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Novel folated and non-folated pullulan bioconjugates for anticancer drug delivery

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ABSTRACT

Two new anticancer polymer therapeutics were designed for tumour cell targeting. The bioconjugates were synthesised by pullulan derivatisation with either doxorubicin or doxorubicin and folic acid. Pullulan was activated by periodate oxidation and functionalised by reductive conjugation with cysteamine and 1.9 kDa PEG(NH_2)$_2$. The cysteamine thiol groups were conjugated to doxorubicin through a pH-sensitive hydrazone spacer while the pending PEG-NH_2 functions of one derivatised pullulan batch were conjugated to folic acid to obtain one of the two polymer therapeutics. The reaction intermediates and the final products were characterised by mass spectrometry, UV–vis analysis and reverse phase and gel permeation chromatography. The folic acid-free derivative [(NH_2 PEG)-Pull-(Cyst-Dox)] contained 6.3% (w/w) doxorubicin while the folic acid-doxorubicin-coupled derivative [(FA-PEG)-Pull-(Cyst-Dox)] contained 6% (w/w) doxorubicin and 4.3% (w/w) folic acid. Photon correlation spectroscopy showed that (NH_2 PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox) assembled into particles of about 150 and 100 nm diameter, respectively. The two bioconjugates displayed similar drug release profiles either at pH 7.4 buffer or in plasma, where less than 20% of doxorubicin was released within three days. At pH 5.5, both conjugates underwent complete drug release in about 40 h. *In vitro* studies carried out with KB tumour cells over-expressing folic acid receptor showed that both free doxorubicin and (FA-PEG)-Pull-(Cyst-Dox) were rapidly taken up by the cells, while the internalisation of the non-folated derivative was significantly slower. Cell viability studies did not show relevant difference between the two bioconjugates. After 72 h of incubation with folic acid receptor non-expressing MCF7 cells, the IC_{50} values of doxorubicin, (NH_2PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox) were 0.3 μM, 1.2 μM and 3.1 μM, respectively. After incubation with KB cells over-expressing folic acid receptor, the IC_{50} values were 0.4 μM, 1.8 μM and 1.1 μM, respectively. Pharmacokinetic studies showed that 4 h after intravenous administration of the conjugates to Balb/c mice about 40% of the administered drug equivalent dose was present in the bloodstream while in the case of unconjugated doxorubicin, 80% of the drug was cleared within 30 min.

These findings suggest that the novel doxorubicin–pullulan bioconjugates possess suitable properties for passive tumour targeting. On the other hand, folic acid conjugation has been found to have limited effect on selective cell up-take.

1. Introduction

In the recent years, many efforts have been made to develop novel colloidal and supramolecular drug delivery systems with enhanced biopharmaceutical properties, increased target selectivity, decreased toxicity and, therefore, improved therapeutic profiles compared to traditional formulations. Accordingly, challenging nanomedicines have been obtained by chemical or physical assembling of materials with different features and functions. Micelles, liposomes, nanoparticles and macromolecular prodrugs with environmentally tuneable properties that can control and selectively release drugs in the disease site have been developed, thus opening new perspectives for rational as well as personalised therapy (Peer et al., 2007; Lammers et al., 2008; Davis et al., 2008).

Polymer bioconjugation is a promising tool for anticancer chemotherapy (Duncan, 2006; Vicent et al., 2009; Li and Wallace, 2008) as it can enhance the biopharmaceutical performance of molecules with poor physicochemical properties and a low therapeutic index. According to Ehrlich's "magic bullet" concept (Strebhardt and Ullrich, 2008), Ringsdorf's macromolecular model (Ringsdorf, 1975) and the enhanced permeability and retention (EPR) effect described by Maeda and Matsumura (Maeda et al., 2000), a variety of polymer therapeutics have been designed to ameliorate the pharmacokinetic behaviour of anticancer drugs,

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favour the tumour targeting either by passive or active mechanisms, promote the cellular uptake into tumours and tumour endothelial cells and finally provide for intra- or extra-cellular drug release depending on the polymer-linker-drug design (Duncan, 2006; Vicent et al., 2009; Li and Wallace, 2008).

So far, polymer therapeutics have been obtained with synthetic and natural macromolecules, with dendrimeric, hyperbranched or linear architectures, that possess the requisites for systemic administration: solubility, biocompatibility and clearance ability (Khandare and Minko, 2006; Satchi-Fainaro et al., 2006; Segal and Satchi-Fainaro, 2009). Additionally, polymers featured with multiple functionalities can be conveniently used to anchor high drug amounts via releasable bonds and, eventually, to conjugate physicochemical or biological modifiers such as solubilising molecules, environmentally sensitive moieties and targeting agents.

Due to their excellent physicochemical properties, polysaccharides have been investigated for developing macromolecular prodrugs with favourable biopharmaceutical properties and improved therapeutic performance compared to parent drugs (Pawar et al., 2008; Kojima et al., 1980; Hashida et al., 1981, 1983, 1984; Caliceti et al., 2010). These materials display, in fact, high biocompatibility and biodegradability, multiple insertion points and biological properties that can be advantageously exploited to endow derivatives with tuneable bioresponsiveness, targeting or environmental triggering properties, or to combine molecules with synergistic therapeutic effect.

Among natural and semi-synthetic polysaccharides, hyaluronic acid, chitosan and dextran have been largely investigated for drug bioconjugation as the therapeutic properties of anticancer drugs can be combined with the biological properties of these polymers (Caliceti et al., 2010; Luo et al., 2002; Park et al., 2000). Tumour targeting and selective anti-cancer drug delivery can in fact be accomplished by receptor-mediated uptake or passive fluid-phase endocytosis of polysaccharide prodrugs. Moreover, both hyaluronic acid and chitosan were found to play a role in cancer biology, and may be exploited for tumour cell targeting or to inhibit tumour angiogenesis and metastasis (Prashanth and Tharanathan, 2005; Itano and Kimata, 2008).

Natural polymers including polysaccharides and polyaminoacids are often degradable thus facilitating metabolic removal from the organism after administration, and this can be advantageous as it prevents accumulation in the body (Duncan, 1992), although the premature degradation of the conjugate due to presence of glycosidases in the circulation may limit its biopharmaceutical performance. However, covalent conjugation of pendent groups has been shown to reduce the susceptibility of normally biodegradable polymers to enzymatic attack, e.g. modification of dextran reduces its rate of enzymatic hydrolysis by dextranases (Vercauteren et al., 1990; Crepon et al., 1991). Other challenges of using polysaccharides include the difficult conjugation chemistry and purification. Nevertheless, most of the reported studies using polysaccharides are limited to pre-clinical investigations while dextran bioconjugates of doxorubicin (AD-70, DOX-OXD) and topoisomerase I inhibitor exatecan (DE-310) failed the clinical trials (Danhauser-Riedl et al., 1993; Wente et al., 2005).

Pullulan is a natural, non-ionic and linear homopolysaccharide formed by repeating units of maltotriose condensed through α-1,6 linkages (α-1,4-linked glucose molecules, polymerised by α-1,6-linkages to the terminal glucose) (Singh et al., 2008). Due to its excellent biological and physicochemical features, namely biodegradability, low immunogenicity and polyfunctionality, together with its fair solubility in aqueous and few organic solvents, this polymer has become an attractive ingredient for many pharmaceutical applications and chemical manipulations (Leathers, 2003). In particular, pullulan has been investigated as a macromolecular platform to construct colloidal drug delivery formulations such as pH-sensitive nanoparticles, assemblies and bioconjugates for anticancer drug delivery (Suginoshita et al., 2002; Na and Bae, 2002; Na et al., 2007). A few examples reported in the literature describe drug conjugation to the primary hydroxyl groups of pullulan via ester bonds or through lysosomal-sensitive peptide spacers (Kim et al., 2003; Nogusa et al., 1997). According to their physicochemical features, these products were found to accumulate into the tumour tissue by a passive EPR mechanism and undergo drug release. Pullulan derivatives were also obtained by polysaccharide derivatisation with targeting agents, which were used to produce self-organised drug loaded nanogels for receptor-mediated cancer cells targeting (Taniguchi et al., 1999; Park et al., 2006; Kim et al., 2008). *In vitro* and *in vivo* studies demonstrated that the drug and the targeting agent conjugation bestows effective cytotoxicity on drug-loaded bioconjugates and make them potentially useful for the treatment of cancer.

With the aim of exploiting the biopharmaceutical properties of pullulan for anticancer drug delivery, we chemically manipulated the polysaccharide structure by introducing anchoring points for selective drug conjugation. The conjugate was designed to achieve passive disposition into the tumour site and site-specific drug release. A conjugate bearing doxorubicin and folic acid was also synthesised, and a comparative study was undertaken to evaluate the effect of the targeting moieties on the biopharmaceutical and pharmacological properties of the macromolecular prodrug. Reproducible multi-step chemical processes were set up and the final products were characterised to examine their composition and structure. Preliminary investigations were performed to evaluate the biopharmaceutical properties, the cell uptake and pharmacological activity. *In vivo* studies were carried out to determine the pharmacokinetic profile after intravenous administration.

2. Materials and methods

Doxorubicin hydrochloride was supplied as commercial formulation named Adriblastin^{TM} 50 mg from Pfizer (Latina, Italy). 3,3′-N-(ε-maleimidocaproic acid)-hydrazide trifluoroacetic acid salt (EMCH) was obtained from Pierce (Rockford, USA). Diaminopolyethylene glycol [1.9 kDa PEG(NH_2)$_2$], ~100 kDa pullulan (Mw/Mn 2.03), folic acid (FA), sodium borohydride ($NaBH_4$), N-hydroxysuccinimide (NHS), N,N′-dicyclohexylcarbodiimide (DCC), and all the solvents were purchased from Fluka (Buchs, Switzerland). Cysteamin (Cyst), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), mannitol, sodium cyanoborohydride ($NaCNBH_3$), 2,4,6-trinitrobenzenesulfonic acid (TNBS), pullulan standard set for gel permeation analysis, [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) and the tissue culture products were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Human nasopharyngeal epidermal carcinoma KB, human breast adenocarcinoma MCF-7 and HeLa cells were cultured as a monolayer at 37 °C in a humidified atmosphere containing 5% CO_2 in Folate-free Dulbecco's modified Eagle's medium (FF-DMEM) supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL of Amphotericin B. Cells were routinely treated with a 500 μg/mL trypsin and 200 μg/mL EDTA solution in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS).

Four-week-old female Balb/C mice, weighing 22–24 g, were obtained from the Department of Pharmaceutical Sciences of the University of Padua. Animal care and handling were performed in accordance with the provisions of the European Economic Community Council Directive 86/209 (recognized and adopted by the

Italian Government with the approval decree D.M. No. 230/95-B) and the NIH publication No. 85-23, revised in 1985. The animal experiments were approved by the Ethical Committee of University of Padua and the official surveillance institutions.

2.1. Pullulan oxidation

Pullulan oxidation was carried out according to the protocol reported in the literature (Bruneel and Schacht, 1993). Briefly, pullulan (2 g, 12.3 mmol glucose units) was dissolved in 200 mL of distilled water and added of 0.9 g of $NaIO_4$ (4.1 mmol). The solution was kept in the dark for 16 h under stirring at room temperature. The unreacted periodate was inactivated using 1.5-fold molar excess of mannitol (1.1 g, 6.1 mmol). The low molecular weight by-products were eliminated by extensive water ultrafiltration using an Amicon system operating with a 10 kDa cut-off membrane. The ultrafiltered solution was lyophilised and the oxidation degree was estimated by reaction with hydroxylamine hydrochloride and potentiometric titration (33). The oxidized pullulan was obtained as white powder in 95% yield (w/w).

2.2. Gel permeation chromatography

Gel permeation chromatography was carried out using G3000SWXL and G4000SWXL columns (Tosoh Bioscience, Stuttgart, Germany) serially operated on an HPLC system equipped with a refractive index (RI) detector. The columns were isocratically eluted with 0.1 M phosphate buffer, 0.15 M Na_2SO_4, 10% methanol, pH 7.4 at a flow-rate of 0.8 mL/min. The apparent molecular weights of native pullulan and oxidised pullulan reduced with $NaBH_4$ were calculated on the basis of a standard curve obtained by eluting 400, 200, 110, 50, 22, 12 and 6 kDa pullulan.

2.3. Synthesis of NH_2 PEG-pullulan-cysteamine (NH_2PEG-Pull-Cyst)

Oxidized pullulan (1.5 g) dissolved in 150 mL of distilled water was added of 1.4 g (18.6 mmol) of cysteamine (Cyst). The solution was acidified to pH 6.0 with 1 N HCl and added of 1.1 g (18.6 mmol) of $NaCNBH_3$. The aqueous solution was maintained under stirring at room temperature for 24 h and then added of 20 mL of water containing 7.1 g (3.7 mmol) of 1.9 kDa PEG$(NH_2)_2$. The acidic pH was maintained by 1 N HCl addition. The solution was kept under stirring at room temperature for 24 h and then basified to pH 9.0 with 1 N NaOH and added of 0.7 g (18.6 mmol) of $NaBH_4$. After 24 h stirring at room temperature, the solution was acidified to pH 6.0 with 1 N HCl and lyophilised. The solid residue was dispersed in 100 mL of CH_2Cl_2 and the organic mixture was filtered. The solid fraction was desiccated under vacuum and then dissolved in water and ultrafiltered with a 10 kDa cut-off membrane. The purification was carried out by serial addition of 100 mL of water and the ultrafiltered solution was continuously tested by the Ellman's assay with DTNB for thiol group detection (Riddles et al., 1979). Following ultrafiltration, the solution was lyophilised. An aliquot of dry product was carefully weighed, dissolved in 10 mM phosphate buffer, 0.15 M NaCl, pH 7.2, and the thiol and amino groups were determined colorimetrically by the DTNB and TNBS assay (Snyder and Sobocinski, 1975), respectively. The SH and NH_2 content was calculated on the basis of titration curves obtained with cysteine and 1.9 kDa PEG$(NH_2)_2$, respectively.

2.4. Synthesis of (folic acid-PEG)-pullulan-cysteamine [(FA-PEG)-Pull-Cyst]

N-hydroxysuccinimidyl ester of folic acid (250 mg, 0.46 mmol) prepared according to the procedure reported in the literature (Leamon and Low, 1991) was dissolved in 20 mL of anhydrous DMSO and added of 500 mg of (NH_2PEG)-Pull-Cyst The reaction solution was maintained in the dark overnight under stirring and then dropped into 200 mL of iced ethyl ether. The precipitate was desiccated under vacuum, dissolved in 100 mL of 0.02 M acetic acid pH 6.8, and centrifuged at 4000 rpm for 5 min. The solution was purified by ultrafiltration using a 10 kDa cut-off filter and lyophilised. An aliquot of the dry product was exactly weighed and dissolved in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.2 for determination of the folic acid content by UV spectroscopy ($\varepsilon_{363} = 6197\ M^{-1}$) (Kranz et al., 1995).

2.5. Synthesis of doxorubicin–EMCH (Dox–EMCH)

Dox–EMCH was synthesised according to the method reported in the literature (Willner et al., 1993). Briefly: 150 mg (0.26 mmol) of doxorubicin (Dox) was dissolved in 50 mL of methanol containing 20 μL of TFA. The alcoholic solution was added of 100 mg (0.29 mmol) of ε-maleimidocaproyl hydrazide trifluoroacetic acid (EMCH) and maintained overnight under stirring at room temperature in the dark. The volume was reduced to 7 mL under vacuum and then added of 43 mL of acetonitrile. The solution was maintained 72 h at 4 °C and the precipitate was recovered by centrifugation, washed with methanol/acetonitrile 1:10 and finally desiccated. The product yield was 90% (w/w). The derivative was analysed by ESI-TOF mass spectrometry (ESI-MS; Applied Biosystems, Mariner Foster City, CA, USA).

ESI-MS [m/z]: 751.29 $(M+H^+)^{1+}$ [calcd for $C_{37}H_{42}N_4O_{13}$: 750.27].

2.6. Synthesis of (folic acid-PEG)-pullulan-(cysteamine-doxorubicin) [(FA-PEG)-Pull-(Cyst-Dox)] and (NH_2PEG)-Pull-(cysteamine-doxorubicin) [(NH_2PEG)-Pull-(Cyst-Dox)]

(FA-PEG)-Pull-Cyst or (NH_2PEG)-Pull-Cyst (300 mg) dissolved in 30 mL of distilled water were added of 10 mg of triscarboxyethylphosphine (TCEP). The pH was adjusted to 8.0 with 0.001 N NaOH and the solution was added of 500 μL 40 mg/mL of Dox–EMCH in DMF. The reaction mixture was maintained under stirring at room temperature in the dark. After 30 min the solution was added dropwise to 50 mL of methanol. The precipitate was recovered by 5 min centrifugation at 5000 rpm, redissolved in the minimum volume of DMSO and re-precipitated in methanol. The free doxorubicin in the alcoholic solutions was estimated by UV ($\varepsilon_{495} = 13000\ M^{-1}$) (Larson and Drummond, 2001). The precipitation procedure was repeated until a doxorubicin-free methanol solution was obtained. The product was dissolved in a minimal volume of DMSO and dropped in 50 mL of ice-cold ethyl ether and the precipitate recovered by centrifugation was desiccated under vacuum. The dry product was dissolved in 30 mL of water containing 46 mg (0.3 mmol) of DTT. After 30 min, the solution was extensively ultrafiltered with a 10 kDa cut-off membrane using 10 mM ammonium bicarbonate, pH 7.8 until complete elimination of DTT, which was monitored in the eluted volume by Ellman's assay. Finally, the solution was lyophilised and the dry product was maintained under nitrogen at −20 °C. The conjugated doxorubicin was determined by dissolving known amounts of the lyophilised conjugate in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.2, and by UV analysis at 495 nm ($\varepsilon_{495} = 9250\ M^{-1}\ cm^{-1}$ for doxorubicin–EMCH) (Di Stefano et al., 2004). The free doxorubicin content in the lyophilised (FA-PEG)-Pull-(Cyst-Dox) and (NH_2PEG)-Pull-(Cyst-Dox) products was evaluated by gel chromatography using a Biogel 40XL column (Bio-Rad, Milan, Italy) isocratically eluted with 0.1 M phosphate buffer, 0.15 M Na_2SO_4, 10% methanol, pH 7.4 at a flow-rate of 1 mL/min.

The UV detector was set at 495 nm. The area under the peak corresponding to free doxorubicin was referred to a standard curve obtained by eluting doxorubicin solutions at known concentrations.

2.7. Dynamic light scattering analysis

(NH_2PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox) samples were prepared by dissolving 0.2 or 5 mg of bioconjugate in 1 mL of 20 mM phosphate buffer, 0.15 M NaCl, pH 7.2. The polymer solutions were vortexed and then centrifuged at 5000 rpm for 5 min. The solutions were analysed using a single angle photon correlation spectrometer Nicomp-380 (Particle Sizing Systems, Santa Barbara, CA) equipped with a 632.5 nm laser.

2.8. Doxorubicin release

Solutions were prepared by dissolving 290 μg of doxorubicin equivalent (FA-PEG)-Pull-(Cyst-Dox) and (NH_2 PEG)-Pull-(Cyst-Dox) in 1 mL or in 25 mL of 20 mM phosphate buffer, 0.15 M NaCl, pH 7.4 or 0.1 M acetate buffer pH 5.5 or plasma obtained by centrifugation of heparinised mouse blood. The solutions were incubated at 37 °C under mild stirring. At scheduled times 50 μL of the concentrated samples (290 μg doxorubicin equivalent/mL) were diluted with 50 μL of 0.1 M phosphate buffer, 0.15 M Na_2SO_4, 10% ACN, pH 7.2, and centrifuged at 10,000 rpm for 3 min, while 1000 μL of the diluted solutions (11.6 μg doxorubicin equivalent/mL) were rapidly iced, lyophilised and then re-dissolved in 80 μL of 0.1 M phosphate buffer, 0.15 M Na_2SO_4, 10% ACN, pH 7.2. The solutions were analysed by gel filtration chromatography using a Biogel 40XL column eluted as reported above. The eluate was analysed using a UV–vis detector at 495 nm. The area of the eluted peaks corresponding to the bioconjugates was elaborated to calculate the doxorubicin released at the experimental times. The experiments were repeated four times.

2.9. Cell uptake

HeLa cells over-expressing folate receptor were seeded in Petri dishes (35 mm diameter) at the density of 2×10^6 cells/dish in FF-DMEM. After 24 h the culture medium was replaced with 100 μL of medium containing 10 μM doxorubicin or doxorubicin equivalent concentrations of (FA-PEG)-Pull-(Cyst-Dox) or (NH_2PEG)-Pull-(Cyst-Dox). At scheduled times (5, 30, 60 and 300 min) the medium was removed and the cells were harvested with trypsin. The cells were centrifuged and washed three times with PBS. Finally, the pellets were resuspended in 100 μL of PBS, and the samples were analysed by an ImageStream multispectral imaging flow cytometer (Amnis Corp., Seattle, WA), which allowed for taking cell images and quantitative analysis of the cell fluorescence that was automatically normalised by the cell number.

2.10. Cell viability studies

The KB and MCF7 cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells/well. After 24 h, the culture medium was replaced with 100 μL of medium containing increasing concentrations (0–40 μM) of doxorubicin or doxorubicin equivalent (FA-PEG)-Pull-(Cyst-Dox) and (NH_2PEG-)Pull-(Cyst-Dox). After 24, 48 and 72 h, 20 μL of a 5 mg/mL MTT solution in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.4, was added to each well. The plates were incubated for 5 h at 37 °C, and then the medium was removed and 200 μL of DMSO was added to each well. The plates were maintained under gentle stirring for 2 h, and the optical absorbance was measured at 570 nm using an EL311SK plate reader (Bio Tek Instruments Inc., Highland, VT, USA). All experiments were replicated six times and data were elaborated to calculate the mean values, standard deviations (SD) and $IC_{50} \pm$ SD. The P (t-Student) values were calculated by elaboration of the data obtained by each single experiment.

2.11. Pharmacokinetic studies

Groups of 24 Balb/c mice were intravenously injected with 100 μL of 0.5 mg/mL doxorubicin or doxorubicin-equivalent doses of (FA-PEG)-Pull-(Cyst-Dox) or (NH_2PEG)-Pull-(Cyst-Dox) in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.2. At scheduled times, 50 μL of blood samples were randomly withdrawn by retrobulbar puncture of anesthetised mice and centrifuged at 1500 rpm for 3 min. The plasma samples (20 μL) were diluted with an equal volume of a 50:50 (v/v) mixture of methanol/40% $ZnSO_4$ in water and centrifuged at 12,000 rpm for 5 min. The supernatant was 1:2 diluted with 0.05% TFA in water, centrifuged at 5000 rpm for 10 min and analysed by reverse phase chromatography using a C18 analytical column (Phenomenex Luna C18, 4.6 mm × 250 mm) isocratically eluted with a 65:35 (v/v) mixture of methanol and 0.01 M phosphate buffer, pH 3.0. The precipitate was suspended in 50 μL of 0.05% TFA and maintained under stirring for 48 h. The mixture was added to 200 μL of ethanol and centrifuged and the supernatant was analysed by RP-HPLC as reported above. The doxorubicin was detected using a fluorimetric detector set at λ_{exc} 475 nm, λ_{em} 580 nm and the area under the peak was elaborated using a standard curve obtained by eluting solutions at different doxorubicin concentrations [y (fluorescence) = 4786.5 × (doxorubicin ng/mL) + 242,558, $R^2 = 0.9989$, detection limit 2 ng/mL]. The obtained values were normalised according to the doxorubicin recovery, which was calculated by preliminary studies performed by addition of different amounts of doxorubicin to plasma volumes and analysis as reported above.

3. Results

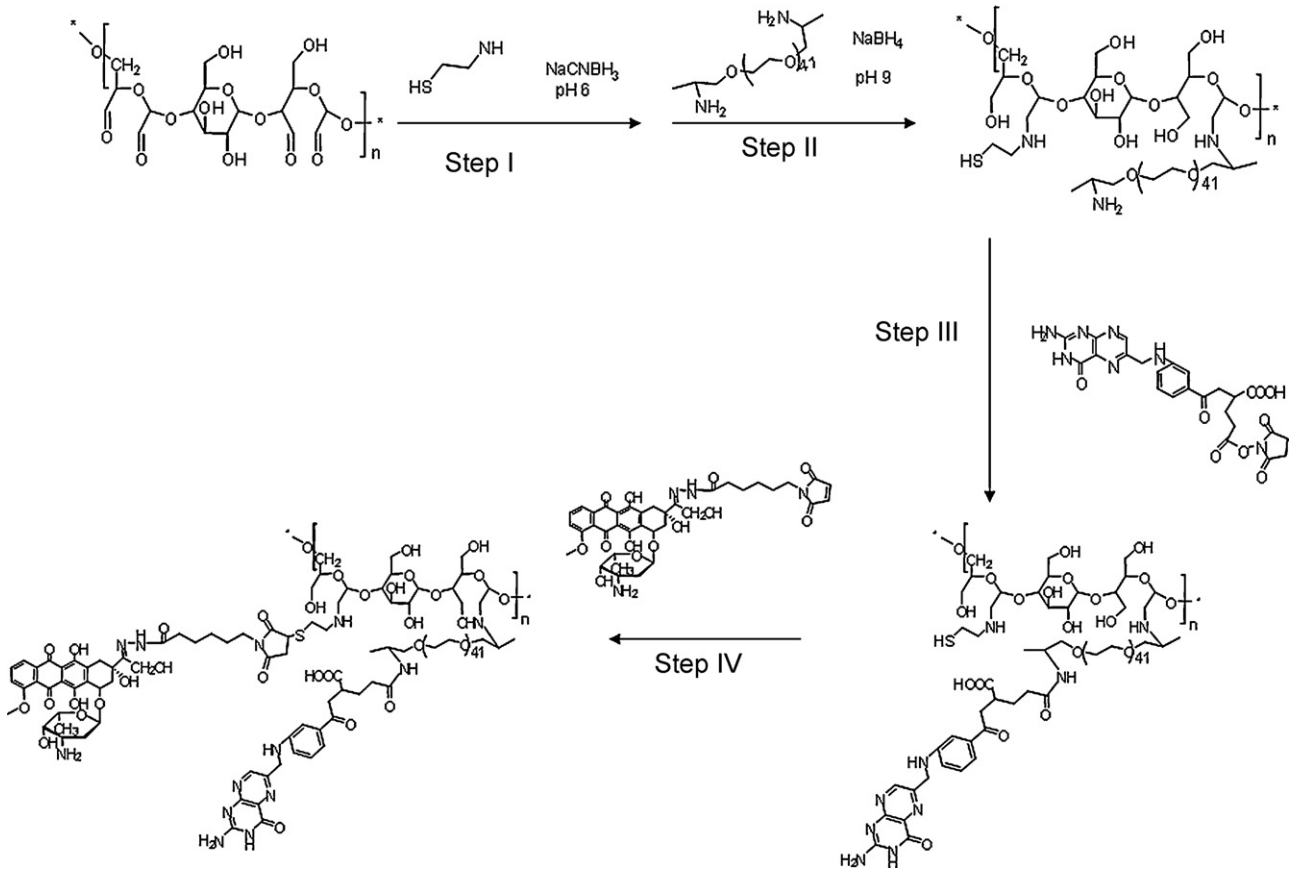
3.1. Synthesis of (FA-PEG)-Pull-(Cyst-Dox) and (NH_2PEG)-Pull-(Cyst-Dox)

The two pullulan bioconjugates, (FA-PEG)-Pull-(Cyst-Dox) and (NH_2PEG)-Pull-(Cyst-Dox), were obtained according to the multistep synthesis protocol described in Scheme 1.

The analysis of the pullulan oxidation with periodate revealed 40 aldehyde groups out of 100 glucose units. However, due to the spontaneous recyclic emiacetal formation reported in the literature (Bruneel and Schacht, 1993) 1/3 of aldehydes could not be revealed. Therefore, the overall oxidation yielded 60 aldehydes out of 100 glucoses corresponding to 30 ± 3% oxidised glucose units.

The cysteamine and PEG(NH_2)$_2$ conjugation to the oxidised pullulan was carried out by a two-step procedure: first cysteamine was reacted with the aldehyde groups and the Schiff bases were reduced to amines with $NaCNBH_3$, then PEG(NH_2)$_2$ was added in large excess with respect to the aldehyde groups and the Schiff bases were reduced with $NaBH_4$.

Unreacted cysteamine and the reducing agents were eliminated by ultrafiltration, while the unconjugated PEG(NH_2)$_2$ was eliminated by extraction in dichloromethane, in which this polymer is fairly soluble. The colorimetric analysis carried out to estimate the thiol and amino group content in the final product showed that about 29 ± 1% and 11 ± 1% of the reactive aldehydes were conjugated with cysteamine and PEG(NH_2)$_2$, respectively, which corresponded to about 67 cysteamine and 25 PEG units per oxidised pullulan chain.



Scheme 1. Synthesis of (NH_2PEG)-Pull-(Cyst) and (FA-PEG)-Pull-(Cyst-Dox): oxidised pullulan functionalisation with cysteamine (step I) and PEG(NH_2)$_2$ (step II), folic acid conjugation (step III), and doxorubicin conjugation (step IV).

Folic acid was activated as succinimidyl ester for conjugation to the amino groups of pendant PEG chains. The reaction yielded 63 ± 2% amino group conjugation, corresponding to 2.8 ± 0.4 folic acid units out of 100 initial glucose units. In the case of (NH_2PEG)-Pull-(Cyst-Dox), folic acid conjugation (step III) was skipped and doxorubicin was directly conjugated to (NH_2PEG)-Pull-Cyst.

The Dox–EMCH intermediate, containing a pH-sensitive hydrazone bond within the linker structure and a terminating maleimide function for conjugation with the thiol groups of the pendant cysteamine, was synthesised according to the protocol reported in the literature (Willner et al., 1993). The reaction resulted in 100% Dox–EMCH conjugation to the pullulan derivative. The doxorubicin content in the final product estimated by UV analysis at 495 nm according to the method reported in the literature (Di Stefano et al., 2004). Preliminary studies showed that at this wavelength neither PEG nor folic acid were found to interfere with the doxorubicin absorption. Furthermore, the doxorubicin content assessed by spectroscopic analysis was found to correspond to the values obtained by acid hydrolysis in the drug release studies, thus demonstrating the suitability of the analytical protocol. The synthesis yielded to 19 ± 0.5% derivatised thiol groups that corresponded to 2.2 ± 0.2 doxorubicin residues out of 100 initial glucose units, indicating that under the selected reaction conditions, the doxorubicin conjugation was quantitative. A final step included the bioconjugate treatment with DTT, which reduces selectively disulphide groups that can be formed during the synthesis yielding cross-linked products. DTT and other by-products were finally eliminated by ultrafiltration. The gel permeation analysis of the bioconjugates showed that the free drug content in the final product was close to the detection limit and the free drug was calculated to be below 0.5% of the conjugated drug indicating that the purification process by extraction was effective in eliminating the small amount of unconjugated drug.

Table 1 reports the weight composition of the plain polymer scaffold (NH_2PEG)-Pull-Cyst and the two conjugates, (FA-PEG)-Pull-(Cyst-Dox) and (NH_2PEG)-Pull-(Cyst-Dox).

Table 1
Pullulan, (NH_2PEG)-Pull-Cyst, (FA-PEG)-Pull-(Cyst-Dox) and (NH_2PEG)-Pull-(Cyst-Dox) composition. The weight content of the different modules in the bioconjugates (w/w, %) and the standard deviation (±SD) were calculated on the basis of the results obtained from five preparations.

	136 kDa pullulan (w/w, % ± SD)	(NH_2PEG)-Pull-(Cyst) (w/w, % ± SD)	(NH_2PEG)-Pull-(Cyst-Dox) (w/w, % ± SD)	(FA-PEG)-Pull-(Cyst-Dox) (w/w, % ± SD)
Glucose	100%	44.4 ± 2.9	41.6 ± 3.3	39.8 ± 2.8
Derivatised glucose	–	19.3 ± 1.3	18.1 ± 1.4	17.3 ± 1.2
1.9 kDa PEG(NH_2)$_2$	–	32.7 ± 1.8	30.7 ± 3.5	29.3 ± 3.5
Cyst	–	3.6 ± 0.2	3.4 ± 0.4	3.2 ± 0.3
Doxorubicin	–	–	6.3 ± 0.7	6.2 ± 0.5
Folic acid	–	–	–	4.3 ± 0.4

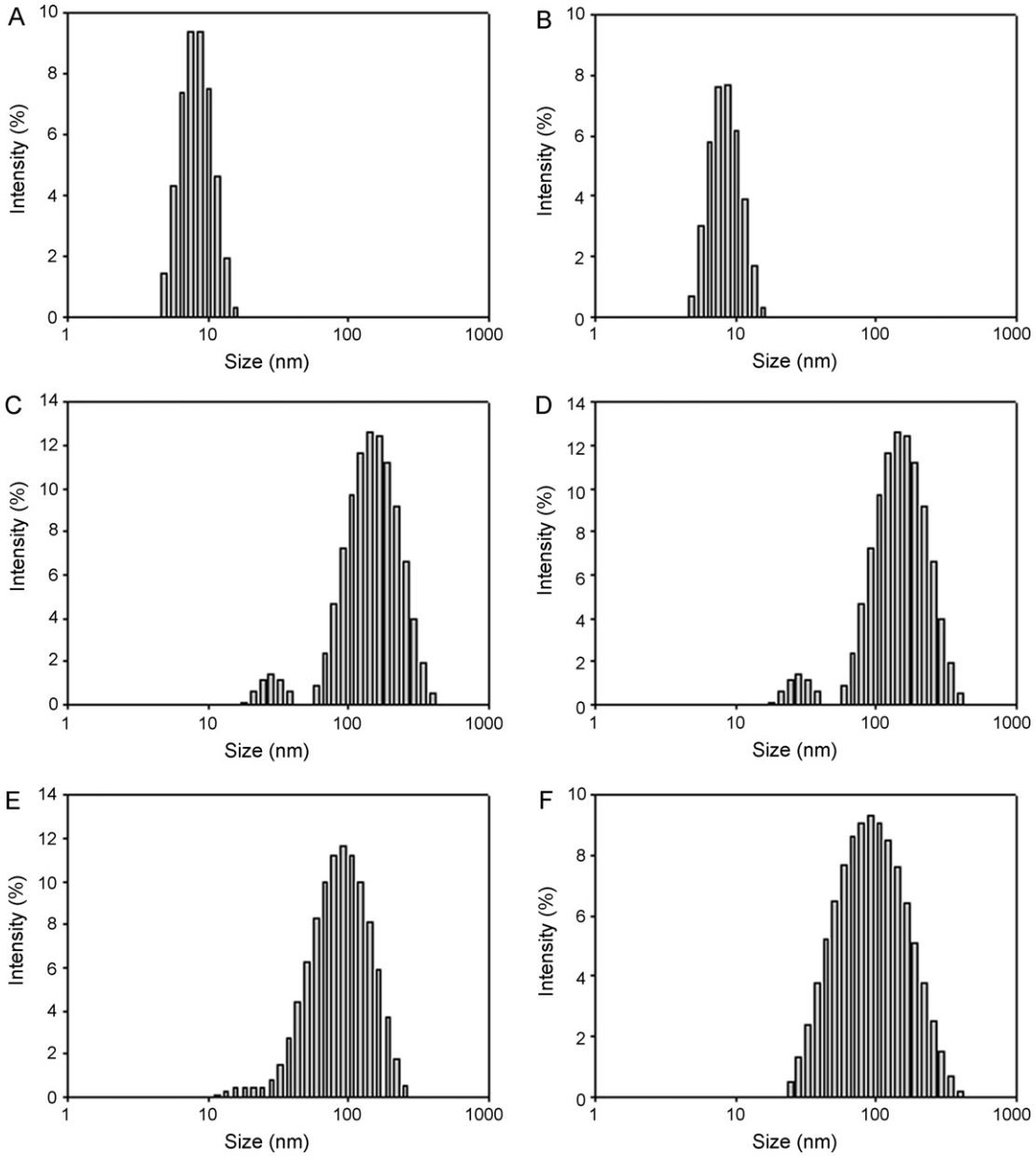


Fig. 1. Dynamic light scattering profiles of (NH_2PEG)-Pull-Cyst (A and B), (NH_2PEG)-Pull-(Cyst-Dox) (C and D) and (FA-PEG)-Pull-(Cyst-Dox) (E and F). The results obtained using 0.2 mg/mL conjugate solutions are reported in panels (A), (C) and (E) and the results obtained with 5 mg/mL conjugate solutions are reported in panels (B), (D) and (F).

3.2. Bioconjugate characterisation

The molecular weight and size of (FA-PEG)-Pull-(Cyst-Dox) and (NH_2PEG)-Pull-(Cyst-Dox) were determined by gel permeation chromatography and dynamic light scattering (DLS).

Pullulan and oxidised pullulan treated with $NaBH_4$ were analysed by gel permeation chromatography to determine their molecular weight. The $NaBH_4$ treatment of oxidised pullulan transformed the aldehyde groups into hydroxyl functions, which produced an hydrophilic polymer with physicochemical properties similar to native pullulan without substantial changes in the oxidised pullulan molecular weight. Gel permeation chromatography showed that the pullulan oxidation reduced the polysaccharide molecular weight from 136 kDa with a polydispersity index (PDI) Mw/Mn of 1.8 to about 93 kDa with a PDI Mw/Mn of 1.7.

Based on the size of the oxidised pullulan determined by gel permeation chromatography and the PEG, cysteamine, folic acid and doxorubicin content determined by the colorimetric assays and spectroscopic analysis, the molecular weight of (FA-PEG)-Pull-(Cyst-Dox) and (NH_2PEG)-Pull-(Cyst-Dox) were calculated to be about 162 kDa and 155 kDa, respectively.

Dynamic light scattering (DLS) analysis were carried out in buffer at two bioconjugate concentrations (0.2–5 mg/mL). The photo correlation spectroscopy analysis showed that, similarly to the species formed by native pullulan (data not shown), pullulan derivatized with PEG and cysteamine has a size of 9.2 ± 3.1 nm (Fig. 1A and B), corresponding to the unimolecular structure of the polysaccharide derivative. The DLS profiles of the pullulan bioconjugates bearing doxorubicin and doxorubicin/folic acid reported in Fig. 1C–E show the presence of small size and large size species that correspond to the unimolecular form and multimolecular assemblies, respectively. Fig. 1C shows that at low concentration (0.2 mg/mL) (NH_2PEG)-Pull-(Cyst-Dox) produced a main specie fraction (96.7 vol.%) with 144.8 ± 65.15 nm diameter

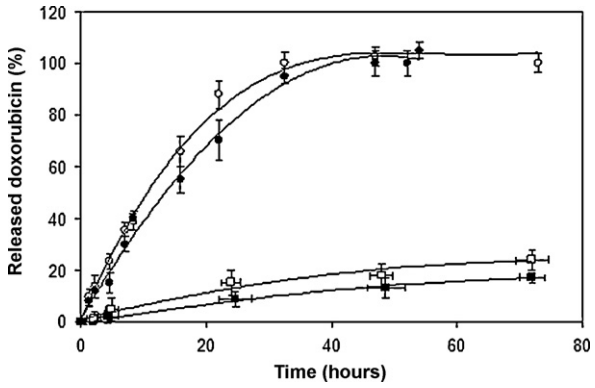


Fig. 2. Doxorubicin release profile from 5 mg/mL solutions of (NH_2PEG)-Pull-(Cyst-Dox) (empty symbols) and (FA-PEG)-Pull-(Cyst-Dox) (full symbols): in plasma (□, ■) and pH 5.5 (●, ○). The drug release at pH 7.4 was not reported. The figure reports the mean values and standard deviation bars obtained from four experiments.

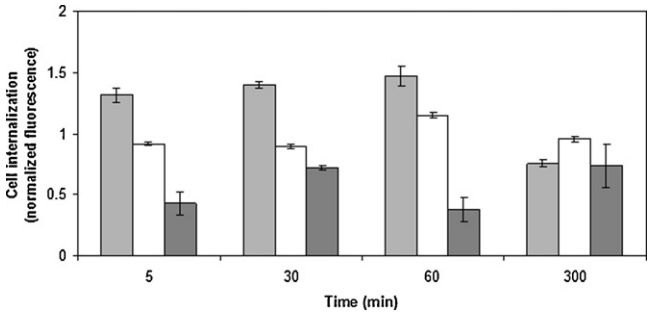


Fig. 4. HeLa cell uptake time courses of free doxorubicin (light grey), (FA-PEG)-Pull-(Cyst-Dox) (white) and (NH_2PEG)-Pull-(Cyst-Dox) (dark grey).

(PDI 0.2) and a small fraction (3.3 vol.%) with 24.3 ± 5.3 nm diameter (PDI 0.048). Fig. 1D shows that these subpopulations and their relative abundance were maintained at a higher concentration (5 mg/mL), as the main fraction (about 95 vol.%) had a diameter of 162.2 ± 66.0 nm (PDI 0.16) and diameter of the small fraction (5.5 vol.%) was 23.3 ± 7.2 (PDI 0.09). The analysis of 0.2 mg/mL (FA-PEG)-Pull-(Cyst-Dox) reported in Fig. 1E shows the presence of 16.8 ± 3.0 nm (1.5 vol.%, PDI 0.03) and 96.8 ± 44.6 nm (98.5 vol.%, PDI 0.21) vesicles. Fig. 1F shows that at 5 mg/mL, only the specie with a larger size (108.2 ± 64.3 nm, PDI 0.35) could be revealed.

3.3. Doxorubicin release studies

The doxorubicin release from the (FA-PEG)-Pull-(Cyst-Dox) and (NH_2PEG)-Pull-(Cyst-Dox) was comparatively investigated in plasma and in buffer at pH 7.4 and 5.5, by using bioconjugate concentrations of about 5 mg/mL. The study was carried out by a validated precipitation method that avoided possible interactions with membranes used in the dialysis protocols.

The results reported in Fig. 2 show that the two bioconjugates undergo similar drug release either in plasma or in buffer. Similar results were obtained by using 0.2 mg/mL bioconjugate concentration (data not shown).

The macromolecular prodrugs were significantly stable in plasma as the drug was slowly released throughout time. About 20% of the conjugated doxorubicin was released in three days from both derivatives. The drug release profiles obtained in plasma substantially overlapped the profiles obtained by the bioconjugate incubation in buffer at pH 7.2 (data not shown), indicating that the drug was released by chemical hydrolysis of the hydrazone bond, whereas enzymatic mechanisms were not involved. At pH 5.5 the doxorubicin release was more rapid than in plasma as about 50% of the conjugated drug was released in 12 h from the bioconjugates and complete doxorubicin release was achieved in about 40 h.

3.4. Cell trafficking and toxicity

The cell internalisation kinetics of doxorubicin, (FA-PEG)-Pull-(Cyst-Dox) and (NH_2PEG)-Pull-(Cyst-Dox) was investigated in HeLa cells, that had been cultured in folate-free medium to yield folate receptor over-expression (Holm et al., 2000).

The images reported in Fig. 3 show that the internal cell fluorescence intensity obtained with the two conjugates increased throughout time. After 60 min of incubation, the (FA-PEG)-Pull-(Cyst-Dox) treated cells (Fig. 3B) displayed higher fluorescence than the cells treated with (NH_2PEG)-Pull-(Cyst-Dox) (Fig. 3D). The elaboration of the data obtained by cytofluorimetric analysis reported in Fig. 4 shows the quantitative cell up-take of free doxorubicin and the two bioconjugates. Free doxorubicin is taken up by cells rapidly, according to its biophysical properties, which facilitates its diffusion across membranes (Holm et al., 2000). The folated bioconjugate was rapidly internalised by the HeLa cells, yielding maximal concentration after 60 min incubation. On the contrary, the non-folated derivative was found to undergo slow cell uptake to achieve maximal levels in 5 h, which were similar to those obtained with free doxorubicin and (FA-PEG)-Pull-(Cyst-Dox).

The cell toxicity of doxorubicin, (NH_2PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox) was evaluated using two cell lines: KB and MCF7, which over-express and do not over-express the folate receptor, respectively.

The cell viability profiles obtained by 72 h of incubation of doxorubicin and the polymer derivatives with MCF7 and KB cells are reported in Fig. 5A and B. The results show that the polymer conju-

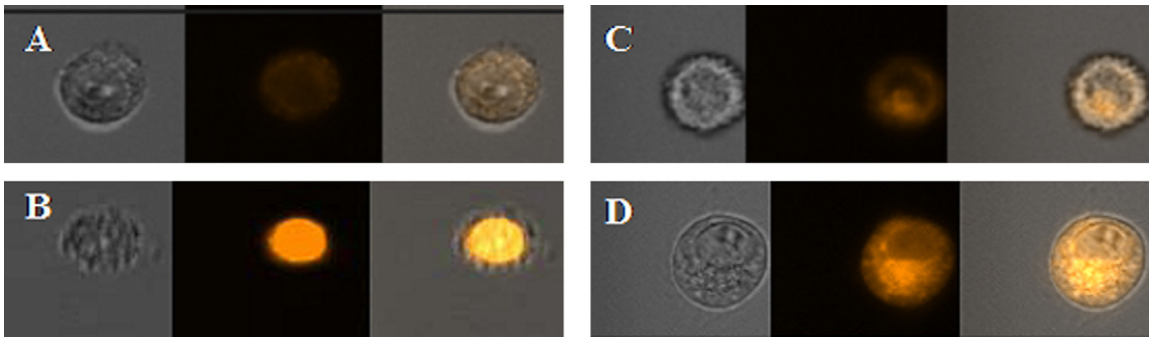


Fig. 3. Cytofluorimetric images of HeLa-HiFR treated with (FA-PEG)-Pull-(Cyst-DOX) (A and B) and (NH_2PEG)-Pull-(Cyst-Dox) (C and D) after 5 min (A and C) and 60 min (B and D) incubation.

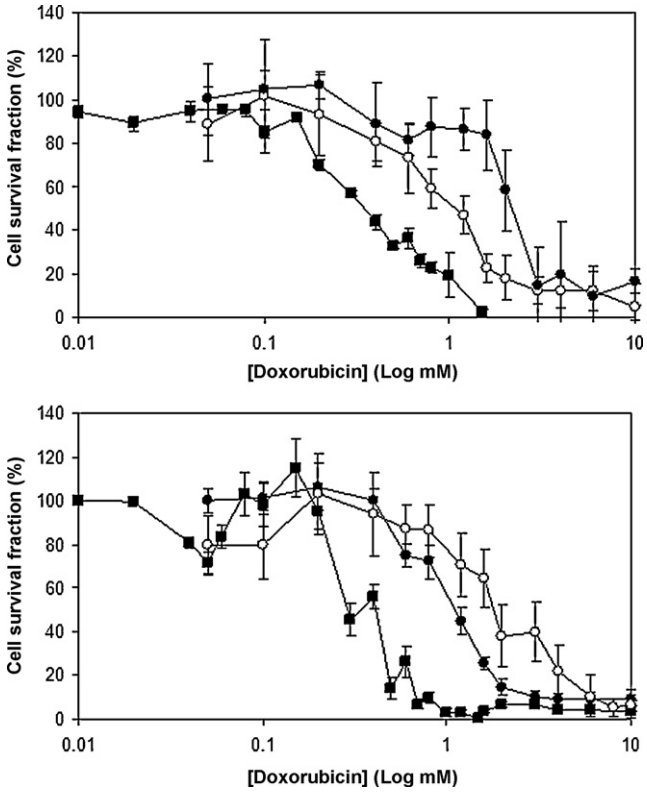


Fig. 5. Cell viability profiles of cells incubated with free doxorubicin (■), (NH_2PEG)-Pull-(Cyst-Dox) (○) and (FA-PEG)-Pull-(Cyst-Dox) (●). Panels (A) and (B) report the cytotoxicity profiles obtained with MCF7 and KB cells, respectively.

gation reduces the doxorubicin activity, in the case of either MCF7 or KB cells that showed slight differences ($P < 0.005$).

In the case of MCF7 cells, the IC_{50} values calculated for doxorubicin, (NH_2PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox) were 0.3, 1.2 and 3.1 μM, respectively. The comparison between the IC_{50} obtained with (NH_2PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox) ($P < 0.01$) indicates that the folic acid conjugation reduced the efficacy of the macromolecular prodrug.

The KB cell viability profiles described in Fig. 5B show an opposite trend when compared to the MCF7 cells as the folated bioconjugate was only slightly more active than the non-folated derivative, with the IC_{50} 0.4 μM in the case of doxorubicin and 1.8 and 1.1 μM in the case of (NH_2PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox) ($P < 0.05$), respectively.

Similar results were obtained by 48 h of cell incubation with doxorubicin, (NH_2PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox) (data not shown).

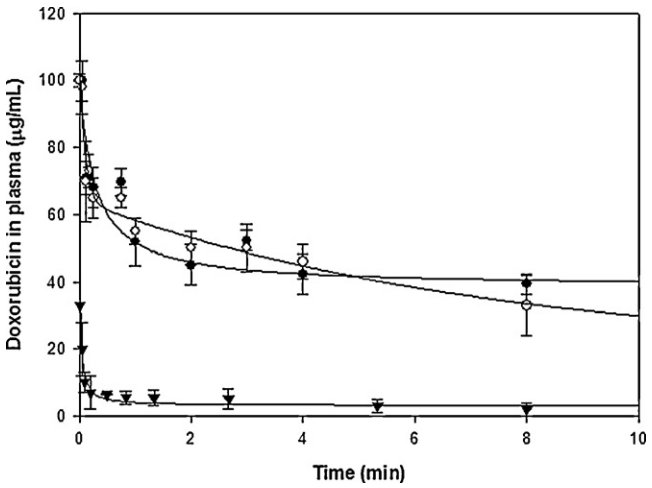


Fig. 6. Pharmacokinetic profiles obtained by intravenous injection of free doxorubicin (▼), (NH_2PEG)-Pull-(Cyst-Dox) (●) and (FA-PEG)-Pull-(Cyst-Dox) (○). The data are referred to the whole doxorubicin recovered from the blood.

3.5. Pharmacokinetic studies

Pharmacokinetic studies were performed by intravenous injection of doxorubicin, (NH_2PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox) in mice. The plasma samples were processed to analyse separately the free and bioconjugated drug. The combination of methanol extraction and acidic water treatment of the blood-derived pellet allowed for the estimation of the whole doxorubicin present in the bloodstream. Preliminary studies showed that this procedure yielded over 89% doxorubicin recovery from the plasma samples. Negligible amounts of free doxorubicin were detected in the plasma samples obtained from mice treated with the bioconjugates.

The pharmacokinetic profiles depicted in Fig. 6 show that doxorubicin and the two conjugates undergo biphasic clearance behaviour and the macromolecular prodrugs display similar profiles. Plasma levels of doxorubicin were significantly higher in the case of the bioconjugates than that obtained with free doxorubicin. Table 2 summarises the main pharmacokinetic parameters calculated by bi-exponential elaboration of the experimental values.

Free doxorubicin displayed short α phase ($t_{1/2\alpha}$) while the higher $t_{1/2\alpha}$ obtained with the two bioconjugates indicate that they undergo slower peripheral distribution. Furthermore, the lower distribution volume (V_{ss}) calculated with the bioconjugates indicates that the derivatives distribute to the peripheral compartment at a lower extent than the free drug. The similar K_{12} and K_{21} values calculated with the bioconjugates indicate that the diffusion between the central and peripheral compartment

Table 2
Main pharmacokinetic parameters obtained by elaboration of the total doxorubicin concentrations in blood after intravenous injection to mice of free doxorubicin and (FA-PEG)-Pull-(Cyst-Dox) and (NH_2PEG)-Pull-(Cyst-Dox): V_c, central compartment volume; AUC, area under the curve; $t_{1/2\alpha}$, alpha phase half life; $t_{1/2\beta}$, beta phase half life; V_{ss}, steady state distribution volume; Cl, clearance; K_e, elimination rate constant; K_{12}, central compartment to peripheral compartment rate constant; K_{21}, peripheral compartment to central compartment rate constant.

	Doxorubicin	(NH_2PEG)-Pull-(Cyst-Dox)	(FA-PEG)-Pull-(Cyst-Dox)
V_c (mL)	1.3	1.1	1.01
AUC (μg mL^{-1} h)	58	1930	1247
$t_{1/2\alpha}$ (h)	0.03	0.32	0.2
$t_{1/2\beta}$ (h)	6.5	26	15.7
V_{ss} (mL)	7.7	1.9	1.7
Cl (mL/h)	0.8	0.05	0.08
K_e	0.7	0.047	0.078
K_{12}	15.9	0.89	2.71
K_{21}	3.0	1.21	3.86

is not restricted. The clearance value (Cl) and the elimination constant (K_e) show that the polymer conjugation dramatically reduces the drug elimination, prolonging its permanence in the bloodstream as observed by the longer β phase ($t_{1/2\beta}$) compared to that calculated for the free drug. The area under the curve (AUC) values underline the enhanced permanence of doxorubicin conjugated to the polysaccharide backbone. The reduced elimination and low distribution into the peripheral compartment of the bioconjugate results in about 10-fold higher AUC than the free drug.

4. Discussion

The chemical modification of pullulan was designed to obtain a novel supramolecular platform with suitable physicochemical and biopharmaceutical properties for tumour targeting. Accordingly, pullulan was activated and derivatised with anchoring agents, which allowed for selective doxorubicin conjugation via a pH-sensitive spacer or folic acid as a targeting agent.

The doxorubicin-derivatised pullulan (NH_2PEG)-Pull-(Cyst-Dox) was designed to obtain a macromolecular prodrug that can passively accumulate in the tumour by the EPR effect and then selectively release the drug into the cell by endosomal drug–polymer bond cleavage.

A folic acid-functionalised bioconjugate was also designed to evaluate whether the active cell recognition could enhance the tumour targeting efficiency of the macromolecular prodrug. Several studies reported in the literature demonstrated, in fact, that the folate receptor is over-expressed by a wide array of cancer cells (Bates et al., 1985; Ross et al., 1999; Elnakat and Ratnam, 2004). Therefore, folate-derivatised systems may benefit from receptor-mediated intracellular uptake, which provides for active drug accumulation into cancer cells by a clathrin-independent potocytosis mechanism (Anderson et al., 1992) and overcome inefficacy due to drug resistance.

In contrast with other chemical procedures described in the literature, which exploit the primary alcohol functions of polysaccharides as anchoring groups (Kim et al., 2008) pullulan was activated under mild conditions via a vicinal diol-selective oxidation that introduces aldehyde groups along the polysaccharide backbone. This chemical procedure is more flexible and efficient as it can produce a polymer with high activation degree than alcohol derivatisation. Controlled oxidation may yield up to two aldehyde groups per glucose unit that can be easily switched into a variety of derivatisable functional groups. Furthermore, the aldehyde conjugation via a reducing reaction may produce more stable systems than the ester conjugates obtained by alcohol derivatisation. This is particularly relevant for drug conjugation through bonds cleavable under specific conditions, namely pH or enzyme sensitive, which allow for site selective drug release, and for conjugation of targeting that must be stably attached to the carrier throughout the delivery pathway.

Based on preliminary studies, pullulan oxidation was carried out under controlled conditions that yielded $30 \pm 3\%$ oxidised glucoses. Cysteamine and PEG(NH_2)$_2$ harnessed the oxidised pullulan to bestow thiol and amino groups on the polysaccharide that were exploited for conjugation with the drug and the targeting moiety, respectively.

The two-step protocol set up for the cysteamine and PEG(NH_2)$_2$ conjugation allowed us to overcome the problems related to the bifunctional nature of PEG(NH_2)$_2$ that could result in polymer cross-linking and low cysteamine derivatisation.

The mild reducing agent ($NaCNBH_3$) used in the first step to stabilise the cysteamine/aldehyde Schiff bond did not quench the unreacted aldehydes. In the second step, performed by using a high PEG(NH_2)$_2$/aldehyde molar ratio to avoid cross-linking, a stronger reducing agent ($NaBH_4$) stabilised the PEG(NH_2)$_2$/aldehyde Schiff bonds and reduced the unreacted aldehydes to alcohols. Therefore, this step resulted in complete elimination of aldehydes as required to obtain non-toxic biocompatible products.

Studies reported in the literature showed that direct folic acid conjugation to the primary hydroxyl groups of pullulan reduces the conjugate solubility and promotes the formation of self-organised nanogels (Kim et al., 2008). Furthermore, these studies showed that extensive folic acid conjugation was not required for the effective targeting of supramolecular carriers. Therefore, in the present study, 16 folate residues per macromolecule were conjugated to the pullulan backbone through a PEG spacer (4.3%, w/w, 2.8 folate residues out of 100 glucose units). PEG also has the advantage of counterbalancing the hydrophobic nature of folic acid, which can produce large aggregates or insoluble products (Prabaharan et al., 2009a). Furthermore, the spacer hydrophilicity and flexibility can facilitate the targeting moiety exposure and folate receptor recognition, while the presence of few folic acid residues along the polysaccharide backbone can provide for a cooperative interaction of the conjugate with the folate receptors located on the cell membrane and enhance the cell uptake of the macromolecule by a caveolar mechanism.

Doxorubicin was conjugated to the thiol groups of pending cysteamine through the pH-sensitive hydrazone. This strategy successfully used for preparation of anticancer macromolecular prodrugs was adopted as it is a simpler conjugation method than the peptide linkers that are intracellularly cleaved by lysosomal enzymes. Nevertheless, due to its pH sensitive stability, hydrazone hydrolysis can provide for specific drug release, though at a different rate, in tumour tissue or in tumour cells (Kratz et al., 2002). For the present study, about 6.2% (w/w) drug loading was obtained, which was comparable to the payload reported in the literature with similar polymer therapeutics (Taniguchi et al., 1999; Nogusa et al., 2000a). The amount of conjugated doxorubicin determined by spectrophotometric analysis was in good agreement with the results obtained after release under acidic conditions indicating that neither the polymer conjugation nor the macromolecular assembly affect the spectroscopic properties of the drug. Based on the evidence that doxorubicin entrapment into hydrophobic micelles changes significantly the spectroscopic properties of the drug (Mohan and Rapoport, 2010), it is possible to conclude that in the case of the pullulan bioconjugates the assembly core provides for a doxorubicin hydrophilic environment. It should be noted that the doxorubicin conjugation via the maleimide activated doxorubicin–EMCH is extremely efficient as it resulted in a nearly 100% yield indicating that higher drug loading could be achieved.

In buffer, both conjugates formed nanosized assemblies according to a concentration-independent behaviour. The unimolecular fraction represented a very small subpopulation as compared to the larger structures and a constant abundance ratio of the two subpopulations was observed in a large range of concentrations. This structural homogeneity of the bioconjugates is paramount to avoid unpredictable and unlike *in vivo* performance. The two bioconjugates yielded nanoassemblies with different size underlying the effect of the physicochemical character of the various modules of the macromolecules on their aggregation properties. Due to its hydrophobic character, folic acid was expected to promote the aggregation of (FA-PEG)-Pull-(Cyst-Dox) as reported in the literature for FA–pullulan and other FA–polymer conjugates (Kim et al., 2008; Prabaharan et al., 2009a). The DLS profiles of the pullulan bioconjugates bearing doxorubicin and doxorubicin/folic acid reported in Fig. 1C–E show the presence of small size and large size species that correspond to the unimolecular form and multi-

molecular assemblies, respectively. The (NH_2PEG)-Pull-(Cyst-Dox) ability to form assemblies indicates that also doxorubicin promotes the macromolecular aggregation according to a dynamic equilibrium dictated by the overall composition, structural and physicochemical properties of the bioconjugates. This aggregation tendency is driven by hydrophobic modules and is expected to increase as the amount of the conjugated folic acid and doxorubicin increases. Since high amounts of doxorubicin or folic acid can cooperatively contribute to obtain insoluble materials, the degree of polymer functionalisation is a key parameter to produce derivative with the required biopharmaceutical properties. On the other hand, the bulky hydrated PEG chains can prevent aggregation phemomena. The absence of large assemblies indicates that PEG can efficiently counterbalance the hydrophobic character of the targeting moiety. The smaller size of the folated derivative seems to indicate that the folic acid favour the formation of more compact structures with respect to the non-folated bioconjugate where the PEG chains may convey a looser structure. The vesicle size differences between the data obtained with these derivatives and the pullulan biconjugates described in the literature may be ascribed to the different composition of the conjugates. The data reported in the literature were in fact generated using pullulan with a higher molecular weight (200 kDa) and absence of PEG in the macromolecular structure. Actually, conjugating folic acid directly to the polysaccharide hydroxyl groups results in partial masking of hydroxyl groups engaged in water coordination and multiple insertion of hydrophobic moieties (folic acid) on the polymer backbone, which may be responsible for the formation of large size aggregates. On the contrary, the strategy used to produce (NH_2PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox) by the PEG chains derivatisation partially counterbalances the hydrophobic effect of folic acid and prevents the formation of heterogeneous products. Finally, the particle size of both conjugates, (NH_2PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox), is below the cut-off size for passive disposition into tumour tissues by EPR (Campbell, 2006).

Concerning the bioconjugate assemblage, it should be noted that, in contrast with the FA–pullulan nanogels reported in the literature that contain drug physically entrapped in the hydrophobic core, in the (FA-PEG)-Pull-(Cyst-Dox) and (NH_2PEG)-Pull-(Cyst-Dox) derivatives doxorubicin is covalently conjugated to the polymer. Therefore, the nanoparticle formation via supramolecular aggregation is not a pre-requisite for drug delivery, whereas the drug release from the conjugates described in this work can be properly controlled as it is not strictly affected by the drug loading and physicochemical stability of the particles. Drug release studies were carried out to investigate the feasibility of using the conjugates as anticancer drug delivery systems, which is strictly related to their stability while circulating in the bloodstream. The drug release behaviours in plasma and in buffer at pH 7.4 and 5.5 were in good agreement with the data reported in the literature (Prabaharan et al., 2009a,b). The hydrazone bond was fairly stable at pH 7.4 and in plasma as required to prevent systemic toxicity due to doxorubicin release in the bloodstream and unspecific diffusion into main tissues and organs. At pH 5.5, which mimics lysosomal conditions, complete drug release was obtained in about 40 h indicating that doxorubicin could be efficiently released into tumour cells.

The cytotoxic effect of the conjugates and their cell selectivity for the tumour cells were examined using folate receptor-overexpressing and folate receptor-non-expressing cell lines, KB and MCF7, respectively.

Both conjugates displayed higher IC_{50} values with respect to those obtained with free doxorubicin with either KB or MCF7 cells. This result was in agreement with the slow drug release from the supramolecular system observed *in vitro* and the colloidal nature of the drug carriers that can slow the cellular disposition of the drug. However, both derivatives displayed high toxicity against the cancer cells indicating that they undergo cell internalisation that is in agreement with the ability of pullulan derivatives to be taken up by cells has been reported in the literature (Nogusa et al., 2000b).

The non-folated bioconjugate displayed slightly higher cytotoxicity against the MCF7 cell line, which does not overexpress the folate receptor, as compared to the folated counterpart. This result suggests that the physicochemical properties of the non-folated conjugate, namely the small size combined with the presence of unconjugated amino groups along the supramolecular structure that convey an overall positive charge to the carrier, can favour its unspecific cell uptake.

Unexpectedly, the folated derivative was only slightly more active than the folate-free conjugate towards the folate receptor overexpressing KB cells, indicating that folic acid conjugation was marginally effective in conveying targeting properties to the drug carrier. Similar behaviour was obtained with folate receptor overexpressing HeLa cells used in the cell trafficking studies (data not shown). This result is apparently in contrast with the cell trafficking data, which showed that folic acid promotes the rapid cell internalisation of folated macromolecular prodrugs into the folate-receptor overexpressing cells. The rapid cell uptake evidenced by the multispectral imaging flow cytometer study is in good agreement with the active folated macromolecule internalisation mechanism described in the literature (Leamon and Low, 1991). This behaviour is also supported by preliminary data that showed that short time incubation of HeLa cells with the folated derivative results in higher cytotoxicity than the non-folated bioconjugation treatment. However, it seems conceivable that the unspecific cell uptake, dictated by the structural features of the supramolecular carriers, predominates the long term internalization overcoming the active folic acid mediated process. The cell uptake data showed, in fact, that after 5 h incubation the two bioconjugates yielded similar intracellular doxorubicin levels. Therefore, although the two carriers may undergo different internalisation pathways and rates, the prolonged cell exposition can minimise the differences between the two conjugates. In order to elucidate the trafficking mechanisms, proper experimental protocols, which include the use of different cell lines, competition investigations, time frame of incubation as well as cell viability studies have been set-up and detailed investigations will be carried out.

Finally, pharmacokinetic studies were undertaken to evaluate the effect of the polymer conjugation on the bloodstream permanence of doxorubicin. A prolonged permanence in the circulation is, in fact, a requisite to achieve effective tumour disposition by EPR.

The polymer bioconjugation was found to prolong the circulation time of the drug *in vivo* according to the typical biexponential behaviour of macromolecules. The alpha-phase may be affected by the product heterogeneity, with the rapid elimination of small size derivatives, as well as by the distribution to the peripheral compartment. Because the study was carried out using healthy mice, the distribution into the peripheral compartment may be ascribed to partial spleen and liver disposition as pullulan derivatives were reported to have tropism for these RES rich tissues (Kaneo et al., 2001). Therefore, despite the chemical modification and the conformational assembling, the polysaccharide maintains structural properties suitable for oligosaccharide-specific receptor recognition on hepatocyte cell membrane. This capacity seems in agreement with the cell culture evidences showing that, despite modification, the conjugate maintains the structural properties that guarantee the cell interaction.

5. Conclusions

The present study shows that pullulan is a versatile macromolecular platform for anticancer drug delivery.

The chemical protocols described here can be properly exploited to selectively introduce into the polysaccharide backbone drugs, targeting agents and any other biological or physicochemical modifier that can modulate the therapeutic performance of the bioconjugate. Accordingly, multifunctional supramolecular structures for combination therapy, passive and active drug targeting and microenvironment sensitiveness can be conveniently designed.

The data obtained with folic acid show that although the straightforward conjugation of a targeting agent conveys to the system recognition properties, the biopharmaceutical and pharmacological performance of the system was substantially unaffected. This observation leads to two conclusions. The first is that sophisticate architectures are not necessarily required to obtain effective drug delivery systems, which is relevant when considering that any additional level of structural complication dramatically increases the problems for product realisation. Secondly, accurate structural design is required to prepare supramolecular systems for active drug targeting to make them therapeutically efficient.

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