the mass fraction of a micelle that is composed of drug (DL = weight of drug in micelle × 100 / weight of drug-loaded micelle).

DOX-loaded PIC micelles were prepared as described above. To determine the EE and DL of DOX, 800 μL of micelles were dialyzed against 200 mL PBS pH 7.4 at 37 °C, for 12 h in the dark. Aliquots of the buffer solution were taken at the end of the dialysis (3 × 150 μL) and placed in a 96-well microplate. Then, 500 mM acetate pH 4 buffer (50 μL) was added to each well and the fluorescence of the samples was measured at a microplate reader (exc. 560, em. 610; Tecan Infinite F200 PRO). Concentration of unloaded DOX in solution was calculated by comparison with a standard calibration curve made from the fluorescence emission of fresh solutions of DOX of known concentrations prepared under identical conditions. An EE of 53% and DL of 15% were obtained.

***In vitro* release study of DOX.** DOX-loaded PIC micelles were prepared and dialyzed as described above (Spectra/Por® 6, MWCO 1 KDa, 800 μL of micelles against 200 mL PBS pH 7.4, 37 °C, 12 h in the dark). Then, dialysis bags were moved to new buffer solutions (200 mL) at 37 °C in the dark: a) PBS pH 7.4, b) 50 mM acetate buffer pH 5.0, 100 mM NaCl. At fixed times, aliquots (3 × 150 μL) were taken and placed in 96-well microplates. Then, 500 mM acetate pH 4 buffer (50 μL) was added to each well and the fluorescence of the samples was measured in a microplate reader (exc. 560, em. 610; Tecan Infinite F200 PRO). The amount of released DOX was calculated by comparison with a standard calibration curve made from the fluorescence emission of fresh solutions of DOX of known concentration, prepared under identical conditions.

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Stability of DOX-Loaded PIC Micelles in Serum. DOX-loaded PIC micelles were prepared as described above. After unloaded DOX was removed by dialysis (Spectra/Por® 6, MWCO 1 KDa) against PBS pH 7.4 (24 h, 37 °C) in the dark, DOX-loaded micelles were incubated with an equal volume of fetal bovine serum (FBS) at 37 °C in the dark for 24 h. Then, the mixture was filtered through centrifugal filters (Amicon Ultra-4, MWCO 10 KDa, 6000 rpm, 40 min, 15 °C). Aliquots of the filtered solution (3 × 50 μL) were taken and placed in a 96-well microplate. Then, 500 mM acetate pH 4 buffer (150 μL) was added to each well and the fluorescence of the samples was measured in a microplate reader (exc. 560, em. 610; Tecan Infinite F200 PRO). An amount of released DOX of 2.6% was calculated by comparing the fluorescence of the samples with a calibration curve made with solutions of known concentrations of DOX processed in the same way as the micelles.

Cell Studies

Cell Cultures. Human adenocarcinoma alveolar basal epithelial (A549) cells were cultured at 37 °C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) with high glucose, containing 10% FBS and supplemented with 50 U/mL penicillin and 50 U/mL streptomycin.

Cytotoxicity Assays. A549 cells were seeded in a 96-well microplate (4000 cells/well) in 100 μL of DMEM supplemented with 10% FBS and maintained at 37 °C in a 5% CO_2 atmosphere during 24 h. Then, growth medium was replaced by fresh medium containing different concentrations of micelles (their constituents or controls) and incubated for selected periods of time. Afterwards, 10 μL of a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (5 mg/mL in PBS) were added to each plate and incubated for 4 h, before 100 μL of solubilizing solution (10% SDS in 0.01 M HCl) were added and the resulting mixtures incubated overnight. Absorbance was read at 570 nm in a microplate reader (Tecan Infinite F200 PRO) and the relative cell viability was calculated according to: $(A_{sample}-A_{blank}/A_{control}-A_{blank}) \times 100$, where A_{sample} corresponds to the absorbance of wells with cells treated with a determined sample, A_{blank} to the absorbance of a well without cells, and $A_{control}$ to the absorbance of a well with untreated cells. IC_{50} values were determined by using GraphPad Prism 5 software.

Cellular Uptake Studies. A549 cells were seeded onto coverslips in a 12-well microplate and incubated overnight in 1 mL of DMEM supplemented with 10% FBS. Then, medium was replaced by solutions of PIC micelles [(i) 100 μL of fluorescently labelled (Alexa Fluor 488) PIC micelles in 900 μL of medium, (ii) 33 μL of DOX-loaded PIC micelles or control free DOX in 967 μL of medium) and incubated at 37 °C for a given time. Afterwards, medium was removed, cells were washed twice with PBS and fresh medium (1 mL) was added before cells were again incubated for a given time. Half an hour before observation, cellular nuclei were stained by adding 10 μL of 2 μM Hoechst 33258 solution to each well, and acidic organelles by adding 10 μL of 5 μM LysoTracker Red (for PIC micelles labeled with Alexa Fluor 488) or 10 μL of 100 μM LysoTracker Green (for free DOX and DOX-loaded PIC micelles). Immediately before observation, medium was removed and cells were washed twice with PBS before fresh medium was added. Intracellular distributions were observed by fluorescence microscopy using an Olympus BX-51 microscope equipped with an Olympus DP-71 camera.

The following parameters of the fluorescent channels were used. Blue channel: ultraviolet excitation U-MWU2: excitation filter 360-370 nm, emission filter 420 nm and dichromatic mirror 400 nm. Green channel: blue excitation U-MWB2: excitation filter 460-490 nm, emission filter 520 nm and dichromatic mirror 500 nm. Red channel: green excitation UMNG2: excitation filter 530-550 nm, emission filter 590 nm and dichromatic mirror 570 nm. Images were processed with Adobe Photoshop software.

Results and discussion

Structural Elements Determining the Stability of PIC Micelles

To address the proposed study, we initially focused on PIC micelles prepared from poly-*L*-lysine [degree of polymerization (DP) 108, PLL_{108}] and a series of anionic PEG-dendritic copolymers of the GATG (gallic acid-triethylene glycol) family,[15] where various structural modifications were adopted: MW of the PEG (PEG_{2000}, PEG_{5000}, PEG_{10000}), G of the GATG dendritic block (G2, G3 and G4; incorporating 9, 27 and 81 terminal groups, respectively), and the nature of the peripheral anionic group (sulfate and carboxylate) (Figure 1 and 2). The synthesis of the PEG-GATG block copolymers is depicted in Figure 2 and described in detail in the Electronic Supplementary Information (ESI). In brief, three G of PEG-GATG block copolymers carrying terminal azides (PEG-[Gn]-N_3; n = 2, 3, 4) and PEG chains of different lengths[28,37] were peripherally decorated at the dendritic block with alkynated anionic residues **1** and **2** *via* triazol linkers generated by Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC).[20,38]

Preliminary experiments on the formation of PIC micelles were performed in 10 mM PB, pH 7.4 with PLL_{108} and PEG_{5000}-[G3]-CO_2^-,[20] a dendritic block copolymer displaying 27 carboxylate groups (Figure 2). Dynamic light scattering (DLS) measurements confirmed the presence of discrete micelles of *ca.* 20 nm with low polydispersity index (PDI 0.11, Figure 3E). To our surprise, however, these micelles resulted unstable after the addition of 150 mM NaCl as evidenced by the appearance of species around 8 nm by DLS after 1 h of treatment, attributed to free block copolymer in solution (Figure 3F). Attempts to obtain more stable PIC micelles by reversing the cation/anion system revealed unfeasible. Mixing a cationic block copolymer PEG_{5000}-[G3]-NH_3^+ (Figure 2)[29] with a negatively charged polyaminoacid, poly-*L*-glutamic acid (PGA_{136}), rendered micelles of even lower stability towards ionic strength (Figure S18). This result led us to hypothesize that the triazol in PEG_{5000}-[G3]-CO_2^- was acting as a non-innocent linker,[39,40] increasing the stability of the micelles by hydrophobic interactions.[41,42] Accordingly, we decided to also address in our study the role of the hydrophobicity of the linkers in the stability of the micelles. A new aliphatic linker was introduced in PEG_{5000}-[G3]-A-CO_2^- by reaction of PEG_{5000}-[G3]-NH_3^+ with glutaric anhydride. In addition, PEG_{5000}-[G3]-PhCO_2^-

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and PEG_{5000}-[G3]-$PhSO_3^-$, incorporating an extended hydrophobic phenyl-triazol linker, were prepared by CuAAC from **3** and **4** (Figure 2). All block copolymers in Figure 2 were isolated in very good to excellent yields by ultrafiltration, and characterized by ^1H and ^{13}C NMR, IR and MALDI-TOF MS (ESI).

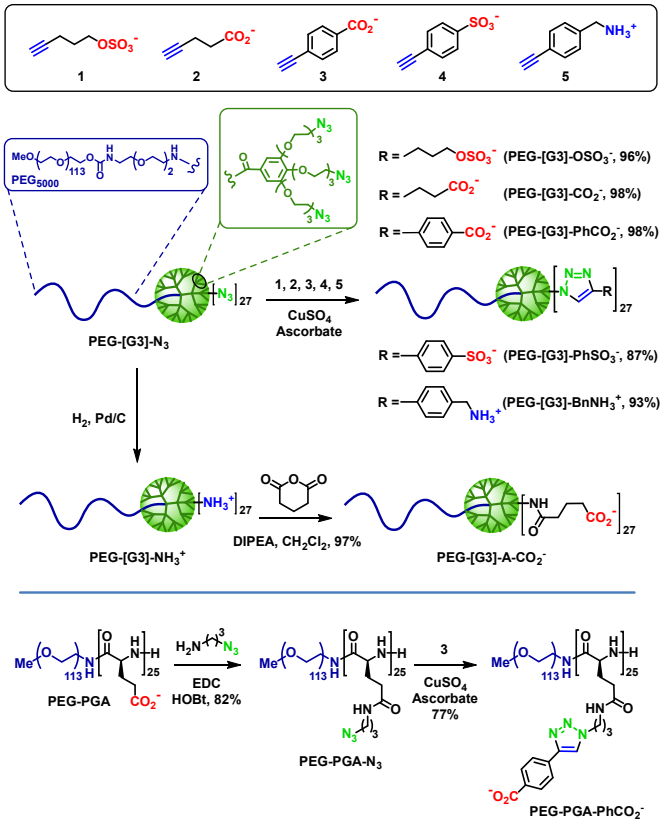

Figure 2. Synthesis of PEG-dendritic (GATG) and PEG-linear block copolymers used in the preparation of PIC micelles (representative structures, see the ESI).

With this sizable collection of anionic PEG-dendritic copolymers in hand, the preparation of PIC micelles was assessed with PLL_{108} (Figure 3, Table 1). The resulting micelles with size in the range 20-30 nm were aged at rt for 3 h and their stability towards ionic strength was analyzed by DLS (25 °C). Increasing concentrations of NaCl (from 150 mM to 1.05 M) were added to the micelles and DLS measurements repeated after 1 h. Finally, those micelles stable at 150 mM NaCl were selected and their integrity tested in simulated physiological conditions (10 mM PB, pH 7.4, 150 mM NaCl, 37 °C) for 12 h (Figures 3, S3-S12). PIC micelles were considered stable in the absence of changes in DLS histograms towards free polymeric components (centered below 10 nm) or aggregation through time. A summary of this stability study is shown in Tables 1, S1 and S2.

The analysis of the data throws several conclusions. First, there is a minimum chain length for the hydrophilic PEG block necessary to stabilize the PIC micelles (for PLL see the ESI). Their formation fails due to precipitation when PEG_{2000} is incorporated to the anionic block copolymer, while PEG_{5000} and PEG_{10000} render micelles of similar stability (Figures S7, S9). Second, variations at the dendritic block reveal G3 as the optimal generation, with G2 (not enough multivalency) and G4 (reduced solubility) copolymers affording less stable micelles (Figures S10, S11). Third, regarding the terminal anionic group, sulfate and sulfonate render more stable micelles than carboxylate groups (Figure 3 and ESI) in agreement with the reduced net charge of the latter at pH 7.4. Fourth, increasing the hydrophobicity of the linker (phenyl-triazol) results in micelles with higher stability due to additional hydrophobic contributions (up to 600 mM NaCl for PEG-[G3]-$PhCO_2^-$/PLL_{108} micelles and higher than 1.05 M for PEG_5-[G3]-$PhSO_3^-$/PLL_{108}; Table 1, Figure 3 and ESI).

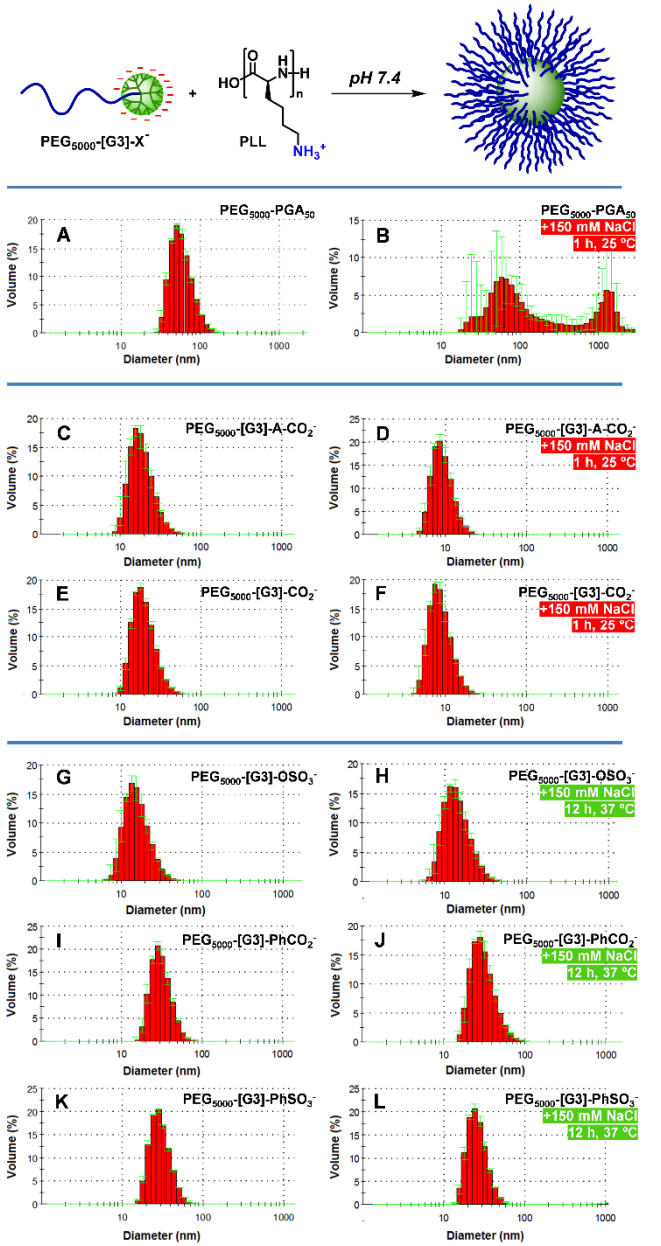

Figure 3. Formation and stability of PIC micelles with PLL_{108}. DLS histograms of micelles as prepared (10 mM PB, pH 7.4, 25 °C) (A, C, E, G, I, K), 1 h after the addition of 150 mM NaCl, 25 °C (B, D, F), and 12 h after the addition of 150 mM NaCl and heating at 37 °C (H, J, L). Hydrodynamic diameters and PDI of micelles in Table S2.

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Table 1. Stability of PIC micelles upon formation and towards increasing concentrations of NaCl and heating at 37 °C (+: stable, -: not stable). Hydrodynamic diameters and PDI of micelles in Table S2.

PIC with PLL_{108}	Upon formation PB 7.4 25 °C	PB 7.4 25 °C 150 mM NaCl	PB 7.4 25 °C 300 mM NaCl	PB 7.4 25 °C 450 mM NaCl	PB 7.4 25 °C 600 mM NaCl	PB 7.4 25 °C 750 mM NaCl	PB 7.4 25 °C 900 mM NaCl	PB 7.4 25 °C > 1 M NaCl	PB 7.4 37 °C 150 mM NaCl, 12 h
PEG_{5000}-[G3]-A-CO_2^-	+	–							–
PEG_{5000}-[G3]-CO_2^-	+	–							–
PEG_{5000}-[G3]-OSO_3^-	+	+	–						+
PEG_{5000}-[G3]-$PhCO_2^-$	+	+	+	+	+	–			+
PEG_{2000}-[G3]-$PhCO_2^-$	–								–
PEG_{10000}-[G3]-$PhCO_2^-$	+	+	+	+	+	–			+
PEG_{2000}-[G2]-$PhCO_2^-$	+	–							–
PEG_{2000}-[G4]-$PhCO_2^-$	+	+	–						–
PEG_{5000}-[G3]-$PhSO_3^-$	+	+	+	+	+	+	+	+ (1.05 M)	+
PEG_{5000}-PGA_{25}	–								–
PEG_{5000}-PGA_{25}-$PhCO_2^-$	+	–							–
PEG_{5000}-[G3]-$PhCO_2^-$ with PEG_{5000}-[G3]-$BnNH_3^+$	+	+	+	+	+	+	+	+ (3 M)	+

We next turned our attention to the influence of the architecture of the charged block: linear *vs* dendritic. To this end, PIC micelles were prepared from PLL_{108} and carboxylated linear copolymers comprising a PEG_{5000} and PGA (DP 25, 50, 100) blocks (Figures 2-3, Table 1). DLS measurements revealed that only PEG_{5000}-PGA_{50} led to discrete micelles, which in addition showed much lower stability with time than any of the dendritic systems (few hours *vs* weeks/months at 25 °C) and were immediately destabilized by addition of 150 mM NaCl (Figures 3, S13-S15). Further comparison between linear and dendritic architectures was obtained by appending the best candidate linker (phenyl-triazol) to PEG_{5000}-PGA_{25}, the linear copolymer with similar number of carboxylates as in G3 (Figure 2). Although micelles were obtained from PEG_{5000}-PGA_{25}-$PhCO_2^-$, they were once again completely disrupted in the presence of 150 mM NaCl even at 25 °C (Figure S16).

Overall, these data not only confirm the superior stability of PIC micelles prepared from dendritic copolymers, but also disclose a unique dendritic effect: the leaning to increase micelle stability *via* hydrophobic interactions by incorporation of peripheral hydrophobic linkers. We hypothesize that the tridimensional orientation of peripheral hydrophobic groups on the globular dendritic block maximizes the stabilization when compared to linear copolymers. As a limiting example of this dendritic effect, micelles prepared from PEG_{5000}-[G3]-$PhCO_2^-$ and PEG_{5000}-[G3]-$BnNH_3^+$ (Figure 2), two dendritic copolymers displaying terminal benzoate and benzylamine groups, resulted in exceptional stability even at 3 M NaCl (the upper concentration used in our experiment), which represents to the best of our knowledge the highest saline concentration attained with PIC micelles (Table 1, Figure S17).[6]

Mixed PIC Micelles: Tuning the Stability of PIC Micelles by Combining Copolymers with Different Peripheral Functionality

Based on the results shown in Figure 3 and Table 1, we found particularly interesting the possibility of fine-tuning the properties of PIC micelles by combining PEG-dendritic block copolymers with different peripheral functionalities. To prove this *mixed micelle* concept, we prepared a series of micelles with PLL_{108} and variable proportions of PEG_{5000}-[G3]-CO_2^- and PEG_{5000}-[G3]-$PhCO_2^-$, two copolymers affording PIC micelles at the low and high end of stability towards ionic strength. As hypothesized, the stability of the resulting micelles could be precisely tuned (up to 600 mM NaCl) by adjusting the copolymer ratio in the blends (Table 2, Figure 4). This mixed micelle concept paves the way towards the facile modulation of diverse properties, either biodegradability, drug loading, or the response to external stimuli (pH, reductive media, enzymes, temperature) by simply combining differently functionalized block copolymers.

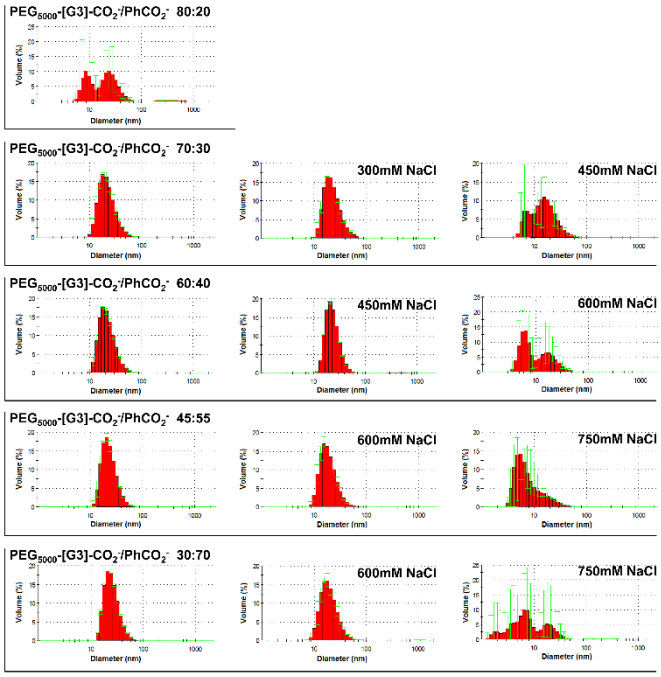

Figure 4. Mixed PIC micelles prepared from PLL_{108} and various proportions of PEG_{5000}-[G3]-CO_2^- and PEG_{5000}-[G3]-$PhCO_2^-$ in 10 mM PB, pH 7.4, 150 mM NaCl, 25 °C. DLS histograms upon formation (left column) and 1 h after the addition of increasing concentrations of NaCl (center and right columns).

Table 2. Stability of mixed PIC micelles towards increasing concentrations of NaCl (+: stable, -: not stable).

PEG-[G3]-CO_2^- /$PhCO_2^-$	150 mM NaCl	300 mM NaCl	450 mM NaCl	600 mM NaCl	750 mM NaCl
100 : 0	-				
80 : 20	-				
70 : 30	+	+	-		
60 : 40	+	+	+	-	
45 : 55	+	+	+	+	-
30 : 70	+	+	+	+	-
0 : 100	+	+	+	+	-

Characterization of Dendritic PIC Micelles and pH-Sensitivity

DDS designed to specifically respond to biological triggers are especially appealing.[43,44] Among them, those with adequate nanometric size to selectively accumulate in solid tumors and release their cargo in response to the slightly acidic pH of tumors (6.6–6.9) and/or at the more acidic cellular compartments endosome (pH ~ 5–6)/lysosome (pH ~ 4–5) have

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great potential for cancer therapy.[45] With the aim of exploring the potential of dendritic PIC micelles in this field, we decided to test their reliability for the controlled delivery of low molecular weight anticancer drugs. To this end, micelles composed of carboxylated copolymers were considered. The characteristic pKa of carboxylic acids around 5.0 promotes the selective disruption of electrolyte complexes in acidic environments by partial protonation. Preliminary studies with PEG_{5000}-[G3]-CO_2^-/PLL_{108} and PEG_{5000}-[G3]-OSO_3^-/PLL_{108} micelles validated this approach. While the carboxylate micelles were immediately disrupted at pH 5.0, the sulfate-derived micelles remained stable for several days at this pH even at 37 °C (Figure S20), confirming the pH-sensitivity granted by the carboxylate groups. Based on the higher stability towards ionic strength of the carboxylate micelle derived from PEG_{5000}-[G3]-$PhCO_2^-$, we selected it as prototype for the development of a pH-sensitive DDS and proceeded to evaluate its properties in detail.

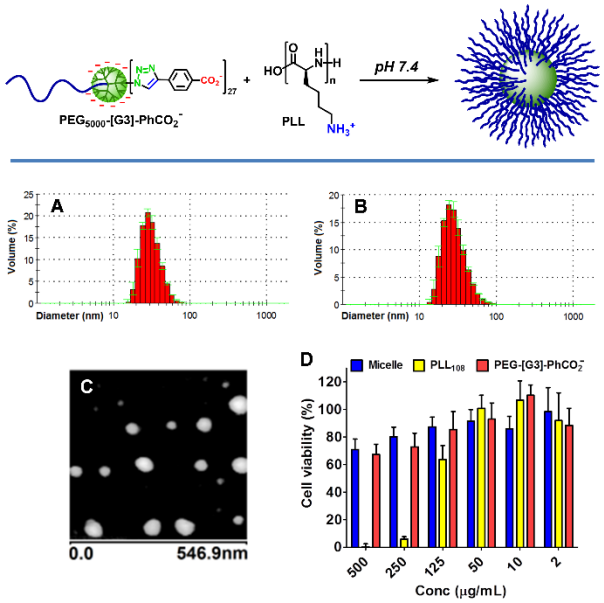

Figure 5. Characterization of PEG_{5000}-[G3]-$PhCO_2^-$/PLL_{108} micelles. DLS histograms of micelles as prepared (10 mM PB, pH 7.4, 25 °C) (A), and after the addition of 150 mM NaCl and heating for 72 h at 37 °C (B). AFM image of micelles (C). Cell viability (MTT, 24 h) of A549 cells in the presence of micelles (shown concentrations refer to micelles; PEG_{5000}-[G3]-$PhCO_2^-$ and PLL_{108} were used as controls at the same concentrations as in micelle) (D).

Figures 5A-B illustrate the extraordinary stability of this system as shown by indistinguishable DLS histograms of the micelles as prepared (32 nm, PDI 0.07) and after the addition of 150 mM NaCl and heating for 72 h at 37 °C (29 nm, PDI 0.18). These micelles were stable for months at 4 °C and could be lyophilized and resuspended without significant variation in size, a relevant property for an easy storage and carriage. The z-potential close to zero (0.15 mV) agrees with the high colloidal stability of this micelle, the PEG corona and charge stoichiometry of the constituents. The efficiency of the micelle formation was determined as *ca.* 94% by quantifying the amount of free PLL in solution after ultracentrifugation. Visualization of the micelles was possible by AFM and TEM, which revealed a spherical shape and mean diameters of 30±8 and 24±4 nm respectively, in complete concordance with DLS measurements (Figures 5C and 6D). Similar stability profile and properties were now obtained when reversing the ammonium/carboxylate system in a micelle prepared from PEG_{5000}-[G3]-$BnNH_3^+$ (Figure 2) and PGA_{136} (31 nm by DLS, 27±5 nm by AFM, z-potential -1.42 mV). As expected, this micelle excels that from PEG_{5000}-[G3]-NH_3^+ and PGA_{136} (*vide supra*) in stability and performance (compare Figures S18 and S19), illustrating once more the great stability of PIC micelles incorporating charged PEG-dendritic copolymers peripherally functionalized *via* hydrophobic linkers.

The pH-sensitivity of PEG_{5000}-[G3]-$PhCO_2^-$/PLL_{108} micelles was confirmed by DLS by appearance of large aggregates (with lower diffusion coefficient) when submitted to acetate buffer pH 5.0 (Figures 6A-C). pH-responsiveness of these micelles was also confirmed by TEM and ^{1}H NMR (Figure 6). For this latter experiment, a solution of micelles was divided into aliquots and pH adjusted to 7.0 and 5.1. After 24 h of incubation at 37 °C, ^{1}H NMR spectra were recorded. Comparison of normalized spectra of the micelles at pH 7.0 with those of PEG_{5000}-[G3]-$PhCO_2^-$ and PLL_{108} revealed at a glance a huge decrease in the intensity of the resonances due to PLL and the GATG block in the micelle (segregated compact core of polyions with reduced ^{1}H T_2 relaxation times).[46] Conversely, when recorded at pH 5.1, the spectrum of the micelles showed a sharp increase in the ^{1}H signals of PLL, consistent with destabilization of the micelles, accompanied by an almost disappearance of the resonances associated to the GATG block because of reduced solvation after protonation.

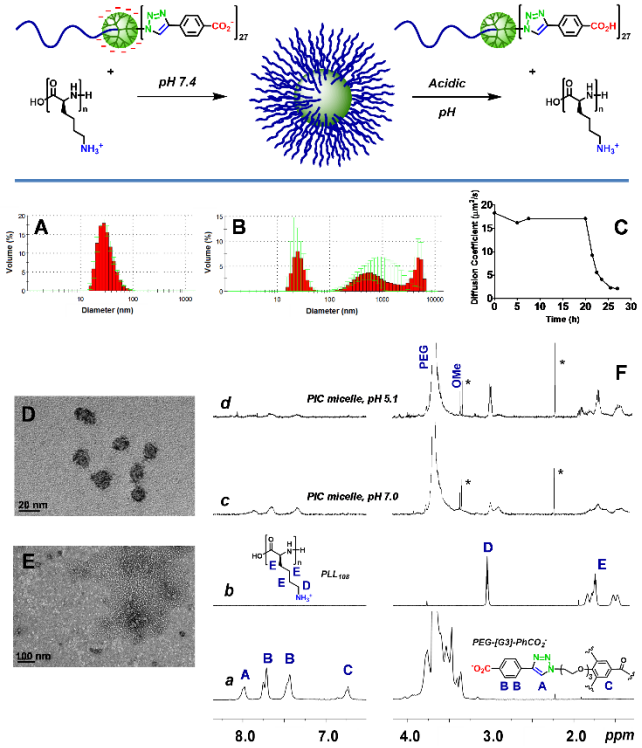

Figure 6. pH-sensitivity of PEG_{5000}-[G3]-$PhCO_2^-$/PLL_{108} micelles. DLS histograms of PIC micelles after 27 h at 37 °C in: 10 mM PB pH 7.4, 150 mM NaCl (A) and 50 mM acetate buffer pH 5.0, 150 mM NaCl (B). Time evolution of the diffusion coefficient of micelles at pH 5.0 (C). TEM images of PIC micelles as prepared in 10 mM PB pH 7.4, 150 mM NaCl (D) and after dialysis against 50 mM acetate buffer pH 5.0, 150 mM NaCl, 24 h, 37 °C (E). ^{1}H NMR spectra (D_2O, 750 MHz, 25 °C) of PEG_{5000}-[G3]-$PhCO_2^-$ (*a*), PLL_{108} (*b*), PEG_{5000}-[G3]-$PhCO_2^-$/PLL_{108} micelles in 10 mM PB, pH 7.0 (80%)/D_2O (20%) (*c*) and after acidification to pH 5.1 (*d*) (*c*, *d* with WATERGATE) (F).

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Validation of Dendritic PIC Micelles for the Intracellular Delivery of Doxorubicin

Having identified PEG_{5000}-[G3]-$PhCO_2^-$/PLL_{108} micelle as the best combination of pH-sensitivity and stability, we decided to validate this formulation for the delivery of the anticancer drug doxorubicin (DOX, Figure 7). DOX is a widely-used chemotherapeutic for the treatment of cancer (bladder, breast, lung, stomach, ovarian), lymphomas and certain leukemias, which unfortunately produces severe side-effects, including cardiac toxicity and myelosuppression.[47] Although improved pharmacokinetic profile and reduced toxicity have been achieved by encapsulation within liposomes (Myocet, Doxil/Caelyx), clinical data do not suggest enhanced antitumor efficacy compared to free DOX,[48] prompting the search of more efficient DDS.

With this aim, cellular uptake studies were first performed with blank PIC micelles fluorescently labeled with the green dye Alexa Fluor 488 (AF488) covalently attached at the distal end of the PEG block (see the ESI). Figure 7E shows fluorescence images of A549 cells treated with these micelles (green) that after cell uptake completely colocalize with LysoTracker Red (red), a well-known endosome/lysosome marker. The final fate of the micelles in organelles with such a distinctive acidic environment encouraged the encapsulation of DOX for a subsequent pH-triggered release following internalization by endocytosis. Before that, the toxicity of the micelles and their components was investigated in the same cell line by a MTT assay. The limited effect of PEG_{5000}-[G3]-$PhCO_2^-$ and micelles on cell proliferation at the highest concentrations analyzed contrasts with the well-known toxicity displayed by PLL (Figures 5D and S21), proving the integrity of the micelles in culture medium supplemented with fetal bovine serum (FBS).

The encapsulation of DOX within the micelles was easily accomplished, favoured by DOX inherent hydrophobicity (DLS histogram in Figure 7B). An encapsulation efficiency of 53% and drug loading of 15% were determined, which compare well with other reported DOX-loaded polymeric nanosystems.[49,50] The selective *in vitro* release profile of DOX at acidic pH was analyzed by fluorescence. As shown in Figure 7C, almost 60% of the drug was released within 48 h at pH 5.0, while a moderate 22% release was observed at pH 7.4. To demonstrate the efficient cellular uptake and subsequent release of DOX by the action of the acidic endosomal pH, DOX-loaded micelles were incubated with A549 cells and the intracellular trafficking of DOX was monitored by fluorescence microscopy (Figure 7E). Free DOX and a crosslinked, non pH-sensitive DOX-loaded micelle were used as controls. After 1 h of incubation, free DOX (red) was exclusively detected in cell nuclei as determined by complete colocalization with Hoechst (blue). Because of its hydrophobic character, DOX is able to internalize cell membranes by diffusion and quickly migrate to the nucleus. Contrarily, DOX in DOX-loaded micelles colocalized exclusively with LysoTracker Green (green) at the same time. It was only after 2 h of additional incubation that in response to the acidic environment of endosome/lysosome, DOX mostly released from the micelles and after organelle escape, migrated to the cell nuclei (red and blue colocalization). Gratifyingly, when non pH-sensitive control micelles were incubated in a parallel experiment, no endosomal/lysosomal escape of DOX was observed (red and green colocalization, Figure S22). Cell viability assays (MTT, 24 h) in A549 cells performed with DOX-loaded micelles and free DOX as control disclosed a slightly higher toxicity for the micelles (IC_{50}: 0.79 *vs* 8.26 μM), probably associated to a more efficient internalization by endocytosis (Figure 7D).[51,52] Finally, when stability studies of the DOX-loaded micelles were performed in FBS, less than 5% of the encapsulated drug was released after 24 h of incubation at 37 °C. These interesting properties connected with the expected longer circulation time and improved biodistribution of the micelles compared to the free drug suggest an attractive future for these dendritic PIC micelles in DD.

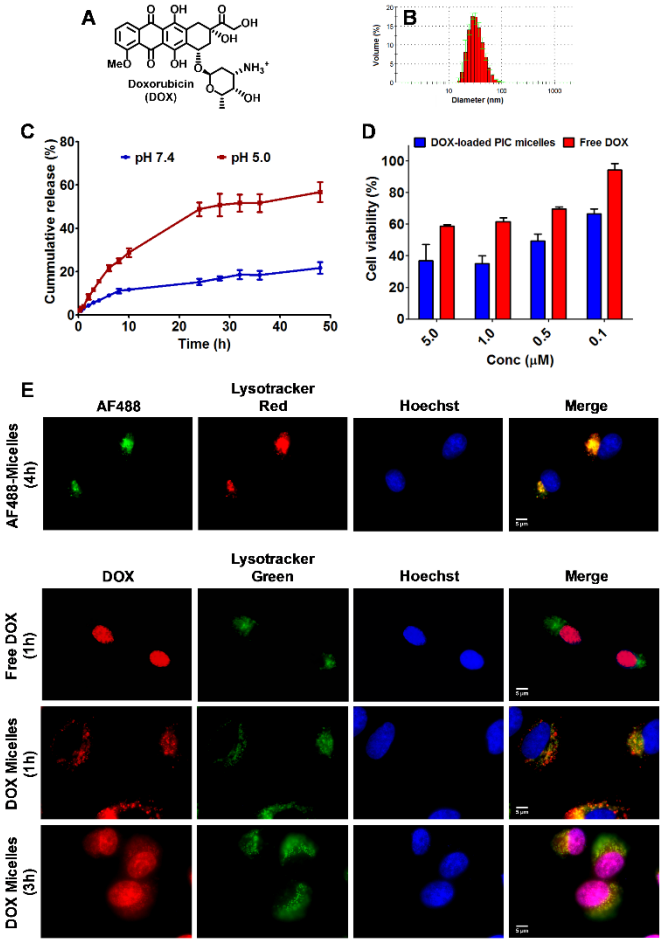

Figure 7. Structure of DOX (A). DLS histogram of DOX-loaded PEG_{5000}-[G3]-$PhCO_2^-$/PLL_{108} micelles (10 mM PBS, pH 7.4, 25 °C) (B). Release profiles of DOX at pH 7.4 and pH 5.0 (C). Cell viability of A549 cells (MTT, 24 h) in the presence of DOX-loaded micelles and free DOX (D). Fluorescence images of A549 cells incubated with AF488-labeled (blank) micelles for 4 h, free DOX (1 h) and DOX-loaded micelles (1 and 3 h) (E).

Conclusions

A thoroughly analysis of the structural elements determining the stability of PIC micelles confirms the superior performance of linear-dendritic copolymers compared to classical linear

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polymers. Even more fascinating, dendritic PIC micelles outclass by unprecedented stability towards ionic strength (concentrations higher than 3 M NaCl) *via* synergistic hydrophobic interactions, an effect presumably associated to a favoured tridimensional orientation of peripheral hydrophobic linkers around the globular/rigid dendritic architecture. The combination of differently functionalized PEG-dendritic block copolymers allows to fine-tune the stability of these micelles, paving the way towards the facile modulation of alternative properties like biodegradability, drug loading, or the response to external stimuli. With this information gained, we succeed in preparing an optimized pH-sensitive PIC micelle for the controlled intracellular release of the anticancer drug DOX, which displays higher toxicity and distinctive mechanisms of cell uptake and intracellular trafficking relative to free DOX. The enhanced stability of these micelles, connected with their expected long circulation time and improved biodistribution, encourage us to further explore their applications in DD and diagnosis.

Acknowledgements

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