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# Self-organized Environment-sensitive Inulin-doxorubicin Conjugate with Selective Cytotoxic Effect Towards Cancer Cells

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An inulin-based random copolymer bearing high dose doxorubicin (18.45 % on a weight basis), INU-EDA-P,C-DOXO, was prepared by coupling doxorubicin with inulin through citraconylamide bridge used as pH sensitive spacer. A further conjugation with pentynoic acid via amidic bond led to the hydrophobization of the copolymer which allow acquiring self-assembling ability at low concentration (0.33 mg mL<sup>-1</sup>) combining both  $\pi$ - $\pi$  stacking and London interactions. Drug release studies were carried out at different pH demonstrating a remarkable pH dependency, where the maximum release rate was observed at pH mimicking cancer tissue and lysosomal environments. Besides, by measuring  $\zeta$ -potential variations as a function of the pH, INU-EDA-P,C-DOXO proved capable of undergoing charge reversal at acidic pH, changing its physicochemical and biological behavior. *In vitro* tests with cancer (MDA-MB 231) and normal (HB-2) breast cells were carried out to verify the conjugate aptitude to follow different routes to enter cells depending on the microenvironment. This finding was supported by quantitative up-take studies, which revealed that INU-EDA-P,C-DOXO released doxorubicin before getting in cancer cells, as the entire copolymer diffused across normal cell membrane without relevant modifications.

## 1. Introduction

Up to now, the laparoscopic surgical removal of tumor mass represents the treatment of choice to efficaciously eradicate cancer.<sup>[1]</sup> Even if several hundred patients take advantage of such clinical routine, those affected by metastases are incurable and the others need to be underwent invasive adjuvant treatments, with the aim of killing circulating neoplastic cells avoiding recidivisms.<sup>[2]</sup> In the last decade, smart nanoscale systems for targeted drug delivery have received great attention as they are the most promising tools to overcome limitations of conventional cancer chemotherapy, mainly due to the lack of drug accumulation into the tumor site and selectivity towards cancer cells as well as poor bioavailability.<sup>[3,4]</sup> At least in principle, such systems can yield to specific drug release and accumulation into the tumor mass by means of the enhanced permeability and retention (EPR) effect<sup>[5]</sup> combined with environment-sensitivity and active targeting mechanisms towards membrane molecules overexpressed by cancer cells (e.g., growth factors, peptides such as RGD or proteins). They are typically endowed with functional groups which make them sensitive to specific endogenous stimuli, such as lowered interstitial pH and a higher glutathione or enzymes concentration, bringing about massive and localized delivery of active molecules

inside cancer cells.<sup>[6]</sup> The higher amount of drug released inside cancer cells, rather than healthy tissues, ensure keeping the local therapeutic window into the tumor site through administering lower dose. As consequence, toxic effects and resistance mechanisms can be significantly reduced, improving the therapeutic effect and patient compliance. Despite its exceptional promise, targeted therapy is still in its early stage, and novel selective tools are necessary to get the goal.

Inulin is a natural, biocompatible, bioeliminable and biodegradable<sup>[7]</sup> polysaccharide consisting of linear chains of  $\beta$ -(2-1) fructose units carrying a glucose unit as reducing end-chain. It is water soluble and exhibits high amount of hydroxyl functional groups available for common coupling reactions.<sup>[8,9]</sup> Inulin derivatives have shown promising results in several *in vitro* and *in vivo* studies aimed for addressing drugs into a specific body district.<sup>[9-11]</sup> However, they largely release drugs too slow and without responding to endogenous stimuli, implying that a very low dose of drug is available for metastatic cells eventually run away from cancer as well as a nonspecific drug release.

This paper report on the synthesis, physicochemical and biological characterization of an environment-sensitive inulin-doxorubicin conjugate which can release the linked anticancer drug in the cancer cell cytoplasm. This finding is related to the conjugate ability

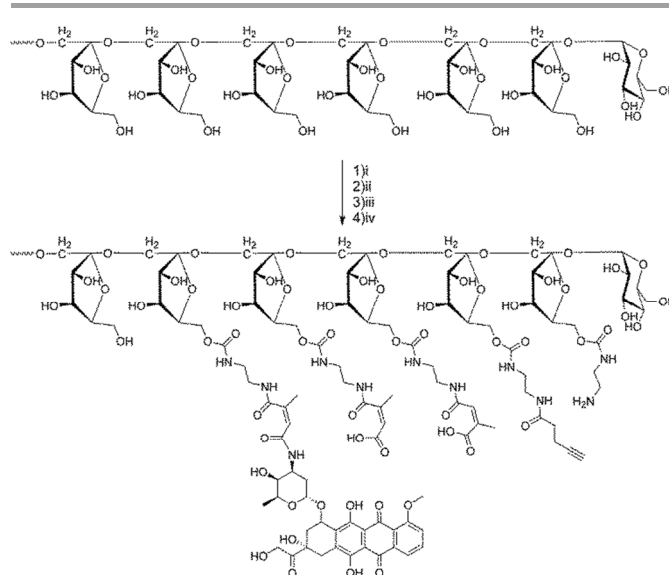
of releasing doxorubicin and reversing its net charge, from negative to positive, once the cancer site is reached, thus improving the preferential release of the drug at the site of action and throughout inside cells. We demonstrated that it is capable of self-assembling into organized nanosystems at low concentration, responding to variations of volume which may occur during the biodistribution at tissue and cellular level, thus protecting doxorubicin from cleavage and limiting drug release at lysosomal level in normal cells. The cytotoxicity of this polymer was systematically investigated using different cancer and physiological cell lines so as to assess any selective effect towards cancer cells instead of the controls. In addition, the cellular up-take and intracellular trafficking of the polymer was investigated on breast cancer cells as model of tumor to understand the intracellular fate of both the main polymer chain and doxorubicin once degraded by cytoplasm or organelles. Our findings shed the light on how "live" polymers, which cleverly respond to several biological stimuli (i.e., pH, volume, ionic strength and temperature changes) might be powerful, safe and cheap solutions for selectively recognize and kill cancer cells minimizing by effects.

## 2. Results and Discussion

### Rational plane

Many research groups have attempted to design systems with outstanding and localized anticancer properties to avoid secondary effects onto healthy tissues. However, so far, those proved capable of reducing tumor mass in mouse model have not shown the same activity in humans, thus remarking that different and more complex approaches in terms of drug release mechanisms should be employed.<sup>[12,13]</sup> This behavior might be related to retro diffusion of drugs ones quickly released inside cells, mainly assisted by the pump efflux of tumor cells, combined with a very low drug payload of the most realized systems (<5% w/w). Herein, a pH sensitive system, named INU-EDA-P,C-Doxo, was synthesized by firstly coupling pentynoic acid to an amine functionalized inulin derivative (INU-EDA) via amidic bond. In tandem, the intermediate was derivatized with citraconic anhydride to obtain pH-sensitive citraconate pendants, which were partially functionalized with doxorubicin affording to a random copolymer carrying the drug and both remaining free carboxyl and amine functions (Scheme 1). These side chains, including doxorubicin, were chosen considering that the main aim of this study was to obtain a drug-inulin conjugate combining pH-sensitivity, self-assembling ability and selective toxic effect towards cancer cells.

INU-EDA is a polycation displaying good cytocompatibility but, as for others formerly studied polycations,<sup>[9]</sup> probably unable to allow suitable bioavailability in vivo being accumulated into high flushed organs after intravenous administration. In this paper, we used citraconic anhydride as a multi-purpose reactant so as to introduce into the main chain free ionizable carboxylic groups, whose ionization degree affect the physicochemical properties of the copolymer, and labile doxorubicin-polymer amidic bond which rapidly hydrolyze under acidic conditions (pH < 6.5) or in the presence of nucleophiles (amine, thiols, etc.).<sup>[14]</sup> It might be expected that the free carboxylates make the system prevalently anionic until the cancer tissue is reached (pH < 6.5), avoiding the typical toxicity profile of common polycations and ensuring that the drug payload is preferentially released into the site of action. The self-assembling property might be expected to increase in the presence of hydrophobic moieties along the polymer chain.



Scheme 1. Synthesis of INU-EDA-P,C-Doxo: i) Bis(4-nitrophenyl) carbonate, 4h, 40°C, 40'; EDA; ii) EDC HCl, NHS, pentynoic acid, pH 6.8, r.t., 18h; iii) Citraconic anhydride, NaOH, pH 8-9, 2h; iv) EDC HCl, NHS, doxorubicin hydrochloride, pH 6.8, r.t., 18h

Besides, the presence of moieties bearing  $\pi$  bonds might provoke  $\pi$ - $\pi$  structuring in water, yielding to either organized macromolecules or supramolecules. Doxorubicin itself and pentynoic pendants provided such interactions, leading to intramolecular and intermolecular structuring. Pentynoic acid was chosen taking as benchmark that known drugs bearing stable and isolated alkyne moiety, such as 17 $\alpha$ -ethynylestradiol and linagliptin, have been shown to have no impact on either intestinal or hepatic CYP3A4 activity.<sup>[15]</sup> We calculate that the volume of a late lysosome is about  $10^5$  lower than that interstitial or cytosolic one. One can deduce that amphiphiles which are present in the interstitial compartment at concentration below their CMC, after cell internalization may get to their CMC and self-assemble into nanosystem at lysosomal level. These properties led us to suppose, later on confirmed by the experiments, that the self-aggregation of the copolymer could occur inside lysosomes even at low pH thereby protecting doxorubicin from cleavage and acidic inactivation inside lysosomes.<sup>[16]</sup> This mechanism was thought to discriminate between healthy and cancer cells on the basis of the status of the copolymer inside lysosomes. An unmodified copolymer should slowly release its drug payload owing to aggregation inside cells, while a partially hydrolyzed copolymer loss the self-assembling ability, being more hydrophilic and prevalently cationic, thus keeping a proper drug dose at cytosolic level and then inside nuclei.

### 2.1. Synthesis and physicochemical characterization of INU-EDA-P,C-Doxo

#### 2.1.1. Synthesis of the INU-EDA-P,C-Doxo conjugate

Inulin ( $\alpha$ -D-glucopyranosyl-[ $\beta$ -D-fructofuranosyl](n-1)-D-fructo furanoside), a linear polysaccharide consisting of glucopyranose end-capped fructose units ( $\beta$ -1,2), was partially functionalized with ethylenediamine (EDA) in order to introduce primary amine pendants available for further functionalization (Scheme 1).<sup>9</sup> A derivatization degree in EDA of 19 mol% was obtained ( $DD_{EDA}$ , related to inulin monomer units) consisting with a random

copolymer bearing an average amount amine pendants of 5.2 per macromolecule. The introduction of C<sub>5</sub>-alkyne moieties into INU-EDA backbone was performed in water by coupling INU-EDA amine pendants with the carboxyl group of pentynoic acid (P) in the presence of a mixture of EDC and NHS as catalyst.

Citraconic anhydride was then employed as heterobifunctional linker capable of easily reacting with EDA amine pendants forming acid-labile amide groups and in the other end a weakly acid carboxyl functional group (pK<sub>a</sub> > 4), made up to react with others amine functions. Under normal conditions the by-products produced in the first step of the reaction, namely isourea and the unreacted monomers, do not interfere in the nucleophilic reaction taking place between the residual amine groups of INU-EDA-Pentyne and citraconic anhydride. However, being water capable of reacting with citraconic anhydride leading to citraconic acid, a certain lowering effect on water pH was expected. Hence, this reaction was accomplished without previous purification, adding a solution of citraconic anhydride in anhydrous THF step-by-step, and in alkaline condition (8 < pH < 9) to keep the amine groups always deprotonated and available for the nucleophilic addition to citraconic anhydride. After purification by means of dialysis, the <sup>1</sup>H NMR spectrum of the product allowed to confirm the proposed structure. Not surprisingly, the addition of INU-EDA amine function to citraconic anhydride was not regioselective, giving rise to both α- and β-methyl citraconylamide pendants. Comparing the integral of the peaks at δ 1.94 and at 1.98, relative to -OOCCH<sub>2</sub>CH<sub>3</sub> and NHCOCH<sub>2</sub>CH<sub>3</sub> pendants respectively, a simple relative quantification of the -OOCCH<sub>2</sub>CH<sub>3</sub> / NHCOCH<sub>2</sub>CH<sub>3</sub> mol ratio was attained (67% vs 33%), revealing that the primary amine preferentially react with the sterically unhindered center in a ratio equal to 2:1. The mol% of pentynoic acid and citraconylamide were also estimated from the integrals of the peaks at 2.48, ascribable to the CH<sub>2</sub>CH<sub>2</sub>CCH (P), and at 1.94-1.98, relative to -OOCCH<sub>2</sub>CH<sub>3</sub> and NHCOCH<sub>2</sub>CH<sub>3</sub> (C), compared with those at 3.50-4.03 relative to the -CH<sub>2</sub>-OH; -CH-CH<sub>2</sub>-OH; -C-CH<sub>2</sub>-O- (INU) hydrogens, respectively. The derivatization degree was found to be 4.1 % for P repeating units (DD<sub>P</sub>%) and 16.1 % for C repeating units (DD<sub>C</sub>%) on a molar basis.

The INU-EDA-P,C intermediate had a large amount of carboxylates useful for doxorubicin conjugation. The reaction involved the

daunosamine amine group of doxorubicin (Doxo) and these free carboxylates conveniently activated by a mixture of EDC and NHS as coupling agents, and employing large excess doxorubicin (moles of Doxo/moles of carboxyl pendant groups in INU-P,C = 2) to maximize the polymer functionalization. DMF was used as co-solvent to avoid precipitation of the polymer conjugate during its formation. Indeed, amide formation owing to the reaction between doxorubicin and inulin implies that very hydrophilic carboxylate groups were replaced with highly hydrophobic citraconylamide-Doxo pendants, affording to drastic changing in polymer solubility in aqueous solution.

INU-EDA-P,C-Doxo was characterized by <sup>1</sup>H NMR spectroscopy, whose assignments were in full agreement with the proposed structure, but with qualifications (Figure S1). Whilst the daunosamine moiety was clearly shown from the peak at δ 1.25 attributable to the CH<sub>3</sub>CH<sub>2</sub>O hydrogens, together with the peak at 5.47 ascribable to the O-CH-O anomeric hydrogen, the doxorubicinone portion was quasi overwhelmed by the noise. The molar derivatization degree of doxorubicin (DD<sub>doxo</sub>, 8.57 %), that is the percentage of linked doxorubicin with respect to the repeating units of inulin, was calculated from the integrals of the peaks at δ 1.25 and 3.50-4.03 relative to the CH<sub>3</sub>CH<sub>2</sub>O (Doxo) and -CH<sub>2</sub>-OH; -CH-CH<sub>2</sub>-OH; -C-CH<sub>2</sub>-O- (INU) hydrogens respectively. It should be noticed that the amount of hydrophobic pendants, namely P and Doxo repeating units, was about 4.5 per macromolecule (about 12.4 mol% of the repeating units), which was sufficient for intramolecular or intermolecular self-assembling of the copolymer. Hence, the desolvation of the aromatic moiety can explain quite well this aberration in the <sup>1</sup>H NMR spectrum, since such desolvated domains cannot be completely detected because of the Zeeman, dipolar and other effects extremely stretch out these signals almost under the threshold of detection.

The possibility that this result was biased by the hydrolysis of the doxorubicinone moiety was ruled out considering that the UV spectrum of the copolymer was nearly overlaid to that of equivalent amount of doxorubicin hydrochloride prepared in the same medium (PBS pH 7.4) (Figure 1).

**Table 1:** Molar derivatization degree values of EDA, P, C and Doxo linked to inulin; molecular weight (Mw), polydispersity and ζ-Potential of the correspondent copolymers.

Sample	#Mw (kDa)	#Mw/Mn	Composition				ζ-Pot (mV)
			*DD <sub>EDA</sub> (%)	*DD <sub>Pentine</sub> (%)	*DD <sub>citric</sub> (%)	**DD <sub>Doxo</sub> (%)	
INU-EDA	4.0	1.87	19.0	-	-	-	18.8±3.9
INU-EDA-P,C	4.3	1.70	19.0	4.1	16.1	-	-24.8±4.1
INU-EDA-P,C-Doxo	13.3	1.62	19.0	4.1	16.1	8.57	-18.2±7.0

\*Calculated by means of <sup>1</sup>H NMR spectroscopy.

\*\*Calculate combining <sup>1</sup>H NMR spectroscopy and UV spectrophotometry.

#Obtained by SEC analysis in 0.1 M LiBr DMF solution.

Figure 1 clearly shows that the structure of doxorubicin pendants, characterized by n → π\* band near 280 nm and a positive π → π\* band near 480 nm, was preserved in INU-EDA-P,C-Doxo. In addition, the π → π\* band near 480 nm seems red-shifted by ~15 nm, confirming that doxorubicin was covalently bonded to the

copolymer giving rise to strong π-π interactions. It is noteworthy that, the amount of doxorubicin in the copolymer evaluated spectrophotometrically endorsed that calculated by <sup>1</sup>H NMR, which correspond to 18.45 % w/w (expressed as doxorubicin hydrochloride weight equivalent).

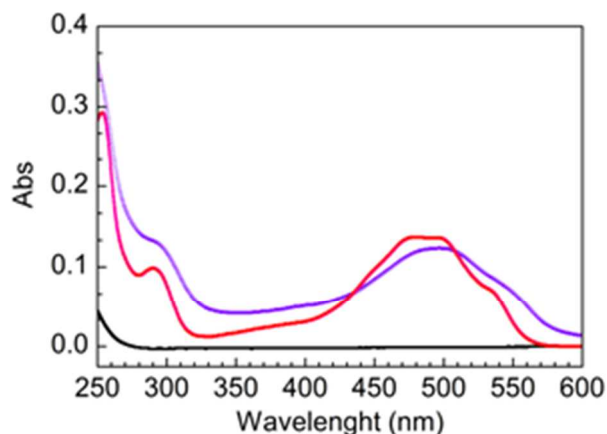


Figure 1. UV spectra of INU-EDA-P,C (black), INU-EDA-P,C-Doxo (magenta) and doxorubicin hydrochloride (red).

Weight average molecular weights ( $M_w$ ) and polydispersity of the synthesized copolymer and their intermediates were obtained by SEC analyses using a calibration curve of monodisperse PEG standards with  $M_w$  ranging from 0.32 to 400 kDa (Table 1).

In detail, although beyond our expectation, the relative molecular weight of the copolymer ( $M_w = 13300$ ) displayed a remarkable increment with respect to the parent polymer (INU-EDA,  $M_w = 4000$ ), proving the feasibility of the conjugation. The occurrence of supramolecular aggregation, ensuing from doxo-doxo and doxo-polyene interactions, may explain the fictitious enormous increasing of the apparent molecular weight. Overall, further confirmation was obtained taking into account that this inconsistency was not observed for the intermediate INU-EDA-P,C ( $M_w = 4300$ ), suggesting that a INU-EDA-P,C-Doxo dimer was revealed in SEC traces.

### 2.1.2. Physicochemical characterization of the INU-EDA-P,C-Doxo conjugate

Viscosimetry in diluted-regimen ( $C < 1 \text{ g dL}^{-1}$ ) is an excellent implement for characterizing polymers, especially to assess supramolecular arrangements or conformational equilibria. Overall, the determination of the inherent viscosity of a polymer, obtained plotting the reduced viscosity of sample at different concentration, enable the calculation of the molecular weight using the Mark-Houwink equation. However, for polymers that display phase segregation or time-dependent conformational changes, as for the amphiphilic one, some deviation from the ideal behavior is usually observed, giving rise to many information on its conformational properties in solution. It has been reported that amphiphilic polymers which self-assemble into micelles by increasing their concentration in aqueous solution showed a reduction of the viscosity curve (reduced viscosity vs concentration) slope near the critical micellar concentration (CMC), ascribable to a volume contraction of the whole polymer chains.<sup>[17]</sup> Tacking these insight as benchmark the self-assembling ability of the copolymer was examined viscosimetrically in water, at 25 °C, and in a diluted regimen (0.01 and 0.125  $\text{g dL}^{-1}$ ) (Figure 2). The plot of the  $\eta_{\text{red}}$  vs  $C$  was bimodal. In particular, it was linear below 0.03  $\text{g dL}^{-1}$  with the intercept ( $[\eta]$ ) equal to 0.12  $\text{dL g}^{-1}$ . The  $[\eta]$  of the copolymer was two times higher than that observed for inulin in water, and comparable to that of inulin in its random coil configuration ( $[\eta]=0.1521 \text{ dL g}^{-1}$ ).<sup>[18-20]</sup> Beyond this concentration the viscosity

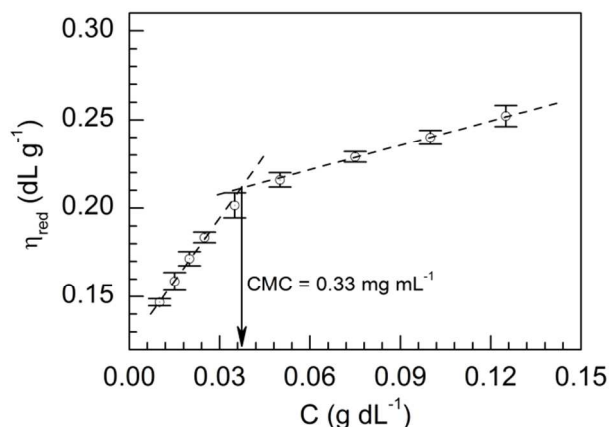


Figure 2. Evolution of the reduced viscosity of INU-EDA-P,C-Doxo ( $\eta_{\text{red}}$ ) as function of its concentration: the critical micelle concentration (CMC) was marked with the arrow

curve roughly decreased in slope, showing that, according with Einstein's equation, aggregation of the system occurred. One can deduce that the polymer molecules, overtaken the critical micelle concentration (CMC), agglomerated forming supramolecules.

The ability of the copolymer to lead to nano-sized aggregates was also assessed by SEM analysis. SEM micrograph (Figure 3) shows that the copolymer assembled into homogeneous nanosystems with a quasi-spherical morphology and dimensions approximately of about 20 nm.

Being INU-EDA-P,C-Doxo prevalently anionic and so unable to efficiently enters healthy cells, but designed to be charge reversible (from negative to positive) at the tumor site, with the aim of understanding if the citraconylamides cleavage was affected by the pH of the medium its  $\zeta$ -potential was examined at different pH (Figure 4). This investigation is motivated by the fact that after an intravenous administration, the usual body trafficking for a drug delivery system involves manifold environment variations, considering that the pH of the medium varies from 7.4 soon after administration (plasma and healthy tissues) to 6.4 in the tumor.

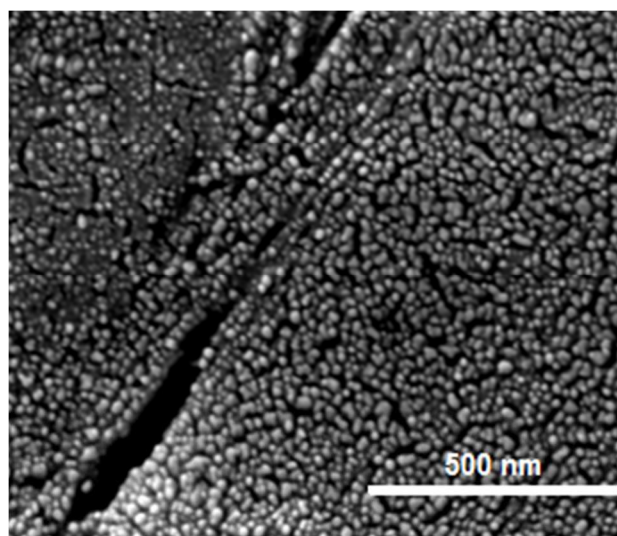


Figure 3. SEM image of INU-EDA-P,C-Doxo

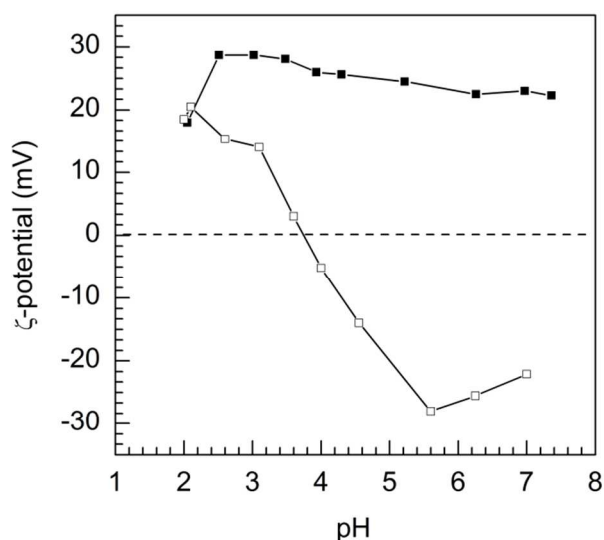


Figure 4.  $\zeta$ -potential plot of INU-EDA (solid symbol) and INU-EDA-P,C-Doxo (open symbol) as a function of the pH of the medium

For comparative purposes the same experiment was performed using INU-EDA as parent compound, whose  $\zeta$ -potential value varied between 19 mV and 29 mV along the whole pH range considered ( $2 < \text{pH} < 7$ ). For the INU-EDA-P,C-Doxo copolymer the observed behavior was more complicated. In particular, the  $\zeta$ -potential increased from  $\approx -21$  mV to  $-29$  mV passing from pH 7 to pH 5.6, than, coherently decreased and reversed its charge at pH 3.9, reaching the same value of the parent INU-EDA. These results point out that in first instance citraconate-Doxo cleavage prevails over INU-EDA-citraconate one, thus increasing the amount of anionic charge. However, at pH below 5.6 this tendency flipped over and citraconylamides carrying carboxylate groups were fast cleaved providing charge inversion.

### 2.1.3. Drug release study

Release experiments were carried out in two media simulating different human body compartments. PBS solution at pH 7.4 and pH 5.5, which mimicked physiological medium and lysosomal environment respectively, were used. The release experiments at the two pH conditions consisted into quantify the amount of released doxorubicin in the external medium, at planned time intervals and until 48 h. The dialysis equilibrium time, previously determined employing equivalent amount of doxorubicin hydrochloride at both pH, was not dependent on the medium and was reached after 1.5 h (Figure 5). Figure 5 shows that INU-EDA-P,C-Doxo proved capable of releasing doxorubicin in both media, providing good evidence that citraconylamide groups, as suggested by the  $\zeta$ -potential experiments, were reversible. However, as expected, a remarkable pH-dependent release profile can be noticed after 2 h of incubation as well, without hinting at "burst effect". In particular, as after 24 h about 13% of doxorubicin payload was slowly released at acidic pH, at the same time only 5% of doxorubicin was released at pH 7.4.

The release experiments were also performed in human plasma to simulate the release after an intravenous administration (See Figure S2). Though an evident increment of doxorubicin release was appreciated by comparison with PBS buffer at pH 7.4, it was always

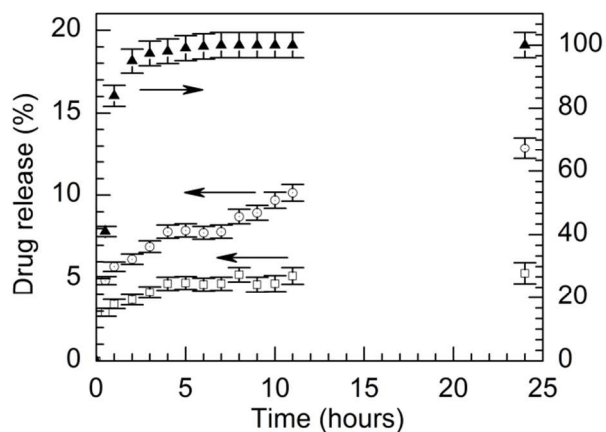


Figure 5. Release profile for INU-EDA-P,C-Doxo at 37 °C in PBS pH 5.5 (open circle) and PBS pH 7.4 (open square) in comparison with doxorubicin hydrochloride alone (solid symbol)

lower than that observed at acidic pH. In particular, after 24 hours only 8% of the doxorubicin payload was released in plasma, providing a good circulating dose of free drug promptly available after drug administration.

## 2.2. Biological characterization

### 2.2.1. Erythrolysis and Cytotoxicity assay for the INU-EDA-P,C-Doxo conjugate

Erythrolysis test was performed at concentration of INU-EDA-P,C-Doxo a thousand time higher than that probably used for future in vivo experiments ( $2 \text{ mg mL}^{-1}$ ), revealing that the conjugate was not hemolytic (data not shown).

Preliminary cytotoxicity studies were carried out on a wide array of cancer (HCT 116, MDA-MB 231 and Sk-Hep-1) and normal (16HBE, HB-2) cell lines, so as to establish the ability of the system to preferentially kill tumor cells instead of the normal one (See Supplemental Data, Figure S3 and S4). Overall, this experiment showed an evident selective cytotoxicity of INU-EDA-P,C-Doxo towards cancer cells, pointing out that the system was potentially capable of focusing its toxic effect on cancer cells independently of the EPR effect.

For comparative purposes, based on the necessity to have a tumor and normal model system of the same tissue, only two cell lines were selected to further study this phenomenon from the biological standpoint. In particular, the INU-EDA-P,C-Doxo cytotoxicity was assessed on MDA-MB 231 and HB-2 cell lines, tumor and normal cells respectively, by CCK-8 assay: the absorbance values were used to metabolically quantify viable cells after the nanoparticles treatment. Figure 6 shows the relative viability of MDA-MB 231 and HB-2 cells after 24 h of incubation in the presence of different amount of INU-EDA-P,C-Doxo or free doxorubicin (50; 25; 5; 0,5  $\mu\text{M}$ ) used as positive control. A dose-dependent reduction for both cell lines treated with INU-EDA-P,C-Doxo was observed, but with some outstanding difference. It might be noticed that the viability of HB-2 cells in the presence of INU-EDA-P,C-Doxo was higher than that of the others samples, even if compared with free doxorubicin treated cells. Differently, the tumor cells treated with INU-EDA-P,C-Doxo showed the lower viability following a non-obvious trend. At doxorubicin equivalent concentration lower than 25  $\mu\text{M}$  the system was significantly less effective than the plain doxorubicin (75-90 %

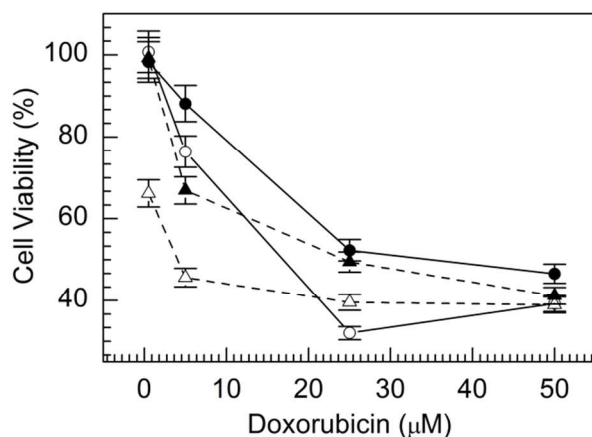


Figure 6. MTS assay on MDA-MB231 (open symbol) and HB-2 (solid symbol) for doxorubicin hydrochloride (dashed lines) and INU-EDA-P,C-Doxo (solid lines)

vs 45-65 % cell viability respectively), whereas at higher concentration the system became about two times more effective, especially at 25 µM of doxorubicin equivalent concentration (28% vs 42 % cell viability). These results showed that the efficacy of the system was significantly higher than doxorubicin alone and somehow selective for the cancer cells, indicating that INU-EDA-P,C-Doxo preferentially target cancer cells in vitro as well.

### 2.2.2. Quantitative uptake studies

The ability of INU-EDA-P,C-Doxo to enter cells across membranes was evaluated by following the FITC-labelled copolymer by cytometry. In addition to a quantitative estimation of the cell uptake, this assay allowed to independently follow in the two model cell examined both drug and the polymer backbone. This thanks to the FITC-labelling of the inulin backbone which make green the system and to the red auto-fluorescence of the doxorubicin pendants. The green fluorescence, that identifies the inulin copolymer, was similar for both cell lines until 8 h from the incubation and has a maximum internalization after 24h of incubation in both cell lines (Figure 7). However, it was roughly greater in the HB-2 cells after 24h of incubation, passing from 5% to 35% and 5% to 58% for the cancer and normal cells respectively. By contrast, the doxorubicin fluorescence in the cancer cell (MDA-MB 231) increased proportionally over time reaching a maximum peak after 24h hours of incubation (Figure 7); as in HB-2 cells the drug was internalized much more slowly, in a fashion which resembles the copolymer, and in a lower amount through time, accumulating in the subsequent 24h. These data are in agreement with a model in which the copolymer behaved in a different manner depending on the surrounding stimuli, in terms of drug release, charge-reversal ratio and self-assembling, giving rise evident divergent biological effects.

It is widely-known that the tumor microenvironment presents a lower pH ( 6.5 ) compared to those of normal tissues (7.4).<sup>[22]</sup> This may selectively trigger a greater release of the drug from the pH-sensitive system. Perhaps doxorubicin was strongly cleaved in the tumor site to independently diffuse across cell membrane, thereby increasing the doxorubicin fluorescence inside cells. On the contrary, in the normal environment INU-EDA-P,C-Doxo-FITC was internalized without any substantial chemical modification thus lea-

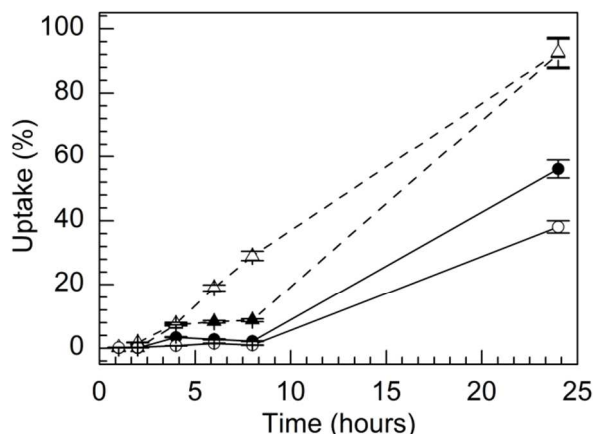


Figure 7. Quantitative uptake of INU-EDA-P,C-Doxo-FITC on MDA-MB231 (open symbol) and HB-2 (solid symbol): FITC (solid lines), Doxo (dashed lines)

ing to a parallel increasing of green and red fluorescence pathway. The enhanced uptake of free doxorubicin released in the cancer microenvironment is more evident in the graph shown in Figure 8, in which the relative uptake is represented as a doxorubicin/FITC fluorescence ratio inside cells. According to this point of view, the uptake of the unmodified system should imply a constant Doxo/FITC ratio through time inside cells, being the fluorescence pendants covalently bonded to the main polymer backbone. In the light of this insight it is quite clear that a greater and faster release of the drug from the polymer occurred outside of tumor cells, which increases proportionally in the first 8h of incubation (Figure 8). After that, the fluorescence of internalized conjugate restored the normal Doxo/FITC ratio at around 3. While, with respect to the curve of the HB-2 cells, only a slight increase of the Doxo/FITC ratio was observed confirming that the system was internalized without releasing its drug payload.

### 2.2.3. Fluorescence microscopy

The data above reported were further confirmed by the cellular uptake of INU-EDA-P,C-Doxo-FITC, observed by fluorescence

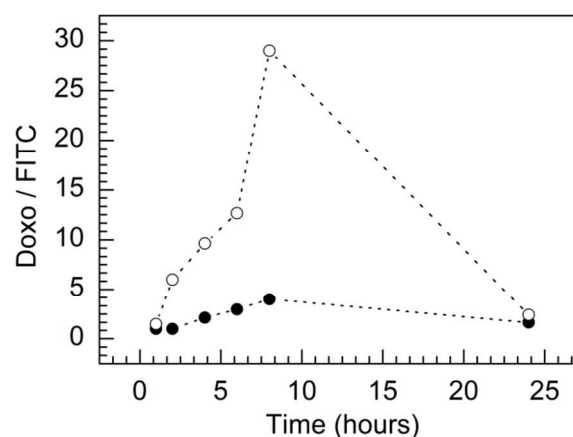


Figure 8. Relative uptake expressed as Doxo/FITC fluorescence ratio on MDA-MB231 (open symbol) and HB-2 (solid symbol)

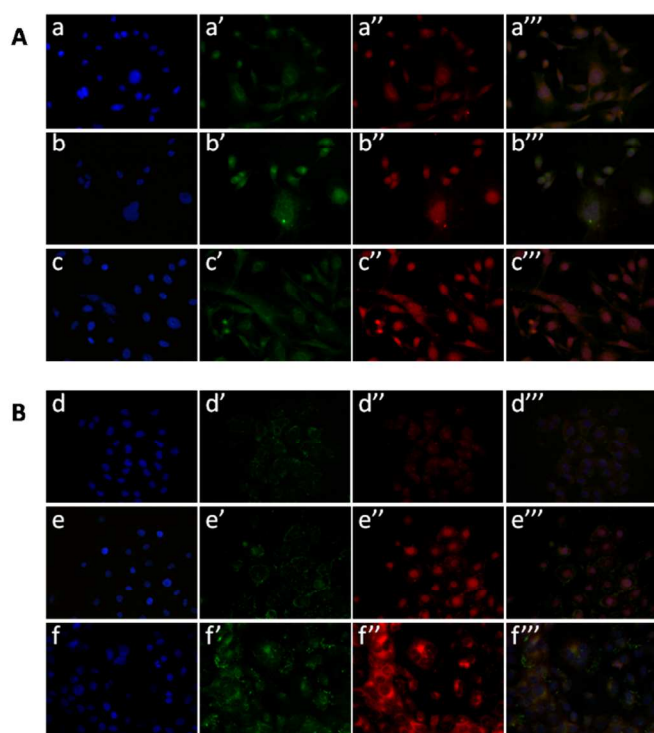


Figure 9. A) Fluorescence microscopy images of MDA-MB 231 incubated with INU-EDA-P,C-Doxo for 1h (a-a'''), 4h (b-b''') and 24h (c-c'''). B) Fluorescence microscopy images of HB-2 incubated with INU-EDA-P,C-Doxo for 1h (d-d'''), 4h (e-e''') and 24h (f-f'''). Blue: Nuclei, Green: Inulin, Red: Doxo, Merge: overlay of 3 fluorescence. Magnificence 40X.

microscopy. Figure 9A shows the images of tumor cells (MDA-MB 231) incubated with INU-EDA-P,C-Doxo-FITC at different time (1h, 4h and 24 h). It can be seen that the green-inulin and the red-Doxo fluorescence enhance inside the cells simultaneously, indicating that the system was efficiently internalized. After 1h of incubation the two fluorescence co-localize inside the cells, but after 4h the doxorubicin appears more into the nuclear compartment. In the panel b (4h) and c (24h), the nuclei begin to appear damaged and cell morphology is altered by the onset of apoptosis phenomena. The INU-EDA-P,C-Doxo-FITC uptake observed in HB-2 cells (Figure 9B) appeared very different. Up to 4 h of incubation the green-fluorescence of the system appeared muffled, punctuate and confined in a specific cellular area, while doxorubicin was localized in the perinuclear region to slowly diffuse into the nucleus (Figure 9B; d, e). Hence, the copolymer and doxorubicin internalized in the early stage were not covalently linked each other and diffuse independently inside cells. After 24 of incubation, where the uptake of the system was much more quantitative (Figure 7), both red and green fluorescence co-localize in a confined region in the cytosol. Both fluorescence increase with time, but the HB-2 cells show a good morphology, even at 24h of treatment unlike the MDA-MB 231 cells. This data are in agreement with the supposed aggregation of the copolymer inside cells before being degraded, yielding to lower cytotoxic effect owing to a slow drug release and high polymer stability inside normal cells.

## 4. Experimental Section

### 4.1. Materials

Inulin-(2-aminoethyl)-carbamate (INU-EDA), used as starting copolymer, was synthesized as previously reported.<sup>[9]</sup> Doxorubicin hydrochloride (DOXO-HCl), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC-HCl), citraconic anhydride, 4-pentynoic acid were purchased from Sigma Aldrich. All reagent were of analytic grade and used as received. SpectraPor dialysis tubing was purchased from Spectrum Laboratories, Inc. (Italy).

Human venous plasma and uncoagulated blood were obtained from healthy voluntary donors and immediately used. These samples were collected from the first co-author with his informed consent.

<sup>1</sup>H NMR spectra were recorded using a Bruker Avance II 300 spectrometer operating at 300.12 MHz. Size exclusion chromatography (SEC) was carried out using a Phenomenex PolySep-GFC-P3000 column (California, USA) connected to a Water 2410 refractive index detector. A solution of 0.1 M LiBr in DMF was used as eluent at 50 °C with a flux of 0.6 mL/min, and PEG standards (400-0.32 kDa, Polymer Laboratories Inc., USA) were used to set up calibration curve ( $R^2 = 0.9958$ ).

Human breast cancer MDA-MB 231 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin G, 100 µg/mL streptomycin at 37°C and 5% CO<sub>2</sub>. Human mammary epithelial HB-2 cells were cultured in Low-glucose Dulbecco's modified Eagle's medium (LG-DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin G, 100 µg/mL streptomycin, hydrocortisone (5 µg/ml) and Bovine Insulin (10 µg/ml) (Sigma Aldrich), at 37°C and 5% CO<sub>2</sub>.

### 4.2. Synthesis of the polymer conjugate

#### 4.2.1 Synthesis of inulin-pentine,citraconate

(INU-EDA-P,C) INU-EDA (150 mg, 0.84 mmol repeating unit) was solubilized in water (4 mL), then NHS (7.71mg, 0.067 mmol) and EDC-HCl (12.9 mg, 0.067 mmol) were added at once under stirring. At this stage 4-pentynoic acid (6.5 mg, 0.067 mmol) was added to the reacting mixture and the pH adjusted to 6.5-6.8 with 0.1 M NaOH. The pH value stabilized after about 30 minutes and the reaction was maintained 15h at room temperature (Min 25 °C, Max 28 °C) with occasional stirring. After that, the pH of the reaction was adjusted to 8.5 with 1M NaOH. Separately, solutions of citraconic anhydride (15.06 mg, 0.134 mmol, in 1 mL anhydrous THF) and NaOH (5.36 mg, 0.134 mmol, in 1 mL milliQ water) were prepared and added to the crude reaction dropwise maintaining the pH within the range 8-9 for 2h. After this time, the product was purified from the by-products and unreacted monomers by means of exhaustive dialysis using a membrane tube with nominal molecular weight cut off (MWCO) 3.5 kDa. It was obtained as a yellowish solid after freeze-drying. Yield 100 mg. <sup>1</sup>H NMR 300 MHz, D<sub>2</sub>O: δ 1.92 (s, 1H<sub>alchylene</sub>, CH), 1.94 (s, 3H<sub>citraconate</sub>, OOCCH<sub>2</sub>CH<sub>3</sub>), 1.98 (s, 3H<sub>citraconate</sub>, NHCOCH<sub>2</sub>CH<sub>3</sub>), 2.48 (br, 4H<sub>alchylene</sub>, CH<sub>2</sub>CH<sub>2</sub>CCH), 3.1-3.57 (m, 4H<sub>EDA</sub>, NCH<sub>2</sub>CH<sub>2</sub>N), 3.50-4.03 (5 H<sub>INU</sub>, -CH<sub>2</sub>-OH; -CH-CH<sub>2</sub>-OH; -C-CH<sub>2</sub>-O-), 4.03-4.45 (2H<sub>INU</sub>, -C-CH-OH; -CH-OH), 5.58 (s, 1H<sub>citraconate</sub>, CHCOO), 5.87 (s, 1H<sub>citraconate</sub>, CHCONH).



#### 4.2.2 Synthesis of inulin-pentyne,citraconate-doxorubicin (INU-EDA-P,C-Doxo)

NHS (7.83 mg, 0.068 mmol), EDC-HCl (13.04 mg, 0.068 mmol) and doxorubicin hydrochloride (39.4 mg, 0.068 mmol) were added to a solution of INU-EDA-P,C (80 mg) in milliQ water/DMF 6:4 (7 mL). The pH of the reaction was adjusted to 6.5-6.8 with 0.1 M NaOH and the reaction kept under these conditions for 15 h at room temperature (Min 25 °C, Max 30 °C). Finally, the reaction was dialyzed through a membrane tubing with MWCO 1 kDa and the pure product retrieved as a deep red powder. Yield 76 mg.  $^1\text{H}$  NMR 400 MHz,  $\text{D}_2\text{O}$ :  $\delta$  1.25 (br,  $3\text{H}_{\text{doxo}}$ ,  $\text{CH}_3\text{CH}_2\text{O}$  sugar ring), 1.90-2.15 (br,  $3\text{H}_{\text{citraconate}}$ ,  $\text{OOCCH}_2\text{CH}_3$  and  $\text{NHCOCH}_2\text{CH}_3$ ), 2.48 (br,  $4\text{H}_{\text{alchyne}}$ ,  $\text{CH}_2\text{CH}_2\text{CCH}$ ), 3.00-3.50 (m,  $4\text{H}_{\text{EDA}}$ ,  $\text{NCH}_2\text{CH}_2\text{N}$ ), 3.50-4.50 (7  $\text{H}_{\text{INU}}$ ,  $-\text{CH}_2-\text{OH}$ ;  $-\text{CH}-\text{CH}_2-\text{OH}$ ;  $-\text{C}-\text{CH}_2-\text{O}-$ ;  $-\text{C}-\text{CH}-\text{OH}$ ;  $-\text{CH}-\text{OH}$ ), 5.47 ( $1\text{H}_{\text{doxo}}$ ,  $\text{O}-\text{CH}-\text{O}$  anomeric sugar ring), 5.52 (s,  $1\text{H}_{\text{citraconate}}$ ,  $\text{CHCOO}$ ), 5.83 (s,  $1\text{H}_{\text{citraconate}}$ ,  $\text{CHCONH}$ ), 8.48 (br,  $\text{H}_{\text{doxo}}$ , aromatic ring).

#### 4.2.3. Synthesis of FITC-labelled inulin-pentyne,citraconate-doxorubicin (INU-EDA-P,C-Doxo-FITC)

FITC-labelled inulin-pentyne,citraconate-doxorubicin was obtained dissolving INU-EDA-P,C-Doxo (8 mg) in milliQ water (5 mL) and adding a solution of FITC (1 mg in 1 mL methanol) dropwise. The reaction was maintained at room temperature 3 hours with stirring and purified through a dialysis tubing with MWCO 12-14 kDa. After freeze-drying a deep yellow solid containing 1 mol% FITC was obtained. Yield 6.5 mg.  $^1\text{H}$  NMR 400 MHz,  $\text{D}_2\text{O}$ :  $\delta$  1.25 (br,  $3\text{H}_{\text{doxo}}$ ,  $\text{CH}_3\text{CH}_2\text{O}$  sugar ring), 1.90-2.15 (br,  $3\text{H}_{\text{citraconate}}$ ,  $\text{OOCCH}_2\text{CH}_3$  and  $\text{NHCOCH}_2\text{CH}_3$ ), 2.48 (br,  $4\text{H}_{\text{alchyne}}$ ,  $\text{CH}_2\text{CH}_2\text{CCH}$ ), 3.00-3.50 (m,  $4\text{H}_{\text{EDA}}$ ,  $\text{NCH}_2\text{CH}_2\text{N}$ ), 3.50-4.50 (7  $\text{H}_{\text{INU}}$ ,  $-\text{CH}_2-\text{OH}$ ;  $-\text{CH}-\text{CH}_2-\text{OH}$ ;  $-\text{C}-\text{CH}_2-\text{O}-$ ;  $-\text{C}-\text{CH}-\text{OH}$ ;  $-\text{CH}-\text{OH}$ ), 5.47 ( $1\text{H}_{\text{doxo}}$ ,  $\text{O}-\text{CH}-\text{O}$  anomeric sugar ring), 5.52 (s,  $1\text{H}_{\text{citraconate}}$ ,  $\text{CHCOO}$ ), 5.83 (s,  $1\text{H}_{\text{citraconate}}$ ,  $\text{CHCONH}$ ).

### 4.3. Chemical and physicochemical characterization of the nanosystem

#### 4.3.1. Determination of drug payload

First, the presence of the anthraquinone moiety in the polymer conjugate was evaluated by comparing the UV spectrum of a solution of doxorubicin hydrochloride ( $0.01 \text{ mg mL}^{-1}$ ) in PBS at pH 7.4 to the spectrum obtained from a INU-EDA-P,C-Doxo solution ( $0.054 \text{ mg mL}^{-1}$ ) carrying equivalent amount doxorubicin. Each absorbance spectral scan was made using a Shimadzu UV-1800 spectrophotometer reading from 200 nm to 600 nm in 1-nm bandwidth.

The amount of doxorubicin linked in the INU-EDA-P,C-Doxo was determined spectrophotometrically measuring the absorbance of the sample at 480 nm. A calibration curve was obtained for serially diluted concentrations of doxorubicin hydrochloride in 0.1 M phosphate buffer at pH 7.4 ( $R^2 = 0.999$ ). For comparative purposes, the content of drug loaded into the system was expressed as the amount of loaded doxorubicin hydrochloride per unit mass of polymer, and resulted to be  $18.45 \pm 0.6 \%$  (w/w).

#### 4.3.2. Aggregation study of the polymer conjugate at different concentration

The ability of the INU-EDA-P,C-Doxo of varying its chain arrangement as function of the concentration was established viscosimetrically in water, evaluating the reduced viscosity ( $\eta_{\text{red}}$ ) of the polymer at diluted regimen. In particular, concentration varied in the range of 0.01 and  $0.125 \text{ g dL}^{-1}$ . Measures were carried out

using an Ubbelohde viscometer immersed in a cryostat bath at temperature settled on  $25 \pm 0.1$  °C. Each point was a medium value of twenty consecutive measures with coefficient of variation (CV%)  $\leq 4\%$ .

#### 4.3.3. Scanning electron microscopy (SEM)

For the morphology studies, a drop of INU-EDA-P,C-Doxo solution ( $0.5 \text{ mg mL}^{-1}$ ) was deposited onto a double sided adhesive tape previously applied on a stainless steel stub. It was then dried under vacuum (40 mbar) for 24h until a thin film was obtained. Hence, the sample was sputter-coated with gold prior to microscopy examination. Images were recorded using a scanning electron microscope, ESEM Philips XL30.

#### 4.3.4. Doxorubicin cleavage studies

The release mechanism of doxorubicin from the conjugate was studied by measuring  $\zeta$ -potential (mV) variations as a function of the pH.  $\zeta$ -Potential measurements were performed at 25°C using a Malvern Zetasizer NanoZS instrument coupled with a MPT-2 accessory, fitted with a 532 nm laser at a fixed scattering angle of 173°. Aqueous solutions of either INU-EDA-P,C-Doxo or INU-EDA ( $0.1 \text{ mg mL}^{-1}$ ) were analysed after filtration through a  $5 \mu\text{m}$  cellulose membrane filter. The pH titration of the polymers was performed potentiometrically using 0.1 M hydrochloric acid, previously filtered through a  $0.22 \mu\text{m}$  cellulose membrane filter, as acid standard. At each pH interval the sample was recirculated inside cells for 2 minutes and then the  $\zeta$ -Potential was measured. The  $\zeta$ -Potential (mV) was calculated from the electrophoretic mobility using the Smoluchowsky relationship and assuming that  $K\alpha \gg 1$  (where K and  $\alpha$  are the Debye-Hückel parameter and particle radius, respectively).

#### 4.3.5. Drug release studies

For drug release studies in artificial medium, INU-EDA-P,C-Doxo (2.5 mg) was dissolved in PBS at pH 7.4 (5 mL) and placed into a dialysis tubing with a MWCO 1 kDa. It was then immersed into PBS at pH 7.4 (35 mL) and incubated at 37°C under continuous stirring (100 rpm) in a Benchtop 808C Incubator Orbital Shaker model 420, for 48 h. Aliquots of the external medium (1 mL) were withdrawn from the outside of the dialysis tubing at scheduled time intervals and replaced with equal amount of fresh medium. The amount of doxorubicin released was evaluated by HPLC using C6-pheryl column and ACN/0.4 M ammonium phosphate buffer at pH  $4 \pm 0.1$  32:68 as mobile phase (flow  $0.8 \text{ mL min}^{-1}$ ). Then, the cumulative release was determined as a function of incubation time. A parallel analysis was carried out using the same procedure above described but employing PBS at pH 5.5 as medium. All release data were compared with the diffusion profile of doxorubicin hydrochloride alone ( $0.5 \text{ mg}$ ) obtained by using the same procedure. Data were corrected taking in account the dilution procedure. Each experiment was carried out in triplicate and the results were in agreement within  $\pm 5\%$  standard error.

### 4.4. Biological characterization of the nanosystems

#### 4.4.1. Erytholysis test

Healthy erythrocytes were collected by centrifuging 2 mL of fresh blood at 2200 rpm for 10 minutes and, then, by washing up the red pellet with PBS at pH 7.4 several times until a colorless aqueous supernatant was observed. A 4% v/v suspension of erythrocytes in PBS pH 7.4 ( $200 \mu\text{L}$ ) was added to a solution of INU-EDA-P,C-Doxo

(200  $\mu\text{L}$ ) at concentration ranging from  $10^{-4}$  -  $2 \text{ mg mL}^{-1}$ . Then, the suspensions were incubated at  $37^\circ\text{C}$  for 1 h, centrifuged at 2200 rpm for 5 minutes and the amount of hemoglobin released in the supernatant was measured spectrophotometrically at 570 nm. Lysis buffer (Sigma Aldrich) was used as positive control in order to normalize all data obtained.

#### 4.4.2. Cytotoxicity of INU-EDA-P,C-Doxo

MDA-MB 231 and HB-2 cells were seeded in 96-well plates at a density of  $1 \times 10^4$  cell/well and maintained using suitable culture medium at  $37^\circ\text{C}$ , in a humidified atmosphere of 5%  $\text{CO}_2$ .

After 24 h from seeding, the cells were incubated for 24 h at different concentrations (final doxorubicin concentration: 50; 25; 5; 0,5  $\mu\text{M}$ ) of INU-EDA-P,C-Doxo. The cells were treated also with the same concentrations of free doxorubicin as positive control; whereas the untreated cells were used as negative control. Cell viability was evaluated using Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich). The water-soluble tetrazolium salt (WST-8) was added to each well (1:10 dilution in complete medium) followed by incubation for 2 h at  $37^\circ\text{C}$ . The mitochondrial dehydrogenases of the living cells, can reduce the WST-8 into soluble formazan dye, therefore, the amount of formazan is directly proportional to the number of living cells. The absorbance at 460nm was determined by a microplate reader DU-730 Life Science spectrophotometer (Beckman Coulter). The percentage of cell viability was calculated as ratio between each sample with respect to the negative control (100% of cell viability).

#### 4.4.3. INU-EDA-P,C-Doxo-FITC uptake measured by flow cytometry

For flow cytometry studies, MDA-MB 231 and HB-2 cell lines were seeded in 6-well culture plates, until the cell confluence. Subsequently, cells were incubated with INU-EDA-P,C-Doxo-FITC for different times (1h, 2h, 4h, 6h, 8h, 24h) at the final doxorubicin concentration of  $25 \mu\text{M}$  in complete medium. Untreated cells served as a negative control for background fluorescence. Afterwards, the cells were washed twice with phosphate buffered saline (PBS) without  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$ , detached by trypsin 1X (Invitrogen), collected by centrifugation at 1000 rpm for 5 min and re-suspended in 0.5 ml of PBS. Therefore, the cells were analyzed using FACS-Canto cytometer (Becton Dickinson, Germany) and the FITC and doxorubicin fluorescence inside the cells was monitored following the green and the red fluorescence emission (respectively 530-585nm). The data for  $1 \times 10^5$  gated events were collected and then further analyzed by using BD FACS Diva software.

#### 4.4.4. Cellular internalization studies of INU-EDA-P,C-Doxo-FITC by fluorescence microscopy

MDA-MB 231 and HB-2 cells were grown for 24 h at a density of  $5 \times 10^3$  cells/well into 12 well culture plates containing a sterile glass covers lip in complete medium, at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . Following, the cells were incubated with INU-EDA-P,C-Doxo-FITC (doxorubicin concentration  $25 \mu\text{M}$ ) for different times (1h, 4h, and 24h). After the incubation, the culture medium was removed and the cells were washed twice with PBS, fixed with 3.7 % formaldehyde for 15 min and washed again with PBS. Cell nuclei were stained with DAPI (1:1000 in PBS) for 30 minutes, at room temperature. The position of the copolymer was monitored by fluorescence microscopy analysis (Leica). Untreated cells served as a negative control for background fluorescence.

## 5. Conclusions

In this paper, the conjugation of inulin with doxorubicin and pentynoic acid to give an environment-sensitive polymeric amphiphile, named INU-EDA-P,C-Doxo, is reported. This copolymer was designed to be capable of self-assembling into nanosystems following volume variations during its intracellular trafficking, thus leading to controlled intracellular drug release. Doxorubicin and pentynoic pendants were introduced into the inulin backbone to confer it upon proper hydrophilic/lipophilic balance and  $\pi$ - $\pi$  interactions, which were proven suitable to lead to self-aggregation varying the copolymer concentration. Doxorubicin was linked to inulin using citraconylamide spacer, which imparted pH sensitivity useful for discriminating cancer and normal microenvironments so as to selectively release doxorubicin in the site of action and buffering the known doxorubicin side effects. This polymer conjugate, besides carrying high dose of doxorubicin, is prevalently anionic and so potentially free to circulate in blood vessel without interacting with plasmatic proteins and reticulo-endothelial system until the cancer site is reached. We demonstrate that in the cancer microenvironment ( $5.5 < \text{pH} < 6.4$ ) INU-EDA-P,C-Doxo may reverse its net charge, from negative to positive, releasing free doxorubicin and, consequently, changing its conformation and biological behavior. These multiple release mechanisms reflect on the cytotoxic effect, which was preferentially expressed in cancer cells instead of the healthy cells, displaying in the meantime a local absolute cytotoxicity higher than doxorubicin alone. This work represents a proof of concept pilot insight which will pave the way for future in vivo studies on such "live polymers".

## Notes and references

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