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Polybenzofulvene derivatives bearing dynamic binding sites as potential anticancer drug delivery systems†

Andrea Cappelli,*[a] Giorgio Grisci,[a] Marco Paolino,[a] Vincenzo Razzano,[a] Germano Giuliani,[a] Alessandro Donati,[a] Claudia Bonechi,[a] Raniero Mendichi,[b] Antonella Caterina Boccia,[b] Mariano Licciardi,[c] Cinzia Scialabba,[c] Gaetano Giammona[c] and Salvatore Vomero[a]

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In order to obtain new advanced functional materials capable of recognizing drug molecules, the polybenzofulvene backbone of molecular brush poly-6-MOEG-9-TM-**BF3k** has been functionalized with a "synthetic dynamic receptor" composed of two 1-adamantylurea moieties linked together by means of a dipropyleneamino bridge as in Meijer's bis(adamantylurea) pincer (**BAUP**). This functional material, bearing synthetic receptors potentially capable of recognizing/loading and then delivering drug molecules, was used to prepare colloidal drug delivery systems (by means of soft interaction with **BAUP**) for delivering the model anti-cancer drug doxorubicin (**DOXO**). The resulting nanostructured drug delivery systems containing the physically loaded drug were characterized in terms of drug loading and release, dimensions and zeta potential, and *in vitro* cell activity and uptake on two different cell lines (*i.e.* the human bronchial epithelial 16HBE and the human colon cancer HCT116). On normal cells, free **DOXO** was found to be more cytotoxic than **DOXO**-loaded nanogels at the higher tested concentration and, only on cancer cells, **DOXO**-loaded nanogels show similar or slightly higher cytotoxicity values than free **DOXO**, suggesting potential advantages in the treatment of cancer. These results were supported by fluorescence microscopy studies, which suggested that **DOXO**-loaded nanogels provide an extracellular reservoir of the drug, which is gradually released and internalized within the cells.

Introduction

During the past 40 years, research in the pharmaceutical field has focused on the development of formulations able to release drugs into an organism at a controlled quantity and speed, since there is a direct relationship between a drug's concentration in the blood and its therapeutic action. For this purpose, non-conventional dosage forms have been developed and implemented with a modified release (Drug Delivery Systems, DDS). In such systems the release of the active ingredient and the relative blood level trends depend on the technological features of the formulations, and not on the drug's chemical and physical characteristics as it occurs in conventional pharmaceutical forms.[1,2]

The most common modified release systems are based on polymeric supports where the drug can be embedded into the polymeric matrix (drug–polymer complexes)[3–5] or covalently bonded to the polymeric backbone (drug–polymer conjugates).[6]

Dendrimers are uniform, nanometer-sized, three-dimensional synthetic polymers, characterized by a branched architecture and spherical shape.[7,8] Owing to their peculiar features (high level of branching, multivalency, clearly defined molecular weight, globular structure), they are widely used as drug carriers of both pharmaceuticals and nucleic acids for gene therapy.[9] Polypropylene imine (PPI) dendrimers are polyalkyl amines which present a core of tertiary amines and primary amines as surface groups. These terminal amino residues can be conveniently functionalized with various chemical groups.[10]

Meijer's research group at Eindhoven Technical University synthesized adamantylurea-functionalized PPI dendrimers by making PPI terminal amino groups react with 1-adamantyl isocyanate. The adamantylurea portion proved to be able to bind ureidoacetic acids through multiple non-covalent interactions.[11] In order to evaluate the possibility of host–guest interactions between these surface-modified PPI dendrimers

[a]*Dipartimento di Biotecnologie, Chimica e Farmacia and European Research Centre for Drug Discovery and Development, Università degli Studi di Siena, Via A. Moro, 53100 Siena, Italy. E-mail: andrea.cappelli@unisi.it; Fax: +39-0577-234333; Tel: +39-0577-234320*

[b]*Istituto per lo Studio delle Macromolecole (CNR), Via E. Bassini 15, 20133 Milano, Italy*

[c]*Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università degli Studi di Palermo, Via Archirafi 32, 90123 Palermo, Italy*

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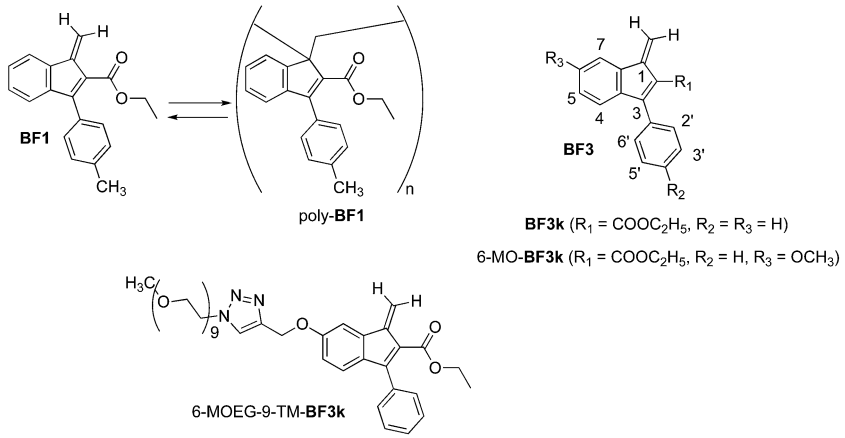

Scheme 1 Structures of some key benzofulvene derivatives.

(host) with small molecules (guest), Meijer and coworkers designed specific ureidoacetic acids capable of establishing simultaneous interactions with the tertiary amine (charge-assisted H-bond) and ureido groups (H-bonds) of these macromolecules. In this way they developed an interesting supramolecular system based on a pincer-shaped "dynamic synthetic receptor" able to incorporate ureidoacetic acids through the establishment of specific and reversible interactions (ionic interactions, hydrogen bonds and van der Waals interactions).[11–14] Subsequently, such a supramolecular system was implemented with bioactive arylpiperazine ureidoacetic acid guests to obtain a prototype of a multivalent supramolecular drug.[15]

The ten-year work performed in our laboratories demonstrates that a wide range of benzofulvene derivatives (Scheme 1) polymerize in the apparent absence of catalyst or initiators by simple removal of solvents to give polymers showing vinyl structures stabilized by aromatic stacking interactions.[16–26]

The corresponding polybenzofulvene derivatives show intriguing features such as rapid and almost quantitative formation without side reactions and byproducts, high molecular weight, thermoreversible polymerization/depolymerization behavior, high solubility in the most common organic solvents, tunable solubility and aggregation behavior in water, liability to generate nanostructured aggregates, and susceptibility to molecular manipulation.[16–26]

Furthermore, we have recently reported that click chemistry reactions such as the copper(I)-catalyzed alkyne–azide 1,3-dipolar cycloaddition (CuAAC) can be used to introduce suitably designed side chains into the polybenzofulvene macromolecular system to obtain polybenzofulvene derivatives tailored for specific applications.[25]

The work described in the present paper was aimed at developing new advanced functional materials by functionalization of preformed polybenzofulvene derivatives ("grafting onto" approach) with synthetic receptors capable of recognizing and then delivering drug molecules. For this aim, doxorubicin (**DOXO**) was used as a model anticancer drug to obtain nanostructured drug delivery systems containing the drug physically loaded by means of interactions with the bis(adamantylurea) pincer (**BAUP**, synthetic dynamic receptor) anchored to the polybenzofulvene backbone.

Experimental section

Synthesis

Melting points were determined in open capillaries in a Gallenkamp apparatus and are uncorrected. Merck silica gel 60 (230–400 mesh) was used for column chromatography. Merck TLC plates, silica gel 60 F_{254}, were used for TLC. NMR spectra were recorded with a Varian Mercury-300, a Bruker DRX-400 AVANCE, or a Bruker DRX-600 AVANCE spectrometer in the indicated solvents (TMS as internal standard): the values of the chemical shifts are expressed in ppm and the coupling constants (J) in Hz. An Agilent 1100 LC/MSD operating with an electrospray source was used in mass spectrometry experiments. The starting polybenzofulvene derivative poly 6-PO-**BF3k** (ref. 25) and the azide **MOEG-9-N_3** (ref. 23) were synthesized as previously reported.

N′-Trityl-*N*‴-[3-(tritylamino)propyl]propane-1,3-diamine (3)

A mixture of bis(3-aminopropyl)amine (**2**, Sigma Aldrich, 1.1 mL, 7.9 mmol) in CH_2Cl_2 (20 mL) containing TEA (3.2 mL, 23 mmol) and trityl chloride (2.2 g, 7.9 mmol) was stirred at room temperature for 1.5 h and then washed with water. The organic layer was dried over sodium sulfate and evaporated under reduced pressure. Purification of the residue by flash chromatography with ethyl acetate as the eluent gave compound **3** (2.3 g, yield 94%, mp 80–82 °C) as a white crystalline solid. ^{1}H NMR (400 MHz, $CDCl_3$): 1.64 (m, 4H), 2.18 (t, J = 6.7, 4H), 2.65 (t, J = 7.0, 4H), 7.16 (m, 6H), 7.25 (m, 12H), 7.46 (m, 12H). MS (ESI): m/z 616 ($M + H^+$).

3-[Bis[3-(tritylamino)propyl]amino]propan-1-ol (4)

A mixture of compound **3** (2.3 g, 3.7 mmol) in CH_3CN (30 mL) was heated under reflux until complete dissolution and then Na_2CO_3 (0.80 g, 7.55 mmol) and 3-bromopropan-1-ol (0.83 mL, 9.2 mmol) were added. The reaction mixture was heated under

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reflux for 4 h and then concentrated under reduced pressure. The residue was partitioned between CH_2Cl_2 and H_2O and the organic layer was dried over sodium sulfate and evaporated under reduced pressure. Purification of the residue by flash chromatography with ethyl acetate as the eluent gave compound **4** (1.5 g, yield 60%) as a waxy solid. ^{1}H NMR (400 MHz, $CDCl_3$): 1.63 (m, 6H), 2.12 (t, $J = 6.9$, 4H), 2.44 (t, $J = 7.7$, 4H), 2.60 (t, $J = 5.5$, 2H), 3.72 (t, $J = 5.2$, 2H), 7.17 (m, 6H), 7.26 (m, 12H), 7.46 (m, 12H). ^{13}C NMR (150 MHz, $CDCl_3$): 28.5, 28.7, 42.4, 52.8, 55.2, 64.8, 71.4, 126.7, 128.4, 129.2, 146.6. MS (ESI): m/z 674 ($M + H^+$).

N′-(3-Azidopropyl)-*N*‴-trityl-*N*′-[3-(tritylamino)propyl] propane-1,3-diamine (5)

To a solution of compound **4** (0.58 g, 0.86 mmol) in CH_2Cl_2 (20 mL), TEA (0.40 mL, 2.9 mmol) and CH_3SO_2Cl (0.20 mL, 2.6 mmol) were added in sequence at room temperature. The resulting mixture was stirred at room temperature for 30 min and then washed with water. The organic layer was dried over sodium sulfate and evaporated under reduced pressure. The residue was dissolved in DMF (15 mL) and NaI (0.50 g, 3.3 mmol) and NaN_3 (0.40 g, 6.15 mmol) were added to the solution. The reaction mixture was stirred at room temperature for 48 h and then concentrated under reduced pressure. The residue was partitioned between CH_2Cl_2 and a saturated NH_4Cl solution. The organic layer was dried over sodium sulfate and evaporated under reduced pressure. Purification of the residue by flash chromatography with ethyl acetate–petroleum ether (3 : 7) as the eluent gave compound **5** (0.39 g, yield 65%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): 1.56 (m, 6H), 2.11 (t, $J = 6.8$, 4H), 2.38 (m, 6H), 3.17 (t, $J = 6.5$, 2H), 7.15 (t, $J = 7.3$, 6H), 7.24 (t, $J = 7.6$, 12H), 7.46 (d, $J = 7.8$, 12H). ^{13}C NMR (150 MHz, $CDCl_3$): 27.2, 28.7, 42.7, 50.1, 51.4, 52.9, 71.4, 126.6, 128.6, 129.4, 146.8. MS (ESI): m/z 699 ($M + H^+$).

1,1′-[3,3′-(3-Azidopropylazanediyl)bis(propane-3,1-diyl)]bis(3-adamantylurea) (1)

A mixture of compound **5** (0.30 g, 0.43 mmol) in TFA (4.0 mL) was stirred at room temperature for 2 h. Then, TFA was removed under reduced pressure and the residue was diluted with $CHCl_3$ and extracted with water. The aqueous phase was concentrated under reduced pressure and the residue was dissolved in THF (8 mL). To this solution, 1-adamantyl isocyanate (Sigma Aldrich, 0.245 g, 1.38 mmol) and K_2CO_3 (0.195 g, 1.41 mmol) were added and the resulting mixture was stirred at room temperature for 24 h. Finally, the reaction mixture was concentrated under reduced pressure, diluted with water and extracted with $CHCl_3$. The organic layer was dried over sodium sulfate and evaporated under reduced pressure. Purification of the residue by flash chromatography with ethyl acetate–methanol (8 : 2) as the eluent gave the expected bis(adamantylurea) synthon **1** (0.19 g, yield 78%, mp 174–176 °C) as a white crystalline solid. ^{1}H NMR (600 MHz, $CDCl_3$): 1.65 (s, 16H), 1.73 (m, 2H), 1.96 (s, 12H), 2.04 (s, 6H), 2.48 (m, 6H), 3.17 (t, $J = 6.7$, 4H), 3.33 (t, $J = 6.7$, 2H), 4.67 (br s, 2H), 5.44 (br s, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): 25.8, 27.2, 29.6, 36.5, 38.5, 42.6, 49.6, 50.7, 50.8, 51.7, 157.9. MS (ESI): m/z 569 ($M + H^+$).

Poly-6-BAUP-TM-BF3k

In a microwave tube, a mixture of poly-6-PO-**BF3k**[25] (73 mg, 0.22 mmol in monomer units) in THF (5.0 mL) containing synthon **1** (250 mg, 0.44 mmol), CuBr (6.0 mg, 0.042 mmol), and DIPEA (0.011 mL, 0.063 mmol) was exposed to microwave irradiation in a CEM Discover instrument for 40 min (4 × 10 min, $T =$ 60 °C, $W = 50$) and finally evaporated under reduced pressure. The residue was dissolved with $CHCl_3$ (20 mL) and washed with water (15 mL) and 33% NH_4OH (5.0 mL). The organic layer was dried over sodium sulfate and concentrated under reduced pressure. The polymer was purified by precipitation with diethyl ether from a solution of the final residue in chloroform and dried under reduced pressure to obtain poly-6-BAUP-TM-**BF3k** as a white solid (0.17 g, yield 86%).

Poly-6-MOEG-9-TM-BF3k-*co*-6-BAUP-TM-BF3k (9 : 1)

In a microwave tube, a mixture of poly-6-PO-**BF3k**[25] (0.13 g, 0.39 mmol in monomer units) in THF (5.0 mL) containing synthon **1** (0.022 g, 0.039 mmol), CuBr (0.011 g, 0.077 mmol), and DIPEA (0.014 mL, 0.080 mmol) was stirred at room temperature for 24 h. Then, 28-azido-2,5,8,11,14,17,20,23,26-nonaoxaoctacosane (**MOEG-9-N$_3$**, see ref. 23, 0.35 g, 0.77 mmol) was added and the resulting mixture was exposed to microwave irradiation in a CEM Discover instrument for 40 min (4 × 10 min, $T = 60$ °C, $W = 50$). Finally, the reaction mixture was concentrated under reduced pressure to give a residue, which was dissolved in $CHCl_3$ and washed with water and 33% NH_4OH. The organic layer was dried over sodium sulfate and concentrated under reduced pressure. The polymer was purified by precipitation with *n*-hexane from a solution of the final residue in chloroform and dried under reduced pressure to obtain poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** as a dark yellow rubbery solid (0.17 g).

SEC-MALS

The MWD characterization of the new polybenzofulvene derivatives was performed by a MALS light scattering photometer connected to a SEC chromatographic system. The SEC-MALS system and the corresponding experimental conditions were identical to those used in our previous studies[16,17] and are not reported in detail here.

Preparation of doxorubicin loaded poly-6-MOEG-9-TM-BF3k-*co*-6-BAUP-TM-BF3k (9 : 1) nanogels

DOXO–HCl (5.0 mg, 0.0086 mmol) was dissolved in 1.0 mL of DMSO and neutralized with 4 equivalents of TEA. The resulting **DOXO** solution was mixed with a polymer dispersion (25 mg in 1.5 mL of DMSO) obtained by using an ultrasonic bath, and the mixture was added drop-wise to 10 mL of double distilled water and sonicated for 10 min. The **DOXO**-poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) dispersion was dialyzed for 24 h against 1.0 L of double distilled water using Spectra Por Dialysis

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floating tubes with molecular weight cut-off (MWCO) of 500 Da. After 24 h, the nanogel dispersion was collected, frozen by immersion in liquid nitrogen and freeze-dried from water in the presence of threalose as a cryoprotector. **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels were obtained with a yield of 90 weight%. Empty nanogels were prepared by using the above reported procedure in the absence of **DOXO**.

Dynamic light scattering (DLS) analysis and ζ potential measurements

The mean diameter, width of distribution (polydispersity index, PDI) and ζ potential of the nanogels were measured at 25 °C using a Zetasizer NanoZS instrument fitted with a 532 nm laser at a fixed scattering angle of 173°. The intensity-average hydrodynamic diameter (size in nm) and polydispersity index (PDI) of **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) and unloaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels (0.2 mg mL^{-1}) were measured in double distilled water. The ζ potential (mV) was calculated from the electrophoretic mobility using the Smoluchowsky relationship and assuming that $Ka \gg 1$ (where K and a are the Debye–Hückel parameter and particle radius, respectively). Each experiment was performed in triplicate.

Scanning electron microscopy (SEM)

Freeze-dried **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels were visualized using a scanning electron microscope ESEM Philips XL30. Samples were dusted on a double sided adhesive tape previously applied on a stainless steel stub. The MNPs-FA were then sputter-coated with gold prior to microscopy examination.

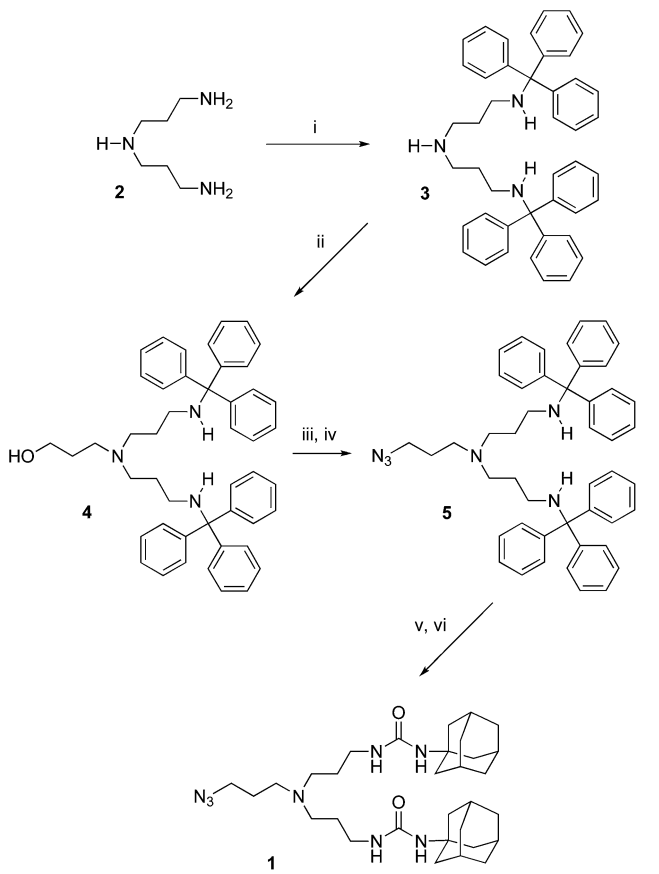
Determination of loaded DOXO amount into nanogels and drug release studies

The amount of **DOXO** loaded into poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels was determined by UV spectroscopy. Samples were prepared by dispersing known amounts of **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels in double distilled water acidified at pH ∼ 4 and measuring the absorbance of **DOXO** at 480 nm. A calibration curve was obtained for serially diluted concentrations of **DOXO**–HCl in bidistilled water at pH ∼ 4. The content of drug loaded into the nanogels was expressed as the amount of loaded **DOXO** per unit mass of dry polymer, and was foundto be 3.5%, while the encapsulation efficiency was calculated to be 70%.

For drug release studies, an appropriate amount (5 mg) of freeze dried **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels and **DOXO**–HCl (0.2 mg) alone (as positive control) were suspended in phosphate buffer PBS at pH 7.4 or 5.5 (5 mL) and transferred inside a floating Spectra/Por dialysis membrane (MWCO 1000 Da). This dialysis membrane was immersed into PBS at pH 7.4 or 5.5 (10 mL) and incubated at 37 °C under continuous stirring (100 rpm) in a Benchtop 808C Incubator Orbital Shaker model 420, for 48 h. At scheduled time intervals, aliquots of the external medium (1 mL) were withdrawn from the outside of the dialysis membrane and replaced with an equal amount of fresh medium. The withdrawn samples were analyzed by HPLC in order to determine the released intact drug amount, using a Waters Breexe System Liquid Chromatograph equipped with a Waters 717 Plus Autosampler (50 μL injection volume), and using a Shimadzu UV-vis HPLC detector connected to a computerized workstation. As column a reversed-phase Gemini C18 Phenomenex (5 μm, 4.6 × 250 mm column with a pre-column H5ODS-10CS) was used. The mobile phase consisted of 10 mM of $(NH_4)HPO_4$ and 5 mL of TEA (pH 4.0 adjusted with orthophosphoric acid) and acetonitrile (68/32 v/v). The eluate was monitored at 485 nm with a flow rate of 1.0 mL min^{-1}. A calibration curve of **DOXO**-HCl was used for drug quantization. Data were corrected taking into account the dilution procedure. Each experiment was carried out in triplicate and the results were in agreement within ±5% standard error.

Cytotoxicity evaluation

The cytotoxicity was assessed by the tetrazolium salt (MTS) assay on both human colon cancer (HCT116) and human bronchial epithelial (16HBE) cell lines (purchased from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia



Scheme 2 Synthesis of bis(adamantylurea) synthon **1**. Reagents: (i) trityl chloride, TEA, CH_2Cl_2; (ii) 3-bromopropan-1-ol, Na_2CO_3, CH_3CN; (iii) methanesulfonyl chloride, TEA, CH_2Cl_2; (iv) NaN_3, NaI, DMF; (v) TFA; (vi) adamantyl isocyanate, K_2CO_3, THF.

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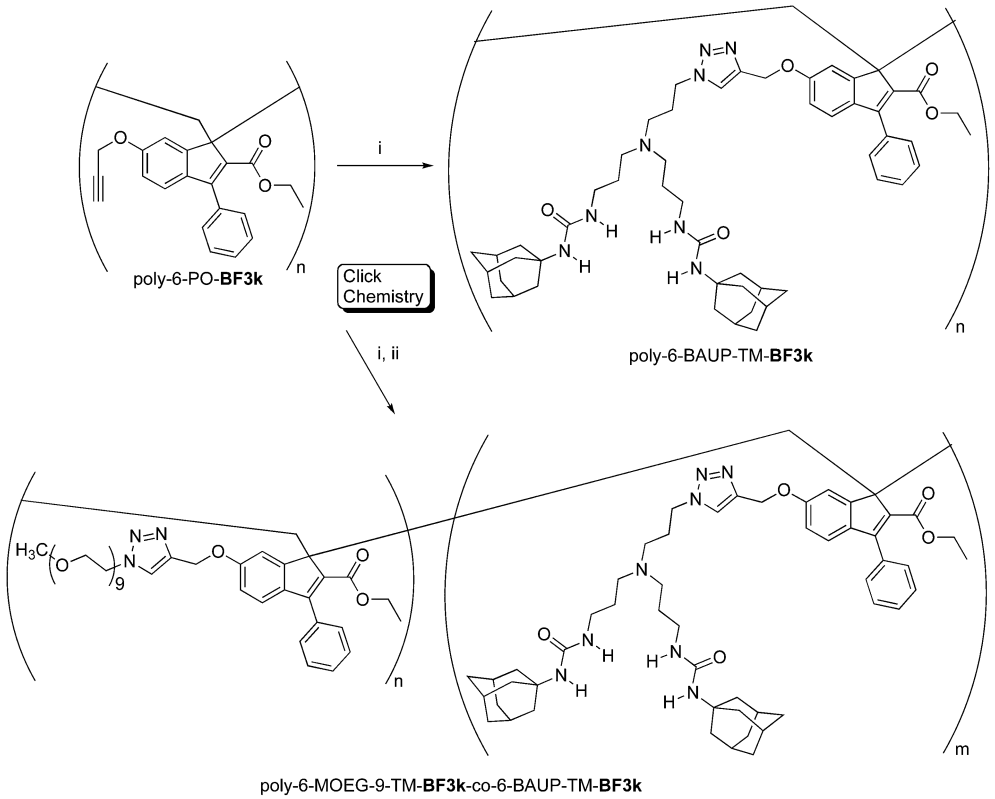

Scheme 3 Click chemistry functionalization of poly-6-PO-**BF3k** with synthon **1**. Reagents: (i) synthon **1**, CuBr, DIPEA, THF; (ii) $CH_3(OCH_2CH_2)_9N_3$, CuBr, DIPEA, THF.

Romagna, Italy) using a commercially available kit (Cell Titer 96 Aqueous One Solution Cell Proliferation assay, Promega). Cells were seeded in 96 well plates at a density of 25×10^4 cells per well and grown in Dulbecco's Minimum Essential Medium (DMEM) with 10% FBS (fetal bovine serum) and 1% of penicillin–streptomycin (10 000 U mL^{-1} penicillin and 10 mg mL^{-1} streptomycin) at 37 °C in 5% CO_2 humidified atmosphere. After 24 h of cell growth the medium was replaced with 200 μL of fresh culture medium containing free **DOXO** or **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels at a drug concentration per well equal to 1, 3, 6, 12 and 30 μM. Empty nanogels were incubated at concentrations corresponding to 10, 50, 100, 200 and 500 μg mL^{-1}. After 4, 24 and 48 h incubation time, DMEM was replaced with 100 μL of fresh medium and 20 μL of a MTS solution was added to each well. Plates were incubated for an additional 2 h at 37 °C. Then, the absorbance at 490 nm was measured using a microplate reader (Multiskan, Thermo, UK). Pure cell medium was used as a negative control. Results were expressed as percentage reduction of the control cells. All culture experiments were performed in triplicate.

Cell drug uptake studies

The cellular uptake of **DOXO** and distribution of the polymer were evaluated by fluorescence microscopy (Zeiss "AXIO Vert. A1" Microscope Inverted) analysis. In particular, for the uptake assay on HCT116 cell lines, 25×10^4 cells per well maintained in normal medium were cultured in a 96 well plate at 37 °C in an atmosphere of 5% CO_2 for 24 h. After 24 h the medium was replaced with 200 μL of fresh culture medium containing **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels and free **DOXO** at a final drug concentration of 30 μM, and cells were incubated for 4, 24 and 48 h. After each incubation period, the medium was removed and the cell monolayer was washed twice with DPBS (Dulbecco's phosphate-buffered saline) and fixed with 4% formaldehyde for 30 min. Subsequently, the formaldehyde solution was removed and the cells were observed by fluorescence microscopy. The images were recorded using an Axio Cam MRm (Zeiss).

Statistical analysis

A one-way analysis of variance (ANOVA) was applied to compare different groups. Data were considered statistically significant with a value of $P < 0.05$ and differences between different groups were compared using *a posteriori* Bonferroni *t*-tests. All values are expressed as the average of three experiments ± standard deviation.

Results and discussion

Synthesis

The synthetic work began with the preparation of azide synthon **1** (Scheme 2) designed starting from the supramolecular system introduced by Meijer and coworkers as a model.[11–14]

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Table 1 Macromolecular features of the new polybenzofulvene derivatives compared with those shown by poly-6-PO-**BF3k** (used in the present work) and by previously reported poly-6-MOEG-9-TM-**BF3k-GO** (obtained by the "grafting onto" approach)

Polymer	M_p (kg mol^{-1})	M_w[a] (kg mol^{-1})	M_w/M_n[b]	R_g[c] (nm)	K[d] (nm)	α[d]
Poly-6-BAUP-TM-**BF3k**	716	1061	3.0	25.4	0.0226	0.48
Poly-6-MOEG-9-TM-**BF3k**-co-6-BAUP-TM-**BF3k** (9 : 1)	283	436	3.6	20.7	0.0596	0.42
Poly-6-MOEG-9-TM-**BF3k-GO**[e]	484	821	8.9	33.6	0.0249	0.50
Poly-6-PO-**BF3k**	1045	874	2.0	33.5	0.0099	0.58

[a] M_w: weight-average molecular weight. [b] Dispersity where M_n denotes the numeric-average molecular weight. [c] R_g: radius of gyration, *i.e.* the dimension of the macromolecules. [d] K and α: intercept and slope of conformation plot. [e] See ref. 25.

Our design was aimed at introducing (by the click chemistry reaction CuAAC) multiple dynamic binding sites (pincer-shaped receptors) on a polybenzofulvene backbone with the goal of creating a polymer host system able to interact with small molecules (guests) through specific and reversible interactions.

Bis(adamantylurea) synthon **1** was prepared from commercially available amine **2**, which was selectively protected with trityl chloride to obtain **3**. Protected amine **3** was alkylated with 3-bromopropan-1-ol in the presence of sodium carbonate as the base to afford alcohol **4**, which was activated with mesyl chloride and reacted with sodium azide in the presence of sodium iodide to give azide **5**. After deprotection with trifluoroacetic acid (TFA), the terminal amino groups of **5** were made to react with adamantyl isocyanate to obtain bis(adamantylurea) derivative **1**.

The bis(adamantylurea) pincer was initially developed and demonstrated to be intriguingly functional on PPI dendrimer surface,[11–14] which is generally recognized to be a particular environment. Moreover, the work performed by Meijer and coworkers suggests a dynamic nature of the dendrimer–guest interaction, in which the proximity of several

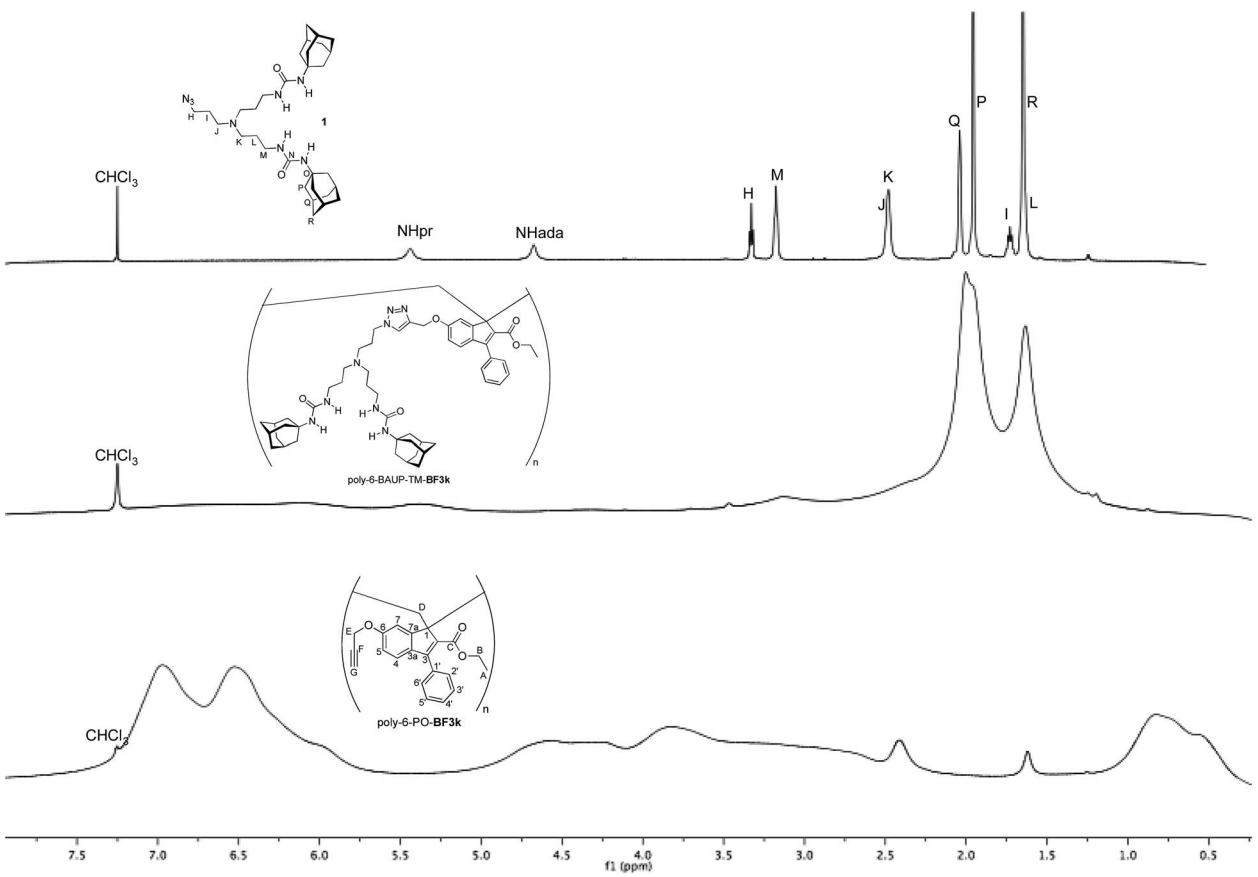

Fig. 1 ^{1}H NMR spectrum ($CDCl_3$) of newly synthesized poly-6-BAUP-TM-**BF3k** compared with those of synthon **1** and of previously reported poly-6-PO-**BF3k**.

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bis(adamantylurea) pincers and the so-called "de Gennes dense packing" may play a significant role.[27] Thus, the evaluation of the effects of the introduction of the bis(adamantylurea) pincer into a different polymer environment required the development of a systematic approach based on the preparation of homopolymers and copolymers, both equipped with synthetic receptors but with different densities.

The functionalization of the polybenzofulvene backbone with **BAUP** was performed by reaction of clickable macromolecular precursor poly-6-PO-**BF3k** with synthon **1** (Scheme 3) in THF in the presence of CuBr and DIPEA to afford the homopolymer poly-6-BAUP-TM-**BF3k**. As previously reported,[25] the optimization of our CuAAC conditions started from the consideration that poly-6-PO-**BF3k** is liable to depolymerize when heated in the presence of solvents. However, in the above-mentioned conditions, the CuAAC reaction proceeded even at room temperature and became virtually complete by microwave exposure without significant depolymerization. The preparation of the designed copolymer poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) was carried out by performing a partial functionalization with synthon **1** at room temperature followed by a final and virtually complete microwave-assisted grafting with a monomethyl oligo(ethylene glycol) (MOEG-9) chain bearing an azide group (**MOEG-9-N_3**).[25]

Properties of the new polybenzofulvene derivatives

Homopolymer poly-6-BAUP-TM-**BF3k** was obtained as a white solid by precipitation with diethyl ether from a chloroform solution and found to retain the good solubility features generally shown by the polybenzofulvene derivatives in the most common organic solvents, but it was virtually insoluble in water. This expected behavior agrees perfectly with the lipophilic character of the grafted side chain and restricted the study of this homopolymer to the organic environment.

On the other hand, the copolymer poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) was obtained as a light brown sticky solid by precipitation with *n*-hexane from a solution of the copolymer in chloroform and, similarly to the homopolymer, showed good solubility in the most common organic solvents. In dichloromethane or chloroform the copolymer dissolved very rapidly, while the interaction with water was found to be the main distinctive feature between the homopolymer and the copolymer. Very interestingly, the latter interacts with water generating a transparent hydrogel, which was believed to constitute an intriguing aggregation state to evaluate the potential of **BAUP** as a synthetic dynamic receptor in the biological environment.

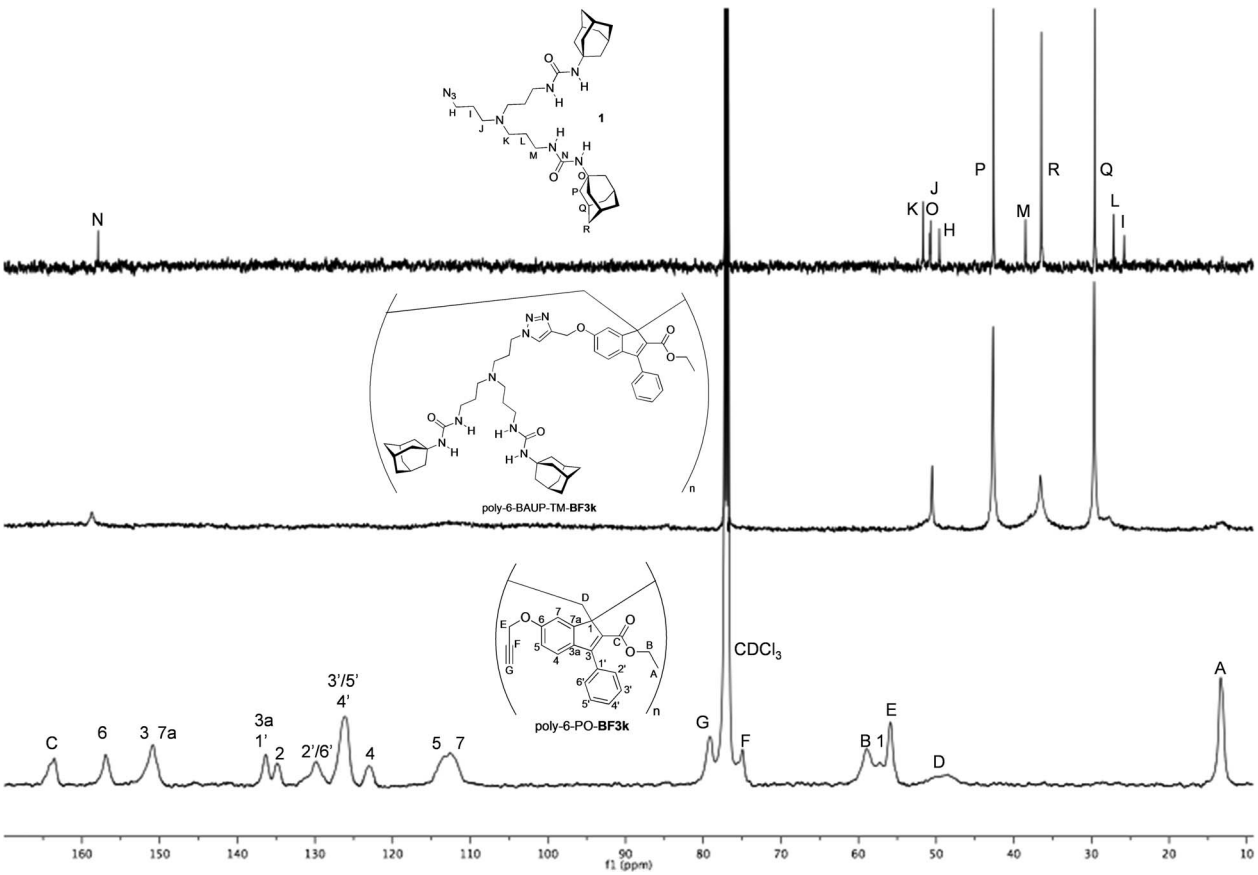

Fig. 2 ^{13}C NMR spectrum ($CDCl_3$) of newly synthesized poly-6-BAUP-TM-**BF3k** compared with those of synthon **1** and of macromolecular precursor poly-6-PO-**BF3k**.

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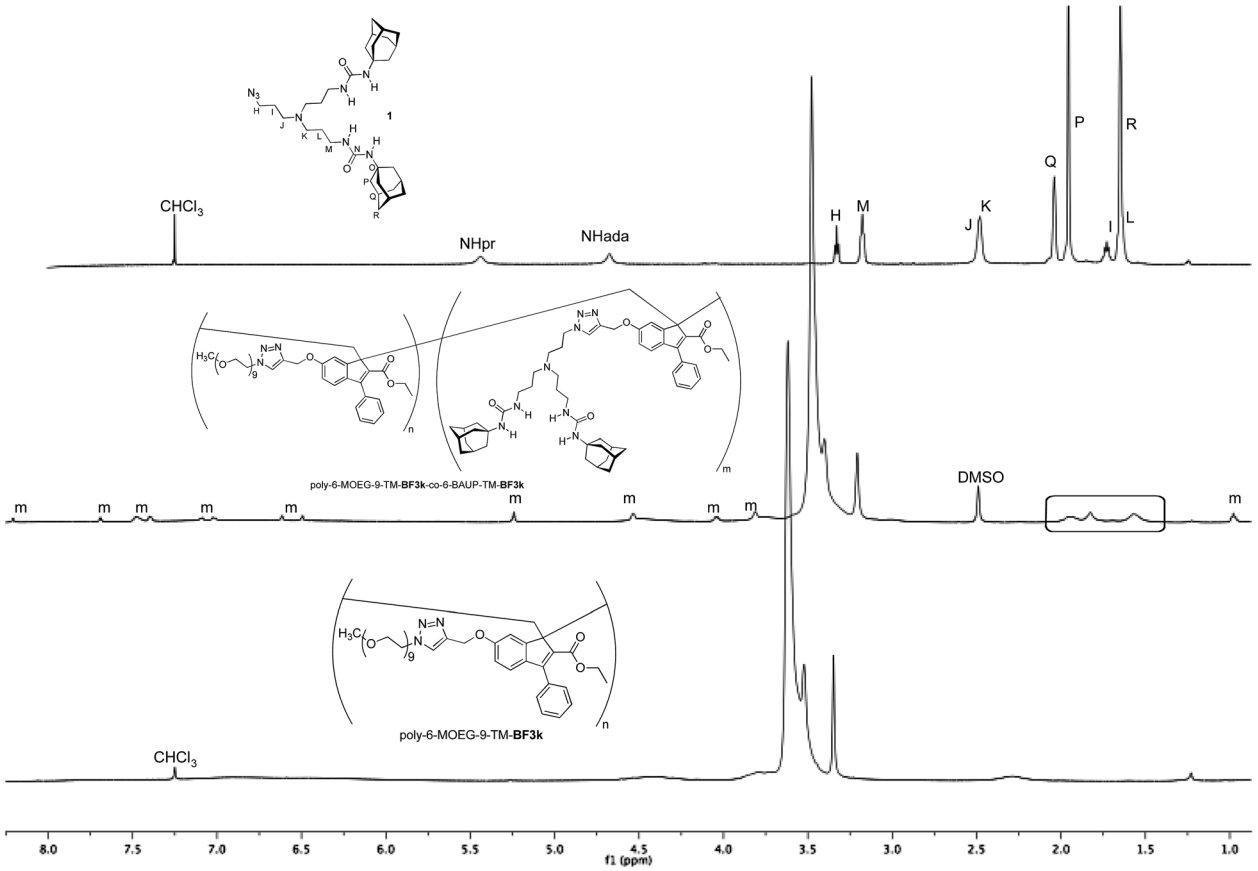

Fig. 3 ^{1}H NMR spectrum (DMSO-d_6) of newly-synthesized poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) compared with those of synthon **1** ($CDCl_3$) and of previously reported poly-6-MOEG-9-TM-**BF3k** ($CDCl_3$). In the spectrum of poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1), m characters mark the signals attributed to low amounts of monomer 6-MOEG-9-TM-**BF3k** produced by depolymerization and the round-cornered box encloses the ones attributed to adamantane moieties.

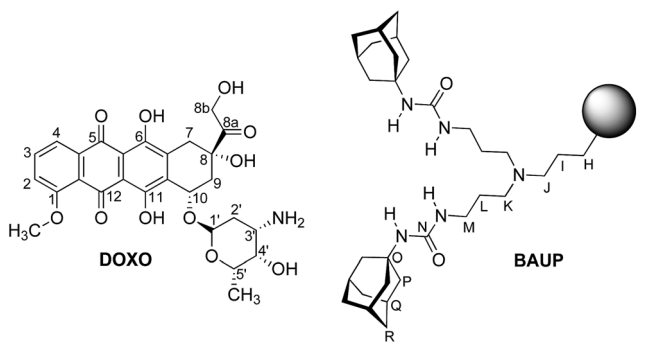

Fig. 4 Structures of doxorubicin (**DOXO**) and bis(adamantylurea) pincer (**BAUP**).

Molecular weight distribution of the new polybenzofulvene derivatives

The molecular weight distribution (MWD) of both newly synthesized poly-6-BAUP-TM-**BF3k** and poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) was determined by means of a multi-angle laser light scattering (MALS) detector connected to a size exclusion chromatography (SEC) apparatus. The presence of hydrogen bond donors and acceptors in the bis(adamantylurea) pincer of the homopolymer and the copolymer required the use of a solvent possessing strong H-bond acceptor features. In particular, DMF was used for the couple of the newly synthesized polybenzofulvene derivatives, while an (8 : 2) mixture of THF–(DMF + 0.01 M LiBr) was employed in the case of poly-6-MOEG-9-TM-**BF3k** and THF was sufficient for macromolecular precursor poly-6-PO-**BF3k**.[25]

The results summarized in Table 1 suggest that the CuAAC reaction of poly-6-PO-**BF3k** with synthon **1** produces small changes in the molecular weight distribution (MWD) of the homopolymer poly-6-BAUP-TM-**BF3k**. On the other hand, a significant decrease in the molecular weight and an increase in dispersity M_w/M_n were observed when poly-6-PO-**BF3k** was reacted in sequence with synthon **1** (at room temperature) and then with **MOEG-9-N$_3$** with the aid of microwaves without the production of significant monomer concentrations. These observations confirm that the polybenzofulvene backbone is susceptible to breaking under the conditions used in the CuAAC reaction,[25] but the physicochemical features of the grafted side chains appear to play a role. We assume that the stability of the polybenzofulvene backbone is

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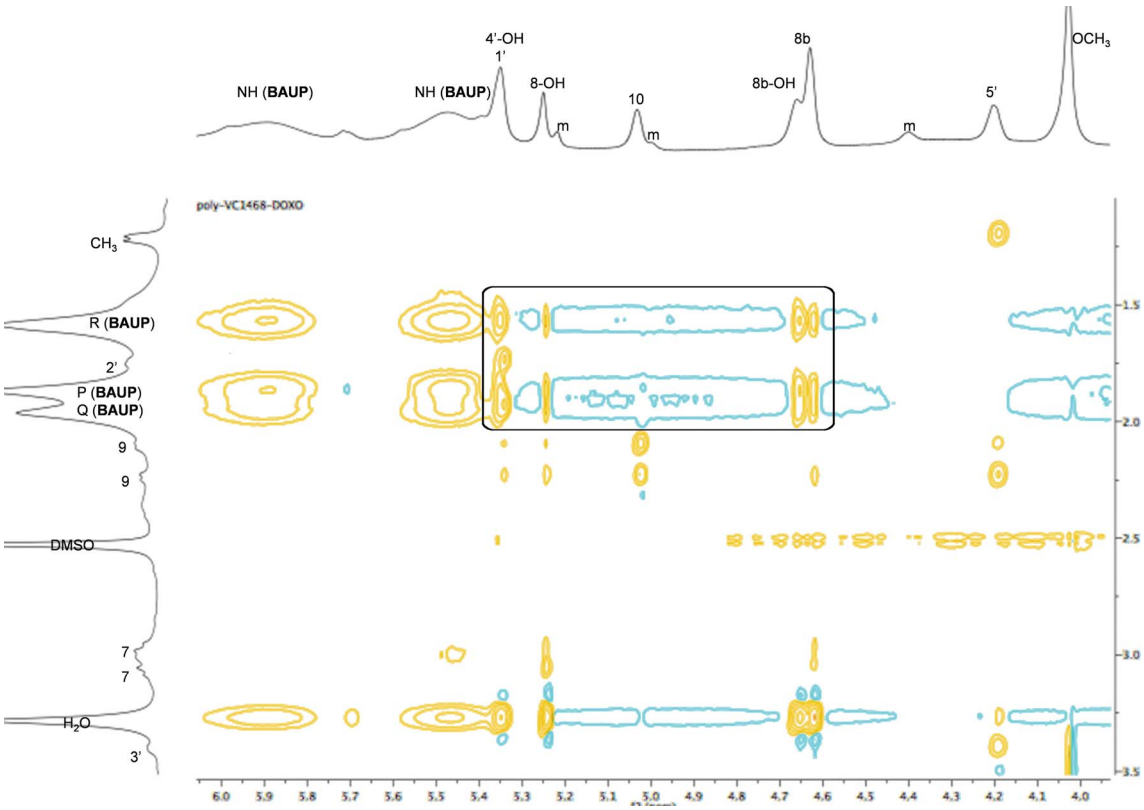

Fig. 5 NOESY spectrum (600 MHz, $CDCl_3$–DMSO-d_6 1 : 1) of **DOXO** and poly-6-BAUP-TM-**BF3k** mixture; m characters mark the signals attributed to low amounts of monomer 6-BAUP-TM-**BF3k** produced by depolymerization and the round-cornered box encloses the relevant cross peaks.

Table 2 Mean diameter (*Z*-average), polydispersity index (PDI) and zeta potential values of **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) and unloaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels as measured in ultrapure water[a]

Sample	*Z*-av. (nm)	PDI	*Z*-Pot. (mV)
DOXO-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1)	213	0.52	3.2
Unloaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1)	266	0.48	3.3

[a] Measurements were performed immediately after the preparation and also after 72 h storage at 25 °C in the same aqueous media to verify the physical stability of the micelles. It is interesting to note that in both cases (loaded and unloaded nanogels) the hydrodynamic diameter of the samples does not change after 72 h of incubation.

affected by the intrachain interactions established by the side chains that are attractive in the case of homopolymer poly-6-BAUP-TM-**BF3k** and mainly repulsive in poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1).

Structures of the new polybenzofulvene derivatives

The structure of the macromolecular precursor poly-6-PO-**BF3k** was previously characterized by NMR spectroscopy.[25] The NMR studies confirmed both the retention of the spontaneous vinyl (1,2) polymerization mechanism and the presence of intact clickable propargyl groups, which were not involved in the thermoreversible polymerization/depolymerization mechanism as confirmed by the depolymerization studies. Moreover, these studies provided evidence about the exhaustive grafting obtained when the appropriate conditions were used in the CuAAC grafting reaction.[25] Thus, the structures of both homopolymer poly-6-BAUP-TM-**BF3k** and copolymer poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) were studied by NMR spectroscopy in order to assess the correct functionalization of the macromolecular precursor poly-6-PO-**BF3k**.

The ^{1}H NMR spectrum of the homopolymer poly-6-BAUP-TM-**BF3k** (Fig. 1) is dominated by the very broad and intense adamantane signals, testifying that our click chemistry CuAAC conditions were successful in functionalizing the polybenzofulvene backbone.

This result was confirmed by comparing the ^{13}C NMR spectrum of the homopolymer with those of parent macromolecule poly-6-MO-**BF3k** and synthon **1** (Fig. 2). In this comparison,

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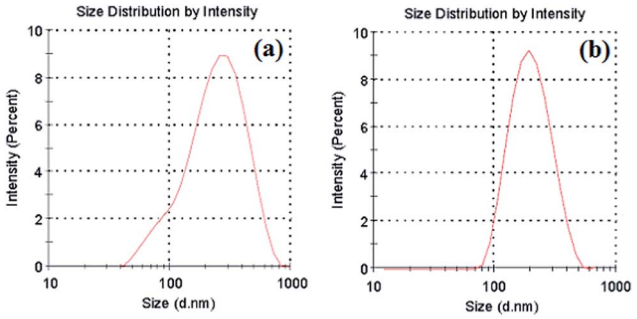

Fig. 6 DLS size distribution histograms of **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) (a) and unloaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) (b) nanogels.

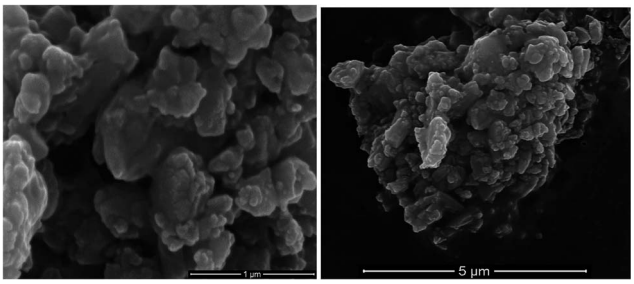

Fig. 7 SEM images of **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels at magnification of 50 000× (left) and 25 000× (right).

poly-6-PO-**BF3k** was used as the model of the polymeric backbone and synthon **1** as the model of the side chains.

The ^{13}C NMR spectrum of homopolymer poly-6-BAUP-TM-**BF3k** shows indeed dominating signals in the up-field region and the comparison with the spectrum of synthon **1** allowed the assignment of these signals to **BAUP**. On the other hand, the signals attributable to the carbon atoms belonging to the polymeric backbone are very broad and appear as baseline modulations, suggesting that homopolymer poly-6-BAUP-TM-**BF3k** undergoes a pronounced self-aggregation that drastically decreased the resolution of carbon atoms belonging to the polymeric backbone. This result can be explained on the basis of the presence of H-bond donors and acceptors in the side chains that may establish both interchain and intrachain interactions.

Conversely, the ^{1}H NMR spectrum of the copolymer poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) is dominated by the signals attributed to the MOEG-9 side chains (Fig. 3), as expected on the basis of the higher proportion with respect to **BAUP**. However, the comparison shown in Fig. 3 demonstrates that the signals attributable to the adamantane moieties are clearly visible in the up-field region of the spectrum, confirming the co-monomeric structure of the copolymer.

Selection of the small drug molecule to be loaded in the newly synthesized polybenzofulvene derivatives

The ability of polybenzofulvene derivatives to complex high molecular weight bioactive molecules, such as immunoglobulin G (IgG), has been already investigated in a physical strong hydrogel obtained with a polybenzofulvene molecular brush of first generation bearing only MOEG-9 side chains.[28] In this system, the protein–polymer interaction was assumed to occur by aspecific adsorption onto the surface of the hydrogel particles. Differently, the copolymer poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) used in the present study bears **BAUP** moieties anchored to the polybenzofulvene backbone that may potentially act as synthetic dynamic receptors for low molecular weight drug molecules.

Although **BAUP** was demonstrated to interact with ureidoacetic acid derivatives when it was expressed on the PPI dendrimer surface,[11–14] doxorubicin (**DOXO**) was envisioned to possess the structural prerequisites (*i.e.* H-bond acceptors and donors and an aromatic moiety, Fig. 4) to interact with the **BAUP** dynamic synthetic receptors linked to the aromatic polybenzofulvene backbone of poly-6-BAUP-TM-**BF3k** and poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k**.

In order to evaluate a possible interaction between the therapeutically relevant **DOXO** and **BAUP** in the newly synthesized polybenzofulvene derivatives, ^{1}H NOESY experiments were performed with homopolymer poly-6-BAUP-TM-**BF3k** as a macromolecular model of **BAUP** in $CDCl_3$–DMSO-d_6 (1 : 1) as the solvent. Preliminary results appeared to support a potentially weak interaction of **DOXO** alcoholic groups with the adamantylurea moieties. In fact, dipolar cross peaks were observed between the signals attributed to **BAUP** adamantane protons and those attributed to the alcoholic hydroxyl protons of

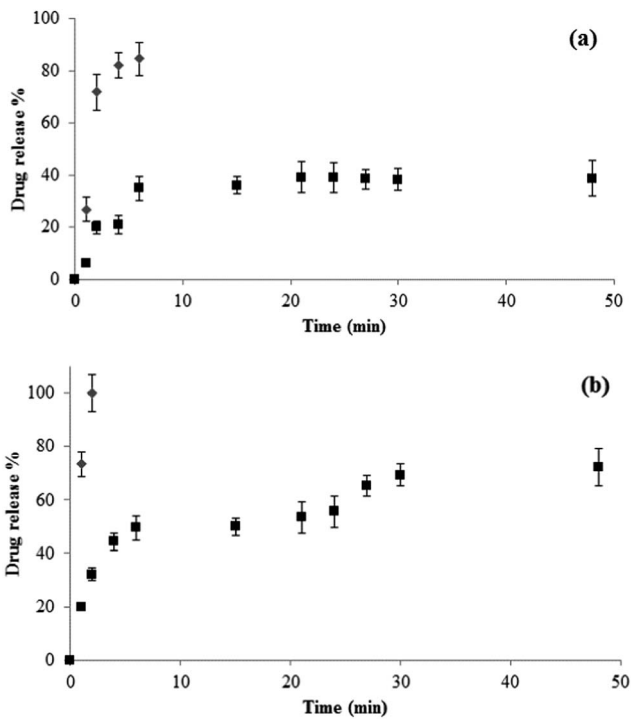

Fig. 8 Drug release profiles of **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels (■) and free **DOXO** (◆) in PBS solution at pH 7.4 (a) and pH 5.5 (b).

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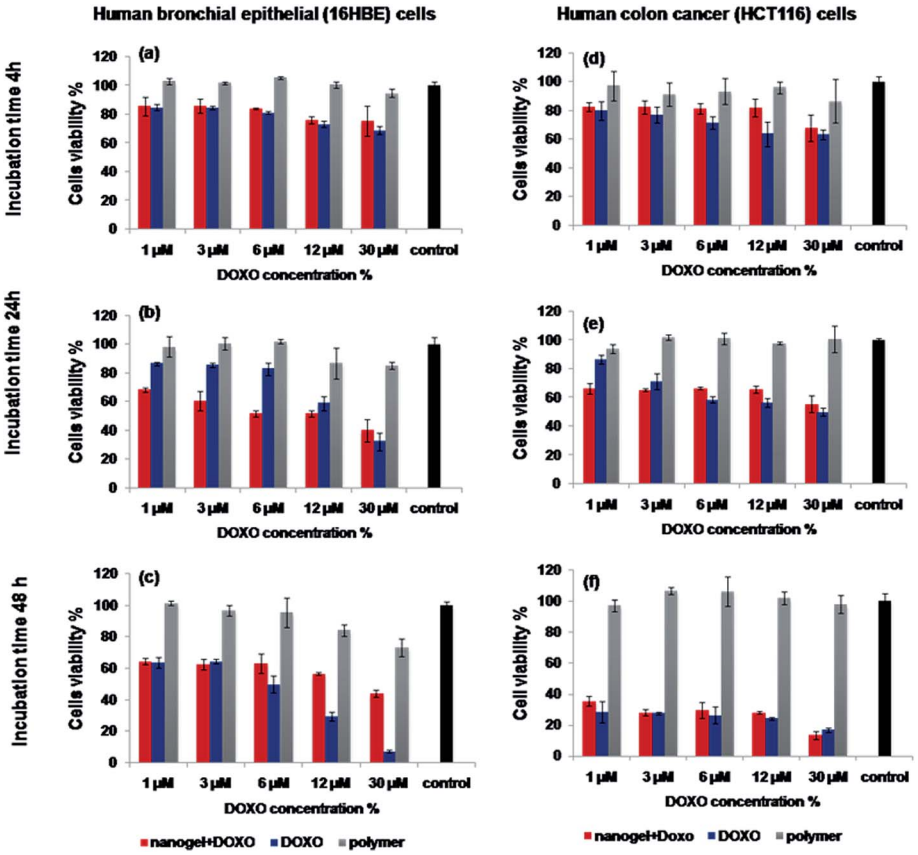

Fig. 9 Cell viability % of unloaded (grey), **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels (red) and free **DOXO** (blue) on human bronchial epithelial (16HBE) cells (a–c) and human colon cancer (HCT116) cells (d–f), at **DOXO** concentrations of 1, 3, 6, 12 and 30 μM. For unloaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels the polymer concentrations correspond to 20, 50, 100, 200 and 500 μg mL^{-1} respectively. Samples were incubated for 4 h (a and d), 24 h (b and e) and 48 h (c and f). The results are reported as the mean ± SD ($n = 6$).

doxorubicin (Fig. 5). It should be emphasized that the system used in these preliminary interaction studies represented a special case and the results could not be generalized, but they were considered however promising enough to proceed with the subsequent steps of the work.

Preparation and characterization of doxorubicin loaded poly-6-MOEG-9-TM-BF3k-*co*-6-BAUP-TM-BF3k (9 : 1) nanogels

In this study, the previously discussed ability of poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) to interact with low molecular weight drug molecules was applied to prepare colloidal drug delivery systems delivering the model anti-cancer drug doxorubicin. The drug loading procedure was carried out by using an already described soft-interaction method (known as dialysis method) consisting of mixing the polymer dispersion with the drug solution in an organic solvent and then placing this mixture into a dialysis membrane against water.[29–31] The slow diffusion of the organic solvent from the internal compartment causes the polymer aggregation into nanogels entrapping the drug molecules. This is related to the lower solubility of the polymer in water than in the organic solvent. On the other hand the drug loading is generated by physical interaction occurring between the drug molecules and the polymeric matrix or, as we presume, with the bis(adamantylurea) pincer system. Actually, the unloaded drug molecules are eliminated by diffusion outside the dialysis membrane. With this method, a loaded drug amount equal to 3.5 wt% was obtained with respect to drug-loaded nanogels. The obtained nanocarriers were characterized by DLS analysis and showed colloidal size (DLS data are reported in Table 2) but, as expected, a large size distribution value (Fig. 6) due to self-aggregation phenomena. In fact, SEM images (Fig. 7) showed micronized aggregates of colloidal particles.

Drug release studies were performed by using the same dialysis method in order to provide experimental proof of the ability of the poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels to release the encapsulated model drug, *i.e.* doxorubicin. Release studies were carried out by simulating pH conditions upon parenteral administration. Thus, nanogels were dispersed in bidistilled water, placed into the dialysis bag, which was immersed in external phosphate buffer PBS solution both at pH 7.4 (mimicking interstitial fluids and plasma pH value) and at pH 5.5 (mimicking the intracellular environment pH value). Then, the amount of released **DOXO** in the external medium, at pre-determined time intervals, was quantified by

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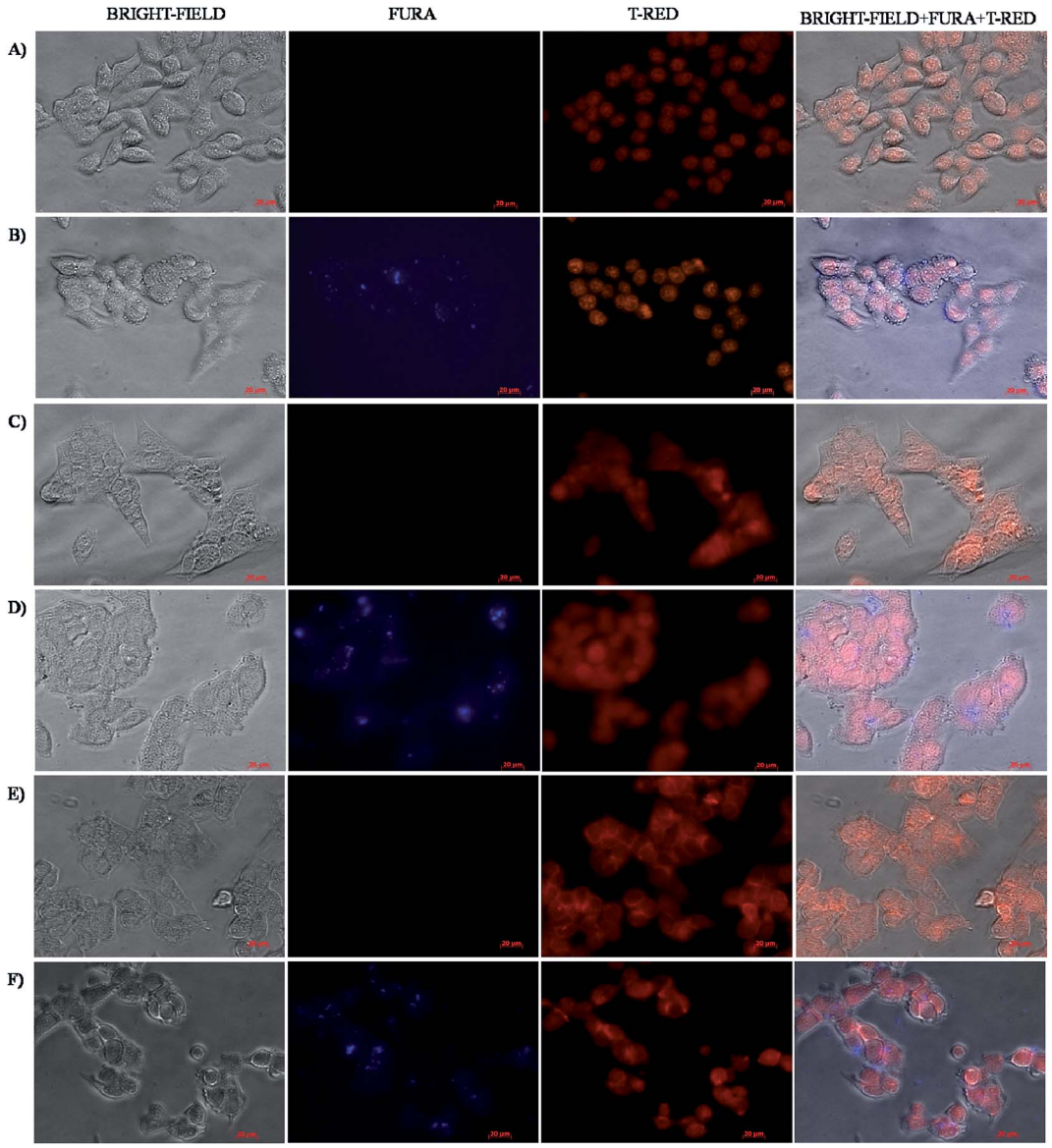

Fig. 10 Fluorescence microscopy images of HCT116 cells incubated with free **DOXO** (A, C and E), and **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels (B, D and F) for 4 (A and B panel), 24 (C and D panel) and 48 h (E and F panel). **DOXO** is visualized with Texas Red filter (red); the polymer is visualized with Fura filter (blue). Magnification is 40× for all images and the scale bar is 20 μm.

HPLC analysis. The drug release profile was detected for 48 h, as shown in Fig. 8, and the amount of released **DOXO** was expressed as the percentage of the total amount of drug loaded into the nanogels. These experiments demonstrated that loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels were able to release **DOXO** in the intact form for a prolonged period and without a first burst release, as observed for free **DOXO**.

In vitro biological evaluation of anticancer activity

Cytotoxicity of unloaded and **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels and of free **DOXO** was evaluated by the MTS assay on two different cell lines, *i.e.* human bronchial epithelial (16HBE) and human colon cancer (HCT116). The former is a non-tumoral cell line extensively used as model normal cells to screen cytotoxicity of novel compounds or drug carriers,[32,33] the latter is a cancer cell line used to investigate the anti-cancer activity of anti-cancer drugs.[34] These cells were incubated with different amounts of **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels corresponding to **DOXO** concentrations of 1, 3, 6, 12 and 30 μM, for 4, 24 and 48 h. The results are shown in Fig. 9a–f, in terms of cell viability (%) as a function of sample concentration.

The obtained results demonstrated that poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1), as a free polymer, is not cytotoxic towards normal and cancer cells at all tested concentrations. Differently, **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels show a cytotoxicity depending on incubation time and concentration of **DOXO** (corresponding

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to the concentration of drug loaded into the nanogels). In particular, on normal cells (16HBE) free **DOXO** (used as positive control) was found to be more cytotoxic than **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels at the higher tested concentration. It is interesting to note that only on cancer cells (HCT116), **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels show similar or slightly higher cytotoxicity values than free **DOXO** (incubation time = 48 h and **DOXO** concentration = 30 μM, Fig. 9 panel f).

These data suggest a potential advantage in the treatment of cancer, with the aim to achieve the optimal pharmacological effect and the lowest secondary effects. Then, in order to investigate a possible correlation between the cytotoxic activity of **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels and the potential ability of this polymer to interact with cell membranes, fluorescence microscopy studies were performed. HCT116 cells were incubated with either **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels or free **DOXO**, at a drug concentration of 30 μM. After pre-determined time intervals, the cells were observed with a fluorescence microscope using different filters (Fura to visualize the polymer and Texas Red to visualize **DOXO**). The images of cells after 4 h (A and B), 24 h (C and D) and 48 h (E and F) of incubation are reported in Fig. 10. They showed that **DOXO** is visible in the cell nuclei of HCT116 cells just after 4 h of incubation when loaded on poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels or as the free drug, and it accumulates progressively after 24 h and 48 h of incubation. Moreover, at the same time we observed the presence of poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels adhered to the cell membrane (visible as blue spots in the brightfield + Fura + T-Red images). Probably, the positive zeta potential values shown by the nanogel particles favoured the interaction with the negatively charged cellular surface. Actually, **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels provide an extracellular reservoir of the drug, which is gradually released and internalized within the cells.

Conclusions

The polybenzofulvene backbone of the previously published amphiphilic polymer brush poly-6-MOEG-9-TM-**BF3k** has been functionalized with a "synthetic dynamic receptor" composed of two 1-adamantylurea moieties linked together by means of a dipropyleneamino bridge as in Meijer's bis(adamantylurea) pincer. To evaluate the effects of the introduction of **BAUP** into the polybenzofulvene environment, homopolymer poly-6-BAUP-TM-**BF3k** and copolymer poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) were prepared, both equipped with synthetic receptors but with different densities. Homopolymer poly-6-BAUP-TM-**BF3k** showed good solubility features in the most common organic solvents, but it was virtually insoluble in water, while the copolymer interacts with water generating a transparent hydrogel, which was considered to be an intriguing aggregation state to evaluate the potential of **BAUP** as a synthetic dynamic receptor in the biological environment. This functional material was used to prepare colloidal drug delivery systems in the form of nanogel particles showing dimensions around 200 nm for delivering the model anti-cancer drug doxorubicin. The resulting nanostructured drug delivery systems containing physically loaded **DOXO** (around 3.5% w/w) were demonstrated to be able to release the drug in the intact form for a prolonged period and without a first burst release. Moreover, cytotoxicity studies performed with human bronchial epithelial (16HBE) and human colon cancer (HCT116) cell lines revealed that the copolymer showed a low toxic potential, whereas the **DOXO**-loaded nanostructured delivery systems show cytotoxicity depending on the incubation time. Interestingly, on normal cells, free **DOXO** was found to be more cytotoxic than **DOXO**-loaded nanogels at the higher tested concentration and, only on cancer cells, **DOXO**-loaded nanogels show similar or slightly higher cytotoxicity values than free **DOXO** suggesting a potential advantage in the treatment of cancer, with aim of achieving the optimal pharmacological effect and the lowest secondary effects. These results were substantiated by fluorescence microscopy studies, which suggested that **DOXO**-loaded poly-6-MOEG-9-TM-**BF3k**-*co*-6-BAUP-TM-**BF3k** (9 : 1) nanogels provide an extracellular reservoir of the drug, which is gradually released and internalized within the cells.

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