Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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ARTICLE

Amphiphilic inulin graft co-polymers as self-assembling micelles for doxorubicin delivery

Cite this: DOI: 10.1039/xoxxooooox

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Received 04th January 2014,
Accepted 00th January 20--

DOI: 10.1039/xoxxooooox

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This paper reports the synthesis and characterization of a new amphiphilic inulin graft copolymer able to self-assemble in water into a micelle type structure and to delivery the anticancer model drug doxorubicin. For this aim, inulin was chemically modified in the side chain with primary amine groups (INU-EDA) and these were used as reactive moieties for the conjugation of poly ethylene glycol 2000 and succinyl-ceramide. The CMC of obtained amphiphilic inulin derivatives (INU-ceramide and INU-ceramide-PEG_{2000}) was measured by means of fluorescence analysis using pyrene as fluorescent probe. The obtained micelles were characterized by DLS and AFM analysis and the ability to release the loaded doxorubicin was studied in different media. Finally cytotoxicity profile on both cancer (HCT116) and normal (16 HBE) cell lines and in vitro ability to deliver drug into cancer cells were evaluated.

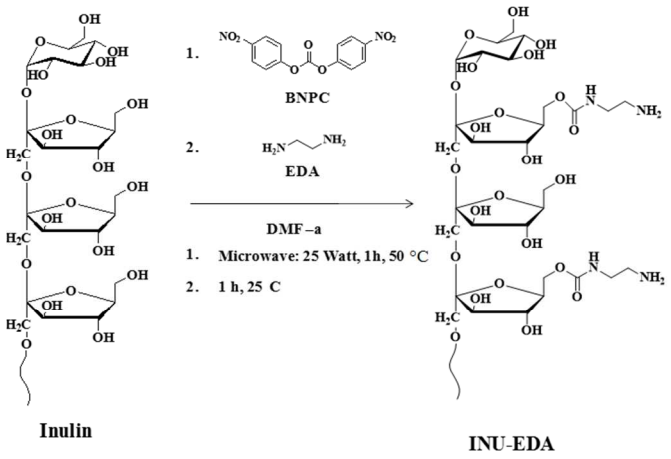
Introduction

Inulin is a natural polysaccharide consisting of linear chains of β-(2-1) fructose units and typically has a glucose unit attached at the reducing end. It is abundant in many plants including garlic, leeks, bananas, and Jerusalem artichokes[1,2] but the main industrial source are chicory roots, which contain ~20−25% inulin on a dry weight basis. For its advantageous properties (absence of toxicity, biocompatibility, water solubility, and prebiotic effect on the intestinal bacterial population), inulin shows potential for widespread use[3]. These attractive characteristics justify the interest in inulin application in the biomedical field. Recently, many studies were concentrated on its chemical modification or derivatization[4] in order to obtain new drug delivery systems (DDS) including hydrogels[5], nanoparticles[6], macromolecular bioconjugates[7,8]. Recent studies report the synthesis of amino derivatives of inulin[9], in order to obtained more water soluble and reactive inulin copolymers, being amino groups more reactive than the polyol functionalities of polysaccharide[10]. However, the potential application of inulin as starting material to develop new drug delivery systems is still underestimated. Taking into account the above considerations, the main goal of this work was to synthesize a new amphiphilic inulin graft copolymer able to self-assemble in water into a micelle type structure and load active molecules. For this aim, inulin was chemically modified in the side chain with primary amine groups used for the conjugation with both hydrophilic chains, such as poly ethylene glycol (PEG), and hydrophobic molecules, like ceramide. Usually, PEGs chains with molecular weight of 2000-5000 Da constitute the hydrophilic outer shell of the polymeric micelles[11] because provide important advantages including the micelles effective steric protection, prevent recognition by the reticuloendothelial system (RES) and prolong bloodstream circulation[12]. On the other hand, ceramide is a member of a panel of bioactive lipids, such as eicosanoids, glycerolipid-derived lipid, sphingolipids, oxidized phospholipids, that play important roles in different signal transduction pathways, including anti- and pro-inflammatory effects, apoptotic effects, growth arrest, proliferation, survival and potent ligands for different G protein-coupled receptors[13,14]. Ceramide is an intracellular lipid that regulates the activity of various biochemical and molecular targets involved in anti-proliferative responses and in cellular responses including oxidative stress and apoptosis[15,16]. In this study, ceramide was chosen primarily to give hydrophobic interaction necessary to form polymeric micelles and, secondarily, to obtain a possible synergistic effect on improving the anticancer activity of the delivered drug[17]. Obtained copolymers were successfully used to prepare polymeric micelles with the aim to delivery the anticancer model drug doxorubicin, and their in vitro biological evaluation was performed.

Results and discussion

Synthesis of inulin-graft co-polymers. Inulin, as common polysaccharides, has in his structure numerous hydroxyl groups

(three for each repeating unit) that can be used as side chain conjugation sites. Nevertheless these hydroxyl groups have different reactivity, the primary one being the most reactive[8]. This fact reduce the reproducibility and limit the number of practicable conjugation reactions on the inulin backbone. In order to increase the reactivity and reproducibility of conjugation reaction on inulin backbone, primary amine groups were conjugated to the inulin by reaction with EDA (Scheme 1). Thus, the first step for the synthesis of the inulin-graft co-polymers was the preparation of the reactive INU-EDA co-polymer. Then, the two amphiphilic inulin derivatives, INU-ceramide and INU-ceramide-PEG_{2000} were synthesized starting from INU-EDA by means of two consecutive single step reactions.

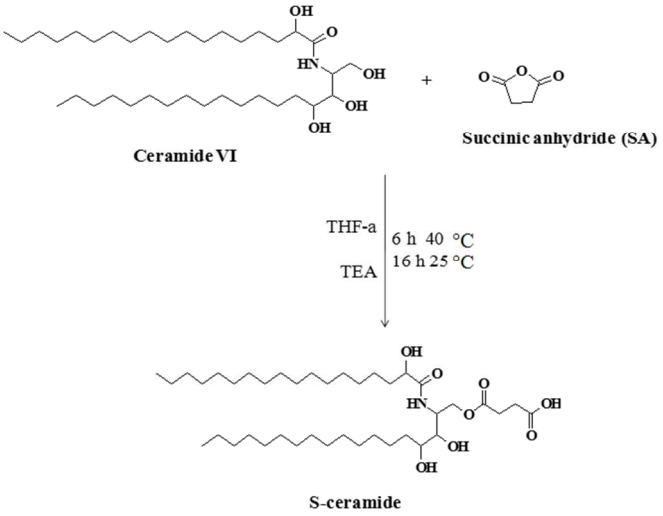


Scheme 1: Synthesis of INU-EDA

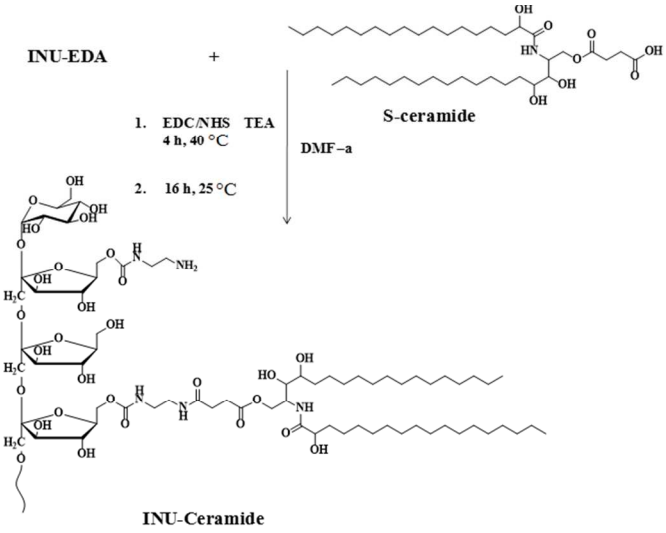
The resulting INU-EDA copolymer, being soluble in water as well as in organic solvents and bearing a programmable number of reactive primary amine groups in the inulin side chain, makes possible its extensive side chain functionalization with different molecules also in consecutive reaction steps. Hydroxyl groups present in the inulin side chain were firstly activated by reacting with BNPC, in anhydrous DMF solution at 60°C by means of microwave reactor (Scheme 1). Molar ratio between moles of activating agent (BNPC) and inulin repeating units (RU), (BNPC/RU) and the microwave assisted synthesis permitted to achieve a precise and repetable activation degree of hydroxyl groups of the polysaccharide. For example, by using a BNPC/RU moles ratio of 0.5 and an activation time of 1h it was obtained an activation degree of hydroxyl groups of inulin equal to 30 mol%. Subsequently, activated inulin reacted with an excess of EDA, obtaining the INU-EDA copolymer with a reproducible EDA derivatization degree of 28 mol%.

The conjugation of ceramide to the INU-EDA via amidic bond was achieved using the succinil derivative of ceramide (S-ceramide), previously synthesized by reaction of ceramide with succinic anhydride, as shown in Scheme 2.

Subsequently, for the conjugation of the ceramide to INU-EDA copolymer, S-ceramide was primarily activated using EDC and NHS as a coupling agents to activate the carboxyl group of S-ceramide (Scheme 3). The amounts of S-ceramide and carboxylic acid activating agents (EDC-HCl and NHS) were used according to: moles of S-ceramide/moles of amino pendant groups in INU-EDA = 0.1; moles of EDC-HCl/moles of S-ceramide = 1.2 and moles of NHS/moles of S-ceramide = 1.2.



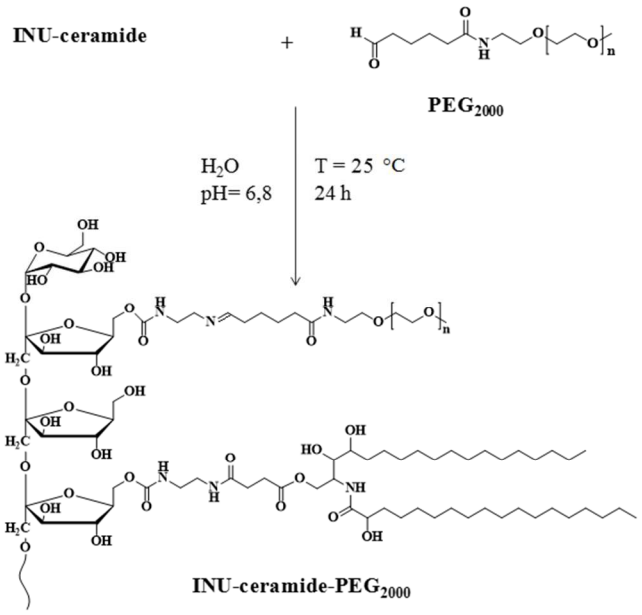
Scheme 2: Synthesis of succinyl-ceramide (S-ceramide).



Scheme 3: Synthesis of INU-ceramide

The ^{1}H-NMR allowed an easy quantification of the ceramide linked to inulin by comparing the integral of the peak attributable to the methylene groups of ceramide at δ1.45 to that belonging to the protons of INU backbone at δ comprised between 3.55 and 4.25. The molar percent of ceramide covalently linked to INU (DD_{CER}%) was equal to 1.8±0.5 mol% referred to the inulin repeating units. We calculated that the number of ceramide molecules covalently linked to INU-ceramide was approximately 1-2 for each 100 repeating units. After this functionalization reaction the INU-ceramide

conjugates still maintain a large number of primary amine groups (about 90 mol%) available for a potential subsequent side chain functionalization with other molecules, such as PEG. Finally, the synthesis of INU-ceramide-PEG_{2000} graft copolymer was performed starting from INU-ceramide by the subsequent reaction of INU-ceramide with an aldehyde functionalized mono-methoxy PEG having an average molecular weight of 2000 Da. The terminal aldehyde functional group of PEG easily reacted with the primary amine groups still present in the inulin side chain in aqueous medium, thus obtaining INU-ceramide-PEG_{2000} graft copolymer (see Scheme 4).



Scheme 4: Synthesis of INU-ceramide-PEG_{2000}

INU-ceramide-PEG_{2000} copolymer was characterized through ^{1}H-NMR spectroscopy, which confirmed the introduction of PEG_{2000} chains on the INU-ceramide backbone and permitted the calculation of molar derivatization degree (DD_{PEG}%). The DD%, indicated as percentage of linked polyethylene chains in comparison with repeating units of inulin, was calculated by comparing the integral of the peak related to protons at δ 3.62 assigned to ethylene protons of PEG with the integral of the peaks related to protons of INU backbone at δ comprised between 3.65 and 4.25. The result of the above calculation was that about 1-2 PEG chain was covalently linked for each 100 repeating units.

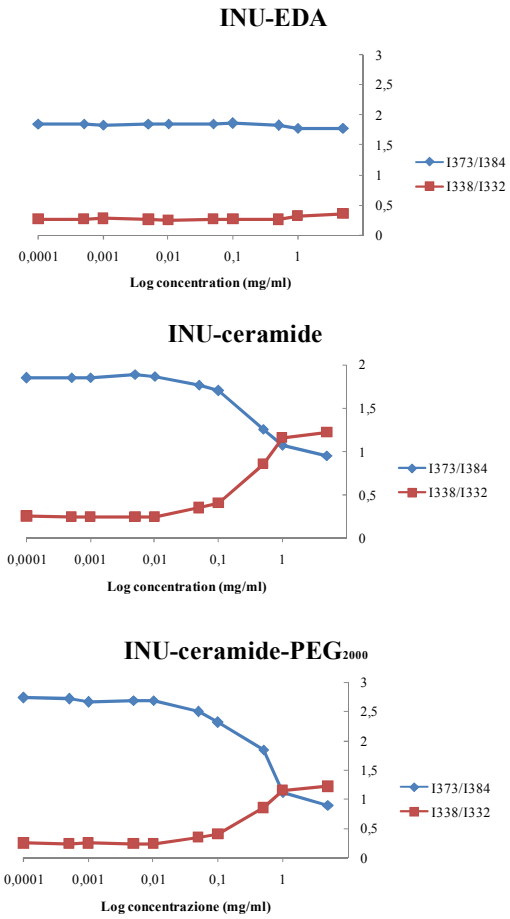
The chemical structures of all synthesized inulin copolymer were exhaustively characterized by ^{1}H-NMR spectroscopy.

Weighted average molecular weights (Mw) and polydispersity of synthesized copolymers were determined by SEC analyses. The average molecular weights (Mw) of INU-ceramide and INU-ceramide-PEG_{2000} were 2.9 and 3.7 kDa respectively. Definitively, the greater molecular weight of INU-ceramide than the starting INU-EDA copolymer can be attributed to the conjugation of ceramide. Similarly, the greater molecular weight of INU-ceramide-PEG_{2000} is certainly due to the conjugation of PEG_{2000}, even if it was expected for INU-ceramide-PEG_{2000}, in theory, a molecular weight around 4.9 kDa (2.9+2.0 kDa), but it must be considered that the obtained experimental error is intrinsic to the SEC analysis approach which is based on a relative calibration curve obtained using molecular weight standards chemically different from the analysed samples (ex. Pullulan). Actually, the increased molecular weight find for INU-ceramide-PEG_{2000} (3.7 KDa) is a confirmation of the PEG conjugation because indicated an increased hydrodynamic radius of the resulting macromolecules and consequently a greater average molecular weight. Conversely, it was find that microwave assisted synthesis in the presence of EDA caused a reduction of the starting inulin Mw, due to secondary hydrolysis reactions on polysaccharide chains. Molecular characterization data are summarized in table 1.

Copolymers	Composition			#Mw (kDa)	#Polydispersity M_w/M_n
	*DD_{EDA} %	*DD_{CER}%	*DD_{PEG}%		
Inulin	-	-	-	4,9	1.3
INU-EDA	28±1	-	-	2,5	1.8
INU-ceramide	28±1	1,8±0,5	-	2,9	1.6
INU-ceramide-PEG_{2000}	28±1	1,8±0,5	1,7±0,2	3,7	1.2

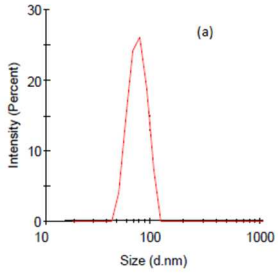
Table 1: Molar derivatization degree values of EDA, ceramide and PEG linked to inulin and molecular weight (Mw) and polydispersity of the correspondent copolymers.
*Calculated by means of ^{1}H-NMR spectroscopy.
#Calculated by SEC analysis.

Preparation and characterization of micelles. The ability of INU-ceramide and INU-ceramide-PEG$_{2000}$ graft-copolymers to



self-assemble into micelles was investigated by means of fluorescence analysis using pyrene as fluorescent probe. This analysis is a very sensitive method for detecting the formation of polymeric micelles and measure the CAC of amphiphilic copolymers[11,18] by plotting both the I_{373}/I_{384} (I_1/I_3) ratio, obtained from the emission spectra, and the I_{338}/I_{332} ratio obtained from the pyrene excitation spectra recorded at 37 ◦C, versus the logarithm of the copolymer concentration, as shown in Figure 1. The CAC was taken from the intersection of the tangent to the curve at the inflection with the tangent of the horizontal tract of the curve. Both INU-ceramide and INU-ceramide-PEG$_{2000}$ empty micelle showed CAC values very similar (6×10^{-2} and 5×10^{-2} mg/mL respectively). As expected, the INU-EDA copolymer did not shown inflection in the curves obtained from the pyrene excitation and emission spectra at all concentrations tested, confirming the absolute hydrophilicity of the copolymer.

Figure 1: I_1/I_3 intensity ratio obtained from pyrene emission spectra and I_{338}/I_{332} intensity ratio obtained from pyrene excitation spectra in the presence of INU-EDA, INU-ceramide and INU-ceramide-PEG$_{2000}$, as a function of the logarithm of copolymer concentration.



The hydrodynamic diameter of the micelles and the polydispersity index (PDI), obtained by using the Zetasizer instrument, was measured on the micelle samples prepared with INU-ceramide and INU-ceramide-PEG$_{2000}$ copolymers at a concentration above the CAC of 0.2 mg/mL, and resulted 76 nm (Figure 2a; PDI = 0,21) and 78 nm (Figure 2b; PDI = 0,19) respectively. Size measurements were performed in ultrapure water. Zeta potential values were also measured indicating a positively charged surfaces with values of 33,2±5,86 mV for INU-ceramide and 25,6±5,3 mV for INU-ceramide-PEG$_{2000}$ micelles. The positive charge found for the micelles is certainly due to presence of free EDA amine groups still present in the polymer side chain.

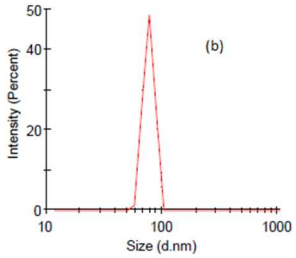


Figure 2: DLS size distribution histograms of INU-ceramide (a) and INU-ceramide-PEG$_{2000}$ (b) micelles.

To evaluate the ability of the obtained micelles to act as a drug-delivery system, they were loaded with doxorubicin (DOXO), used as model anti-cancer drug. Moreover, the fluorescence properties of DOXO is an advantage employed to evaluate its uptake into cells by means of fluorescence microscopy. The drug loading procedure was carried out by dispersing an organic solution of copolymer and proper amount of DOXO in water, into a dialysis tube, as reported in the Experimental Part[19,20]. This technique permitted to obtaining a high amount of loaded drug, equal to 16-17 weight % with respect to drug-loaded micelles, with an encapsulation efficiency of 70%.

Incubation conditions	Micelle sample	Hydrodynamic diameter (nm)	PDI	Zeta potential (mV)
H_2O time = 0	INU-ceramide/DOXO	101.2	0.41	+3.77 ± 3.5
	INU-ceramide-PEG$_{2000}$/DOXO	237.8	0.17	-0.749 ± 1.8

H_2O time = 72 h	INU-ceramide/DOXO	86.8	0.43	+28.6 ± 7.5
	INU-ceramide-PEG_{2000}/DOXO	195.2	0.12	+3.5 ±2.9
PBS pH 7.4 time = 0	INU-ceramide /DOXO	97.2	0.37	+3.8 ±0.2
	INU-ceramide-PEG_{2000}/DOXO	242.4	0.17	+3.29 ± 0.4
PBS pH 7.4 time = 72 h	INU-ceramide/DOXO	80.6	0.34	+3.66 ± 0.5
	INU-ceramide-PEG_{2000}/DOXO	207.7	0.1	-1.5 ± 0.12
PBS pH 5.5 time = 0	INU-ceramide/DOXO	95.2	0.37	+1.47 ± 0.5
	INU-ceramide-PEG_{2000}/DOXO	262.9	0.17	+0.54 ± 0.4
PBS pH 5.5 time = 72 h	INU-ceramide/DOXO	70.5	0.42	-0.35 ± 0.2
	INU-ceramide-PEG_{2000}/DOXO	209.7	0.17	+1.13 ± 0.4
PBS pH 7.4 + 10% FBS time = 0 h	INU-ceramide/DOXO	130.2	0.76	+0.497 ± 0.2
	INU-ceramide-PEG_{2000}/DOXO	218.2	0.32	+0.277 ± 0.1
PBS pH 7.4 + 10% FBS time = 72 h	INU-ceramide/DOXO	73.86	0.67	-2.62 ± 1.2
	INU-ceramide-PEG_{2000}/DOXO	145.9	0.24	-0.8 ± 0.4

Table 2: Mean diameter, polydispersity index (PDI) and zeta potential values of DOXO-loaded INU-ceramide and INU-ceramide-PEG_{2000} micelles as measured in ultrapure water (H_2O), PBS at pH 7.4 and PBS at pH 7.4 with 10% of FBS. Measures were performed immediately after the preparation and also after 72 h storage at 25°C in the same aqueous media.

Size measurements of DOXO-loaded micelles were performed by DLS in three different aqueous media, both immediately after the preparation and also after 72 h storage time at 25°C to verify the physical stability of the micelles. In particular, the size and the stability studies were performed by measuring the hydrodynamic diameter and the zeta potential of the micelles in ultrapure water, PBS at pH 7.4 and PBS at pH 7.4 with 10% of FBS. Obtained data are reported in Table 2.

As shown in Table 2, DOXO-loaded INU-ceramide micelles had a slightly larger diameter than unloaded micelles, and this as a consequence of the encapsulation of DOXO molecules into the whole micelles structure[21]. In particular, DOXO-loaded INU-ceramide-PEG_{2000} micelles shown mean diameters significantly higher than the INU-ceramide micelles, probably due also to the presence of a hydrated PEG chains corona onto the micelle surface that increases the hydrodynamic diameter of the carrier. However, the micelles are still small enough to be administered for all administration routes, including intravenous. On the other hand, the presence of the PEG corona gives the advantage to reduce the adsorption of the serum proteins and the capture by the RES, with a rapid elimination from the blood stream[22]. Moreover, it is interestingly to note that in both cases the hydrodynamic diameter of the micelles decrease after 72h. This fact can be explained supposing a micelles contraction as a consequence of the release of the loaded drug[21]. Finally, the zeta potential of DOXO-loaded micelles undergo a drastic reduction inferring that encapsulated drug molecules are presumably enclosed into the micelle periphery as well. AFM observation of DOXO-loaded micelles dried from water (Figure 3), gave clear 3D morphological images that confirmed the size obtained by DLS analysis and highlighted an approximate spherical shape and no aggregation or adhesion among micelles.

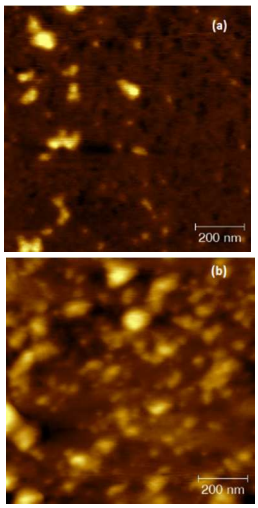
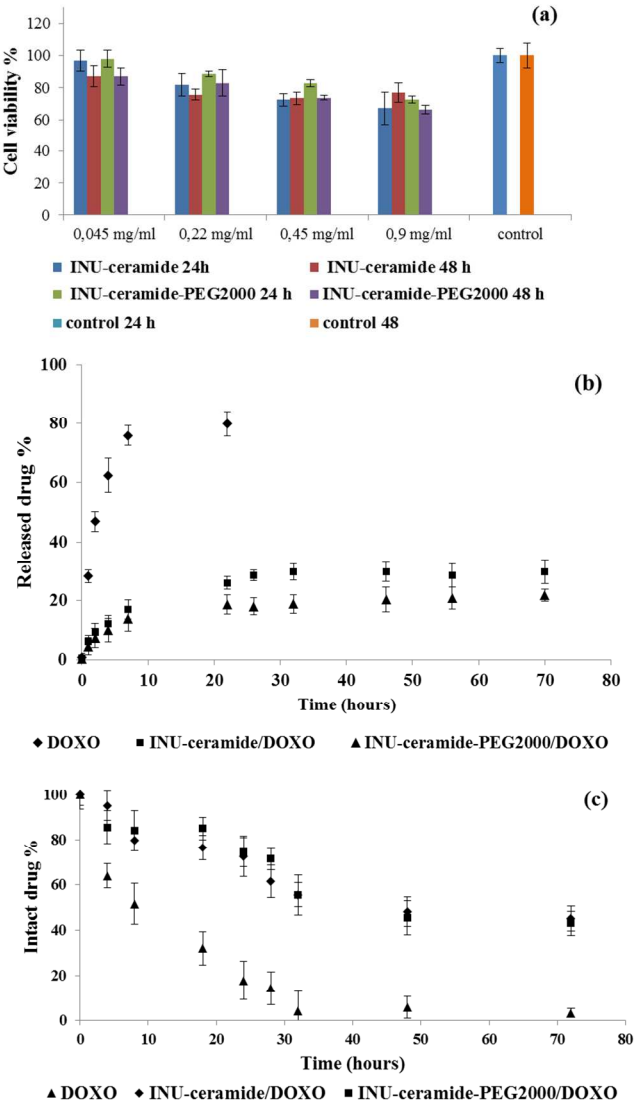


Figure 3: AFM images of DOXO-loaded INU-ceramide (a, a') and INU-ceramide-PEG_{2000} (b, b') micelles.

Drug release and stability studies. Drug release and stability studies were performed in order to demonstrate the ability of the micelles to release the loaded model drug in the intact form. To this aim, release experiments were carried out in different media simulating physiological pH or body compartments. In the first case, as incubating media were used PBS solution at pH 7.4 (Figure 4a) and 5.5 (Figure 4b), mimicking interstitial fluids and intracellular pH respectively; in the second case, as incubating medium was used human plasma (Figure 4c). In the last case, a stability evaluation of the carrier upon parenteral administration can also be extrapolated. Independently from the incubation medium used, the release experiments at the two pH conditions consisted into quantify the amount of released and intact DOXO in the external medium, at pre-determined time intervals and until 72 h. In Figures 4a and b are shown the amount of released DOXO referred to the total amount of DOXO loaded into the micelles, in function of incubation time.

These experiments demonstrated that prepared micelles were able to release DOXO in the intact form for a prolonged period and without a first burst release. Differently, stability studies in human plasma were carried out by quantifying the total amount of intact DOXO still solubilized in the serum in function of incubation time and until 72 h. In order to be sure that the



detected doxorubicin was in the intact form, a high-performance liquid chromatography (HPLC) analysis was used for quantization. As shown in Figure 4c, this experiment demonstrated that prepared micelles were able to protect DOXO against protein binding and to reduce the drug inactivation, if compared with the free DOXO. In fact, intact free DOXO disappears from human plasma in about 30 h, while DOXO-loaded INU-ceramide and INU-ceramide-PEG$_{2000}$ micelles maintain the concentration of DOXO in the plasma, in the intact form, at very high values (above 50 % of the administered dose, 0.85 mg) until 72 h.

Figure 4: Drug release and stability study profiles of DOXO-loaded INU-ceramide and INU-ceramide-PEG$_{2000}$ micelles and free DOXO in PBS solution at pH 7.4 (a), at pH 5.5 (b) and human plasma (c).

In vitro biological evaluations. Cytotoxicity of unloaded and DOXO-loaded INU-ceramide and INU-ceramide-PEG$_{2000}$ micelles and free DOXO was evaluated by the MTS assay on two different cell lines; one, the human bronchial epithelial (16HBE) is a non tumoral cell line extensively used as model normal cells to screen cytotoxicity of novel compounds or carriers[23,24]; the other one, the human colon cancer (HCT116), is a cancer cell line used to investigate the anti-cancer activity of drugs and the associated mechanism of action[25]. These cells were incubated with the micelles at concentrations of 0.045, 0.22, 0.45 and 0.9 mg/mL, for 24 and 48 h. The results, in term of cell viability (%) as a function of samples concentration are shown in Figure 5a and b.

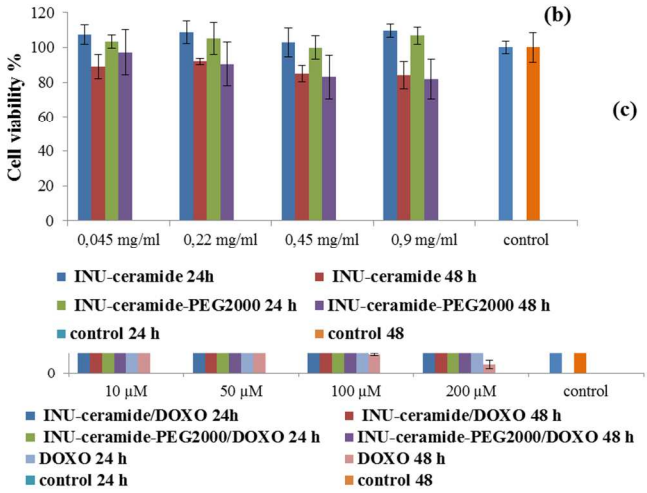
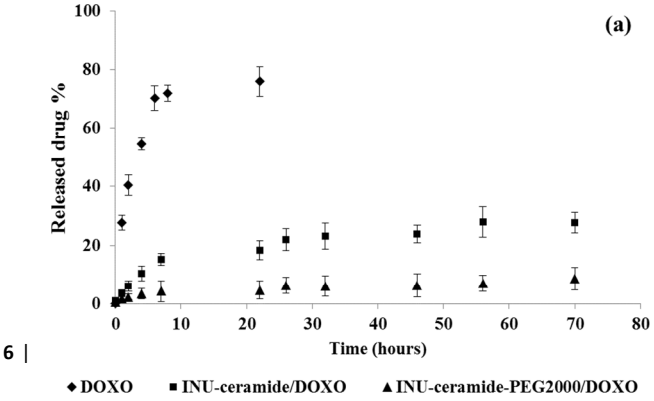
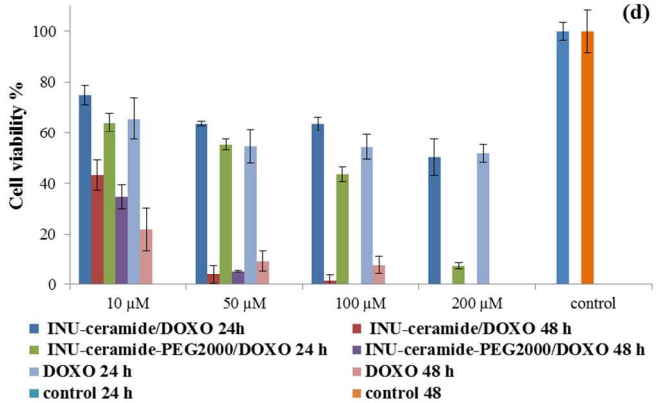


Figure 5: Cell viability % (MTS assay) of: unloaded INU-





ceramide and INU-ceramide-PEG$_{2000}$ micelles on human bronchial epithelial (16HBE) cells (a) and human colon cancer (HCT116) cells (b), at polymer concentration of 0.045, 0.22, 0.45 and 0.9 mg/mL; DOXO-loaded INU-ceramide and INU-ceramide-PEG$_{2000}$ micelles and free DOXO on human bronchial epithelial (16HBE) cells (c) and human colon cancer (HCT116) cells (d) at the concentrations corresponding to 10, 50, 100 and 200 μM of DOXO loaded into micelles, at 24 and 48 h of incubation. The results are reported as the mean±SD (n = 6).

The data show that for both INU-ceramide and INU-ceramide-PEG$_{2000}$ micelles, cell viability was always above 80% for all the tested concentrations on normal and cancer cells, thus indicating a very low cytotoxicity of empty micelles. Differently, DOXO-loaded micelles at DOXO concentration of 10, 50, 100 and 200 μM (corresponding to the concentration of drug loaded into micelles) show a cytotoxicity depending on incubation time and concentration of DOXO. In particular, on normal cells (16HBE) free DOXO (used as positive control) is always more cytotoxic than DOXO-loaded micelles, but it is interesting to note that on cancer cells DOXO-loaded micelles show cytotoxicity values at 48 h of incubation that are higher than free DOXO, at the concentrations of 50, 100 and 200 μM. This preferential cytotoxic activity of DOXO-loaded micelles versus cancer cells rather that normal cells, is a very important advantage in the treatment of cancer, in view to reach the optimal pharmacological effect and the lowest secondary effects. Actually, considering the lack of cytotoxic effects attributable to the empty micelles, we can suppose that the higher activity of DOXO loaded into micelles is attributable to a more productive micelle uptake by the tested cancer cells respect the free drug. Moreover, we cannot exclude that this ability is probably due to the presence of ceramide acting as permeability enhancer agent[26]. For this reason, the potential ability of these micelles to increased drug uptake was evaluate. HCT116 cells were incubated with DOXO-loaded micelles, at a drug concentration of 50 μM. After predetermined intervals, the cell membranes were stained with DAPI and observed with a fluorescence microscope. Figure 6 shows the image of cells after 24 (A, B, C) and 48h (D, E, F) of incubation. Our findings showed that the free DOXO is visible in the cell nuclei of HCT116 cells just after 24h (Figure 6A) of incubation, while the red fluorescence of DOXO-loaded INU-ceramide (Figure 6B) and INU-ceramide- PEG$_{2000}$ (Figure 6C) micelles was mainly located in the cytoplasm.

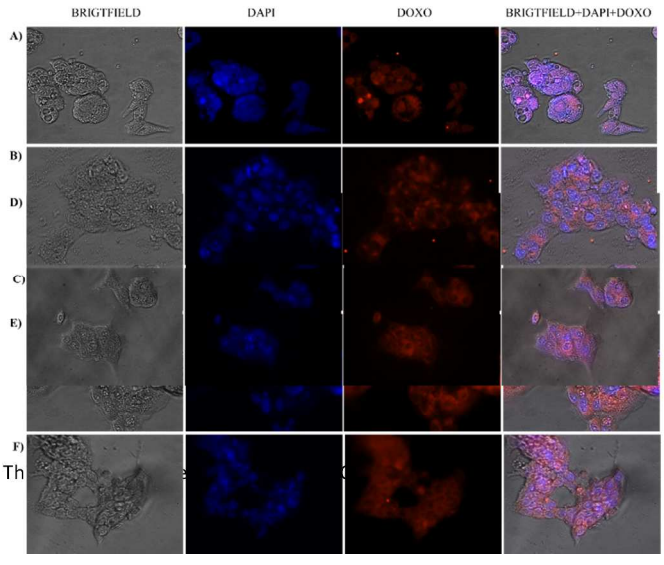


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Figure 6: Fluorescence microscopy images of HCT116 cells incubated with free DOXO (A and D), DOXO-loaded INU-ceramide (B and E) and INU-ceramide-PEG$_{2000}$ (C and F) micelles for 24 (A-C panel) and 48 h (D-F panel). DOXO is visualized in red; Cell nuclei were stained with DAPI (blue). Magnification is 40X for all images.

After 48 h of incubation, an evident red fluorescence of DOXO appeared in both the cytoplasm and nucleus, only for cells treated with DOXO-loaded INU-ceramide (Figure 6E) and INU-ceramide-PEG$_{2000}$ (Figure 6F) micelles. Actually, the fluorescence of the cells treated with the micelles is always higher than that of cells treated with free DOXO. These data suggest that INU-ceramide and INU-ceramide-PEG$_{2000}$ micelles may facilitate DOXO uptake by cancer cells, compared with free drug and agree with the in vitro cytoxicity results.

Obtained results and literature as well[26], support the hypothesis that the action mechanism of the DOXO-loaded micelles is mainly based on the preliminary carrier internalization via endocytosis and hence the cytoplasmic release of the DOXO from the micelles. Considering that the DOXO antiproliferative activity is due to its DNA intercalating feature, only the free drug that reaches the nuclei of the cancer cells can ensure the DNA intercalation, and this fact justifies the retarded antiproliferative activity observed in the case of DOXO-loaded micelles. However, this characteristic may have a significant advantage over the free drug with respect to the phenomenon of the multi-drug resistance in anticancer chemotherapy. The cytoplasmic efflux pumps that easily recognize free drug molecules as substrate, providing their elimination commonly mediate anticancer chemotherapy resistance. When the drug is loaded into micelles, it cannot be recognized by the efflux pumps and expelled. In this case, micelles provide an intracellular reservoir of the drug, which is gradually released and internalized within the nuclei.

Experimental

Materials and methods. Ceramide VI was obtained from Evonik Industries (USA). Inulin, anhydrous tetrahydrofuran (a-THF), triethylamine (TEA), ethylenediamine (EDA) succinic anhydride (SA), Bis(4-nitrophenyl)carbonate (BNPC), doxorubicin hydrochloride (DOXO-HCl) were purchased from Aldrich (Milan, Italy). N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC-HCl), O-[2-(6-oxocaproylamino)-ethyl]-O'-methylpolyethylene glycol 2000 (PEG-COH$_{2000}$), Sephadex G-15, anhydrous dimethylformamide (a-DMF), dimethyl sulfoxide (DMSO) were purchased from Fluka (Switzerland). The purity of all reagents was of analytical grade.

The ^{1}H-NMR spectra were recorded using a Bruker Avance II 300 spectrometer operating at 300 MHz. Fluorescence spectroscopy was performed by using a Shimadzu RF-5301 PC

spectrofluorimeter. Centrifugations were performed using a Centra MP4R IEC centrifuge. Size exclusion chromatography (SEC) was carried out using a Phenomenex PolySep-GFC-P3000 column (California, USA) connected to a Water 2410 refractive index detector. Phosphate buffer pH 6.5/methanol 9:1 (v/v) solution was used as eluent at 35 °C with a flux of 0.6 mL/min, and pullulan standards (112.0-0.18 kDa, Polymer Laboratories Inc., USA) were used to set up calibration curve.

Synthesis of succinyl-ceramide (S-ceramide). Succinil-ceramide was obtained by reaction of ceramide with succinic anhydride, according to the following procedure. Ceramide (100 mg, 0.167 mmol) and TEA (10 µL, 0.072 mmol) were dissolved in 5 mL of a-THF. Then a solution of SA (25 mg, 0.25 mmol) in a-THF (1 mL) was added to the ceramide solution. The resulting mixture was kept at 40°C for 6 h followed by 16 h at 25 °C under stirring. The reaction mixture was concentrated under vacuum, and the obtained S-ceramide derivate washed several times with double distilled water. After purification the S-ceramide residue was freeze dried from water; S-ceramide was obtained with a yield of 100% based on the starting Ceramide. The obtained Ceramide derivative was characterized by ^{1}H-NMR (300 MHz, $CDCl_3$/$DMFd_7$ 8:1): δ 0.89 (6H, **CH_3**-CH_2-), 1.20-1.90 (54H, CH_3-(**CH_2**)^{26}-), 2.40-2.60 (4H$_{Succinyl}$, -O(CO)-**CH_2**-**CH_2**-(COOH) 3.36 (4H, -CH_2-**CH_2**-CH(OH)-), 3,46-4.20 (4H, -O**H**), 4.35 (1H, -CH_2-**CH**-(OH)-), 4.70-4.90 (2H, -**CH**-(OH)-; -**CH**-CH_2-OH), 5.0 (1H, -**CH**-(OH)-CO-), 7.05 (1H, -N**H**-).

Microwave-assisted synthesis of inulin-(2-aminoethyl)-carbamate (INU-EDA) copolymer. BNPC (236 mg, 0.775 mmol), dissolved in 1 mL of a-DMF, was added drop-wise to 4 ml of inulin (previously dried at 70°C for 24 h) solution in a-DMF (62.5 mg/mL). The reaction mixture was transferred into a cylindrical glass reaction vessel and irradiated for a 1h with microwave at a power of 25W in a CEM Discover Microwave Reactor. The reaction temperature was monitored and maintained at 60°C by cooling with external compressed air flow. After the activation time, the reaction mixture was added drop-wise to an EDA solution prepared by dissolving 250 µL of EDA in 1 mL of a-DMF. The reaction mixture was kept under stirring for 1 h at 25°C. After this reaction time the obtained product was precipitated in a diethyl ether/dichloromethane mixture (2:1 vol/vol) and the suspension was centrifuged at 5°C for 5 min, at 9,000 rpm. The obtained solid residue was recovered and washed three times with acetone to remove excess of unreacted EDA and BNPC. Then, the solid product was dissolved in 2 ml of bidistilled water and further purified by gel permeation chromatography using Sephadex G-15 as separating gel. After purification the INU-EDA solution was freeze dried from water; the pure product was obtained with 100% yield based on the starting inulin. The obtained copolymer INU-EDA was characterized by ^{1}H NMR (300 MHz, D_2O): δ 2,90-3.40 (4H$_{EDA}$, -NH-**CH_2**-**CH_2**-NH_2), 3,60-3.90 (5 H$_{INU}$, -**CH_2**-OH; -**CH**-CH2-OH; -C-**CH_2**-O-), 3.92-4,30 (2H$_{INU}$, -C-**CH**-OH; -**CH**-OH).

Conjugation of succinyl-ceramide to INU-EDA (INU-ceramide). EDC-HCl (3.6 mg, 0.019 mmol) in 400 µL of a-DMF, NHS (2.2 mg, 0.019 mmol) in 200 µL of a-DMF and 3µL of TEA in 100 µL of a-DMF were added drop-wise to 1 mL of a S-ceramide solution (11 mg, 0.0158 mmol) in a-DMF. The solution was allowed to react for 4 h at 40°C. After the activation time, the resulting solution was added drop-wise to an INU-EDA solution (25 mg/mL) in 4 mL of a-DMF and left to react at 25°C for 16 h.
The INU-ceramide conjugate was then precipitated in diethyl ether/dichloromethane mixture (2:1 vol/vol) and collected by centrifugating at 5°C for 5 min, at 9,000 rpm. The solid residue was then washed four times with diethyl ether/THF mixture (2:1). The obtained solid product was dried under vacuum and weighted. INU-ceramide was obtained with a yield of 80% (w/w) based on the starting inulin. The obtained copolymer was characterized by ^{1}H NMR (300 MHz, D_2O/$DMF-d_7$ (2:1): δ 1.06 (6H$_{ceramide}$, **CH_3**-CH_2-), 1.45 (54H$_{ceramide}$, CH_3-(**CH_2**)^{26}-), 2.64 (4H$_{Succinyl}$, -O(CO)-**CH_2**-**CH_2**-(CO)O-), 2.80-3.34 (4H$_{EDA}$, -NH-**CH_2**-**CH_2**-NH-), 3.35-3.50 (4H$_{ceramide}$, -CH_2-**CH_2**-CH(OH)-), 3.55-3.88 (5 H$_{INU}$, -**CH_2**-OH; -**CH**-CH2-OH; -C-**CH_2**-O-), 3.90-4.25 (2H$_{INU}$, -C-**CH**-OH; -**CH**-OH). 4.37 (1H$_{ceramide}$, -CH_2-**CH**-(OH)-), 4.80-5.01 (2H$_{ceramide}$, -**CH**-(OH)-; -**CH**-CH_2-OH), 5.32 (1H$_{ceramide}$, -**CH**-(OH)-CO-).

Synthesis of INU-ceramide-PEG$_{2000}$ graft copolymer. INU-ceramide previously synthesized (120 mg) was dissolved in double distilled water (4 mL) and the pH of this solution was adjusted to 6.8 with HCl 0,1 N solution. Then, 35 mg of PEG-COH_{2000} (0.0175 mmol) were added. The pH of the reaction mixture increase slowly and HCl 0.1 N was added to maintain pH at 6.8. The mixture was left under stirring at room temperature overnight. The reaction mixture was then dried by vacuum and the solid residue was washed three time with diethyl ether/dichloromethane mixture (2:1 vol/vol). The solid product was dried under vacuum and weighted. INU-ceramide-PEG$_{2000}$ was obtained with a yield of 98% (w/w) based on the starting inulin. The obtained graft copolymer was characterized by ^{1}H NMR (300 MHz, D_2O/$DMF-d_7$ (2:1): δ 0.90 (6H$_{ceramide}$, **CH_3**-CH_2-), 1.20 (54H$_{ceramide}$, CH_3-(**CH_2**)^{26}-), 2.60 (4H$_{Succinyl}$, -O(CO)-**CH_2**-**CH_2**-(CO)O-), 2.85-3.25 (4H$_{EDA}$, -NH-**CH_2**-**CH_2**-NH-), 3.35-3.50 (4H$_{ceramide}$, -CH_2-**CH_2**-CH(OH)-), 3.62 (176H$_{PEG2000}$, -O-**CH_2**-**CH_2**-O-), 3.65-3.88 (5 H$_{INU}$, -**CH_2**-OH; -**CH**-CH2-OH; -C-**CH_2**-O-), 3.90-4.25 (2H$_{INU}$, -C-**CH**-OH; -**CH**-OH). 4.37 (1H$_{ceramide}$, -CH_2-**CH**-(OH)-), 4.80-5.01 (2H$_{ceramide}$, -**CH**-(OH)-; -**CH**-CH_2-OH), 5.32 (1H$_{ceramide}$, -**CH**-(OH)-CO-).

Determination of critical aggregation concentration (CAC). The CAC of INU-EDA, INU-ceramide and INU-Ceramide-PEG$_{2000}$ were determined by fluorescence analysis, using pyrene as probe[11,18]. A stock solution of pyrene (6.0×10^{-5}M in acetone) was prepared and then aliquots of 20 µL were placed into vials and evaporated to remove acetone in an orbital shaker at 37°C. Subsequently, 2 mL of aqueous copolymer solution at

concentrations ranging from 1×10^{-5} to 5 mg/mL were added to the pyrene residue; the final concentration of pyrene was 6.0×10^{-7}M in each sample. The solutions were kept at 37°C for 24 h under continuous stirring to equilibrate pyrene with micelles. Pyrene excitation and emission spectra were recorded at 37°C using an emission wavelength of 373 nm and an excitation wavelength of 333 nm.

Preparation of doxorubicin loaded micelles. Encapsulation of DOXO into INU-ceramide and INU-ceramide-PEG_{2000} micelles was carried out through membrane dialysis method, as elsewhere reported[19,20]. Briefly, 10 mg of DOXO-HCl was dissolved in 1 mL of DMSO and neutralized with 4 moles excess of TEA. The DOXO solution was mixed with the polymer solution (50 mg in 1 mL of DMSO) by vortexing, and the mixture was added drop-wise to 10 mL of double distilled water and sonicated for 15 minute. The micelles dispersion was dialyzed for 24 h against 1 L of double distilled water using Spectra Por Dialysis Tubing with molecular weight cut-off (MWCO) of 1,000 Da. After 24 h the micelles dispersion was collected, frozen by immersion in liquid nitrogen and freeze-dried from water. INU-ceramide and INU-ceramide-PEG_{2000} DOXO loaded micelles were obtained with a yield of 90 and 96 weight % respectively. Empty micelles were prepared by using the above reported procedure in the absence of DOXO.

Characterization of micelles

Dynamic light scattering (DLS) analysis and ζ potential measurements. The mean diameter, width of distribution (polydispersity index, PDI) and ζ potential of the micelles were mesured at 25°C using a Zetasizer NanoZS instrument, fitted with a 532 nm laser at fixed scattering angle of 173° The intensity-average hydrodynamic diameter (size in nm) and polydispersity index (PDI) of INU-ceramide and INU-ceramide-PEG_{2000} micelles (0.2 mg/mL) whether loaded with DOXO or empty, were obtained in three different media; double distilled water, phosphate buffer solution (PBS) at pH 7.4 and PBS at pH 7.4 with 10% of foetal bovine serum (FBS). The mean diameter and PDI were measured after the dissolution of samples and also after 72 h of samples's incubation in the three different media. 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.

Atomic force microscopy (AFM). For AFM a Multimode V Nanoscope Veeco microscope, driven by a nanoscope controller was used. For samples preparation a drop (20 µL) of micelle dispersion in double distilled water (pH~6) at 0.1 mg/mL concentration was deposited onto freshly cleaved mica and allowed to dry freely in air, then observed with the microscope.

Determination of loaded drug amount into micelles and drug release studies. The amount of DOXO loaded into INU-ceramide and INU-ceramide-PEG_{2000} micelles was determined by UV spectroscopy. Samples were prepared by dispersing known amounts of DOXO loaded micelles in double distilled water at pH~6 and measuring the absorbance of DOXO 480 nm. A calibration curve was obtained for serially diluted concentrations of DOXO-HCl in bidistilled water at pH~6. The content of drug loaded into the micelles was expressed as the amount of loaded DOXO per unit mass of dry micelles, and resulted to be between 16–17%, while the encapsulation efficiency was calculated to be 70%.

For drug release studies, an appropriate amount (5 mg) of freeze dried DOXO loaded INU-ceramide and INU-ceramide-PEG_{2000} micelles and DOXO-HCl alone (as positive control) were suspended in phosphate buffer PBS at pH 7.4 (5 mL) and transferred inside of a floating Spectra/Por dialysis membrane (MWCO 1,000 Da). This dialysis membrane was immersed into PBS at pH 7.4 (15 mL) and incubated at 37°C under continuous stirring (100 rpm) in a Benchtop 808C Incubator Orbital Shaker model 420, for 72 h. At scheduled time intervals, aliquots of the external medium (1 mL) were withdrawn from the outside of the dialysis membrane and replaced with equal amount of fresh medium. The withdrawn samples were analysed by HPLC in order to determine the released intact drug amount. Profile releases were determined by comparing the amount of released drug as a function of incubation time with the total amount of drug loaded into the micelles. The same analysis was carried out using PBS at pH 5.5. Data were corrected taking in account the dilution procedure. Each experiment was carried out in triplicate and the results were in agreement within ±5% standard error.

Stability studies in human plasma. For this study, aliquots of 5 mg of DOXO loaded INU-ceramide and INU-ceramide-PEG_{2000} micelles were dispersed in 1 mL of human plasma and kept at 37±0.1 °C under orbital stirring as above reported. DOXO-HCl alone (0.85 mg) was also incubated as negative control. At suitable time intervals, ranging from 1 to 48 h, 2 mL of 10% (v/v) trifluoroacetic acid solution were added in order to precipitate plasma proteins. After immediate mixing and centrifugation for 5 min at 10,000 rpm at 4 °C, supernatants were filtered through a 0,45 µm regenerated-cellulose (RC) membrane filter and analysed by HPLC, 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 on line with a computerized workstation. As column a reversed-phase Gemini C18 Phenomenex (5 µm, 4.6×250mm 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 monitoring at 485 nm with a flow rate of 1.0 mL/min. A calibration curve of DOXO-HCl was used for drug quantification.

Cytotoxicity assay on human colon cancer (HCT116) and human bronchial epithelial (16HBE) cells. The cytotoxicity

was assessed by the 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 Romagna*, Italy) using a commercially available kit (Cell Titer 96 Aqueous One Solution Cell Proliferation assay, Promega). Cells were seeded in 96 well plate at a density of 2×10^4 cells/well and grown in Dulbecco's Minimum Essential Medium (DMEM) with 10% FBS (foetal bovine serum) and 1% of penicillin/streptomycin (10000U/mL penicillin and 10 mg/mL 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 empty and DOXO loaded INU-ceramide and INU-ceramide-PEG_{2000} micelles at a concentration per well equal to 0.045, 0.022, 0.45 and 0.9 mg/mL, corresponding to 10, 50, 100 and 200 µM of loaded DOXO. After 24 and 48 h of 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). Doxorubicin solution containing the same drug concentrations used in the tested drug loaded micelle samples, i.e., 10, 50, 100 and 200 µM, were used as a positive control, while pure cell medium was used as a negative control. Results were expressed percentage reduction of the control cells. All culture experiments were performed in triplicates.

Cell drug uptake studies. The cellular uptake and distribution of DOXO of the prepared micelles were evaluated by fluorescence microscopy (Zeiss "AXIO Vert. A1" Microscope Inverted) analysis. In particularly, for the uptake assay on HCT116 cell lines, 2×10^4 cells/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 INU-ceramide and INU-ceramide-PEG_{2000} micelles, and free DOXO at final drug concentration of 50 µM, and cells incubated for 24 and 48 h. After each incubation period, the medium was removed and the cell monolayer was washed twice with DPBS and fixed with 4% formaldehyde for 30 min. Subsequently, the formaldehyde solution was removed, the cells washed with DPBS and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). After incubation, DAPI solution was removed, the cells were washed with DPBS and observed by fluorescence microscope. 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*-test. All values are the average of three experiments ± standard deviation.

Conclusions

A new amphiphilic inulin graft copolymer able to self-assemble in water into micelle was synthesized starting from the inulin copolymer INU-EDA, bearing reactive primary amine groups in the side chain. Succinyl-ceramide firstly, and poly ethylene glycol 2000 secondly, were conjugated to INU-EDA producing the two amphiphilic inulin derivatives INU-ceramide and INU-ceramide-PEG_{2000}. These new inulin graft copolymers were able to self-assemble into micelles with CMC values of 6×10^{-2} and 5×10^{-2} mg/mL respectively and load the anticancer drug doxorubicin with 16-17 weight% of drug loading. The release experiments in different media demonstrated that prepared micelles were able to release DOXO in the intact form for a prolonged period and without a first burst release. Differently, stability studies in human plasma demonstrated that prepared micelles were able to protect DOXO against protein binding and to reduce the drug inactivation, if compared with the free DOXO. Finally, cytotoxicity evaluation on both cancer (HCT116) and normal (16 HBE) cell lines evidenced a preferential cytotoxic activity of DOXO-loaded micelles versus cancer cells rather that normal cells. Moreover, results obtained by uptake studies on cancer cells let us to suppose that the higher activity of DOXO loaded into micelles may be attributable to a more productive micelle uptake by the tested cancer cells respect the free drug. This preferential cytotoxic activity of DOXO-loaded micelles versus cancer cells rather that normal cells, is a very important advantage in the treatment of cancer, in view to reach the optimal pharmacological effect and the lowest secondary effects.

Acknowledgements

The authors would like to thank Dr. G. Buscarino from the Department of Fisica e Chimica of the University of Palermo for AFM analysis. The authors also thank the MIUR and the University of Palermo for funding.

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