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Redox-stimuli responsive micelles from DOX-encapsulating polycaprolactone-*g*-chitosan oligosaccharide

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ABSTRACT

Chitosan-based amphiphilic graft copolymers are commonly obtained by modification of chitosan backbones with synthetic polymers hampering both bioactivity and biodegradability. In this work, we report the preparation of a series of chitosan oligosaccharide-grafted copolymers (PCL-*g*-COs) from coupling reactions between azide-pendent polycaprolactones (PCL-N_3) and reducing-end alkynyl-modified chitosan oligosaccharides (COs-alkynyl). The resulting PCL-*g*-COs self-organized in water into nanoscale micelles (R_h < 20 nm) having a COs shell and a PCL core. Locking of the core-micelles structure employing a disulfide-containing bis-alkyne cross-linker resulted in the formation of nano-vehicles which can be degraded in response to physiological (redox) stimuli. This feature was advantageously exploited to preferentially release an anticancer drug, doxororubicin, in response to the intracellular glutathione level.

1. Introduction

The development of biodegradable and biocompatible stimuli-responsive drug delivery systems has been extensively raised over the past decades. A large library of "smart" polymer micelles have already been developed in order to effectively deliver a drug to a target site and thus increase the therapeutic benefit while minimizing side effects (Kaditi, Mountrichas, Pispas, & Demetzos, 2012; Mura, Nicolas, & Couvreur, 2013; Zhang, Koa, & Oh, 2012). Internal stimuli-responsive (pH, ions, glucose, enzymes, temperature, redox, etc.) systems have gained wider attention compared to systems responding to external stimuli (light, ultrasound, magnetic and electric field, etc.) due to their self-controlled drug release and facile application in clinical settings.

Drug delivery systems employing a redox cleavage of disulfide bonds are of particular relevance because cytosol and cell nuclei contain 100–1000 times higher concentration of reducing glutathione (GSH) tripeptide than body fluids including blood and extracellular medium (0.5–10 mM *versus* 2–20 μM GSH) (Lee et al., 2013; McCarley, 2012) and because cancer cells often exhibit even higher levels of glutathione (Manickam et al., 2010). Such redox-sensitive nanovehicles are generally prepared from self-association of amphiphilic block or graft copolymers, containing hydrophobic and hydrophilic segments, linked or cross-linked through a disulfide bridge (Haradaa & Kataoka, 2006).

A wide variety of biodegradable redox-sensitive amphiphilic copolymers has been described to incorporate and release drugs. Hydrophobic polymers such as poly(caprolactone) (PCL), poly(lactic-*co*-glycolic acid), poly(lactic acid), polyphosphoesters and polypeptides have been coupled, for example, with hydrophilic poly(ethylene glycol) (Khorsand, Lapointe, Brett, & Oh, 2013; Wang, Wang, Sun, & Wang, 2011) or polysaccharides (Huanli et al., 2010; Minghui et al., 2013). Polysaccharides are of particular interest due to their renewability, non-toxicity, biodegradability, and for some of them, their biological and physicochemical properties (Gref, Rodrigues, & Couvreur, 2002).

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Chitosan, a linear copolymer of *N*-glucosamine and *N*-acetylglucosamine with β-1,4 linkage derived from partial de-*N*-acetylation of chitin, is certainly one of the most studied polysaccharides in the biomedical and pharmaceutical fields (Shukla, Mishra, Arotiba, & Mamba, 2013), particularly in drug delivery (Meng et al., 2013; Sanyakamdhorn, Agudelo, & Tajmir-Riahi, 2013) and gene transfection (Richard, Thibault, De Crescenzo, Bushmann, & Lavertu, 2013; Zonghua, Ziyong, Changren, & Yanpeng, 2010). The significant attention paid to chitosan-based materials stems from the attractive biological properties of this polysaccharide including analgesic, antitumor, hemostatic, hypocholesterolemic, antimicrobial and antioxidant activities. Chitosan also acts as a permeation enhancer by opening epithelial tight junctions in acidic environments (Artusso, Lindmark, Davis, & Illum, 1994) and strongly interacts with mucus or negatively charged mucosal surfaces (Sogias, Williams, & Khutoryanskiy, 2008). These features are mostly related to the ionizable character of chitosan due to the presence of amino functions at C-2, and to a lesser extent, to the molecular weight of the polysaccharide chains.

Aiming at generating nano-scale chitosan-based drug delivery systems, numerous studies have described the preparation of amphiphilic chitosan-based copolymers able to self-assemble in water. In this context, amphiphilic chitosan-based graft copolymers containing a chitosan backbone and hydrophobic pendent blocks have been extensively studied owing to their straightforward preparation (Novoa-Carballal, Riguera, & Fernandez-Megia, 2012). For instance, biodegradable polyesters have been anchored on chitosan chains through "grafting from" procedures (Qu, Wirsen, & Albertsson, 1999) or through "grafting onto" procedures by amidation, esterification or urethanization reactions with the numerous reactive amine or hydroxyl groups of the polysaccharides, respectively (Feng & Dong, 2006; Gao, Li, Lu, Wu, & Fuhrhop, 2008; Haijun, Wenshou, Xuesi, Chao, & Xiabin, 2006; Moyuan et al., 2012). These grafting strategies are undeniably of interest to prepare biohybrids but unfortunately are surely inadequate to maintain the original biological and biodegradable properties of chitosan; in order to do so it may be necessary that the chitosan backbone remains intact. Obviously, the preparation of bioactive chitosan-based copolymers should thus rely on the (more challenging) chemical modification of chitosan chain ends.

We recently reported an effective route to reducing end-functionalized chitosan oligosaccharides (COs) through aniline-catalyzed reductive amination (Guerry et al., 2013). It was shown that aniline derivatives such as 4-propargyloxyaniline readily react with chitosan reducing end groups to generate "clickable" COs in high isolated yield. A brief study showed the successful grafting of COs-alkynyl onto one well-defined PCL-N_3 backbone.

In this work, we take advantage of these preliminary findings to expand our study on a series of chitosan oligosaccharide-grafted polycaprolactones which are prone to self-assemble in aqueous environment as characterized by diffusion light scattering and transmission electron microscopy pictures. *In situ* core cross-clicking with a disulfide-containing cystamine linker allowed the study of the encapsulation and the release properties of doxorubicin (DOX)-loaded redox stimuli-sensitive micelles.

2. Experimental

2.1. Materials

Chitosan oligosaccharides (trade name FACOS) exhibiting low molecular weight (inferior to 2 kDa and DP = 4.8 estimated by ^{1}H NMR) (Guerry et al., 2013) and having an acetylation degree of 10% were kindly provided by Kitto Life Co., Ltd. The catalyst CuI·P(OEt)$_3$ was synthesized as reported in the literature (Ziegler, Fowler, Rodgers, & Wester, 1987). All other chemicals were obtained from commercial suppliers and used without further purification. Water was purified by a Milli-Q water purification system (Billerica, MA, USA). Dialysis membranes (Spectra/Por® – MWCO: 10,000 and 1000) were purchased from Spectrum Laboratories, Inc. 2,2-dibutyl-2-stanna-1,3-dioxepane (DSDOP) (Kricheldorf & Eggerstedt, 1998), reducing-end alkynyl-modified chitosan oligosaccharides (COs-alkynyl; DP = 4 estimated by ^{1}H NMR) (Guerry et al., 2013) and poly(α-azido-ε-caprolactone-*co*-ε-caprolactone) (PCL-N_3) were synthesized as reported in the literature (Riva, Schmeits, Jérôme, Jérôme, & Lecomte, 2007). The synthesis of the disulfide-containing bis-alkyne cross-linker, bis[(propargyl carbamate)ethyl] disulfide (Cys-alkynyl) (Liu et al., 2010), was adapted from a previously reported procedure. (Sinha, Ilankumaran, & Chandrasekaran, 1999). Briefly, to a solution of cystamine dihydrochloride (1 g, 4.4 mmol) dissolved into 12 mL dioxane was added aq. NaOH (0.01 M, 8 mL) to reach pH 9, and 4 equivalents of propargyl chloroformate (1.73 mL, 17.6 mmol) were dropwise added at r.t. and allowed to react for 2 h. The obtained Cys-alkynyl was purified by chromatography on silica gel (eluent: ethyl acetate/petroleum ether, 2/8, v/v) to give a colorless oil (1.2 g, 81% yield).

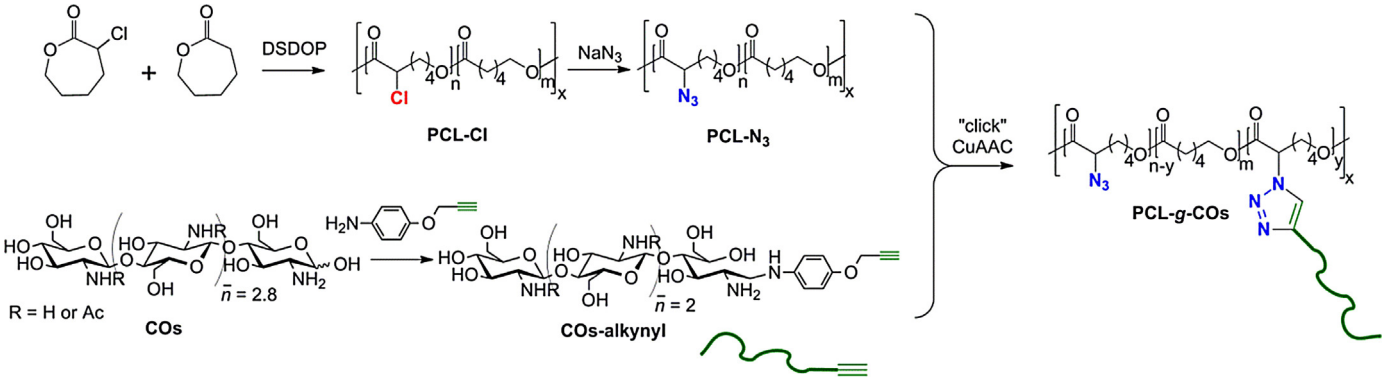
2.2. Instrumentation

^{1}H NMR spectra were recorded using 400 MHz Bruker Avance DRX400. Size exclusion chromatography (SEC) was performed at 40 °C using a Agilent 390-MDS system (290-LC pump injector, ProStar 510 column oven) equipped with a Varian 390-LC refractive index detector and a Knauer Smartline UV detector 2500 and two Agilent PolyPore PL1113–6500 columns (linear, 7.5 mm × 300 mm; particle size, 5 μm; exclusion limit, 200–2,000,000) in DMF/LiCl (0.01 M) at the flow rate of 1.0 mL min^{-1}. Infrared (IR) spectra were recorded using a Perkin-Elmer Spectrum RXI FTIR spectrometer. Transmission electron microscopy (TEM) was carried out using a CM200 Philips microscope. TEM images of the micelles were conducted using a CM200 Philips transmission electron microscope operating at 80 kV with Kodak SO163 film. One drop of micelle suspension was placed on a copper mesh covered with nitrocellulose membrane and dried at room temperature before being stained with uranyl acetate solution (2%, w/v in water). Scattering measurements were performed using an ALV laser goniometer, which consists of a 35 mW HeNe linearly polarized laser operating at a wavelength of 632.8 nm, an ALV/LSE-5004 multiple τ digital correlator with a 125 ns initial sampling time, and a temperature controller. Data were collected typically for a counting time of 300 s at 90° using the digital ALV correlator control software. Each sample was analyzed in triplicate. The aqueous solutions of PCL-*g*-COs were directly filtered into the glass cells through a 0.45 μm Millipore Millex MCE (Mix Cellulose Ester) filter. The relaxation time distribution was obtained using CONTIN analysis of the autocorrelation functions $C(q,t)$. All fluorescence measurements were performed using a Perkin Elmer LS50B spectrofluorometer. Excitation and emission were set at 480 nm and 520 nm respectively with bandwidths of 2.5 and 10 nm, respectively.

2.3. Methods

2.3.1. Synthesis of PCL-g-COs

Typically, 50 mg of PCL-N_3 was dissolved into 5 mL of DMF and allowed to react with 100 mg of COs-alkynyl in the presence of 58 mg of CuI·P(OEt)$_3$ under argon atmosphere. 300-500 μL of water was added to facilitate the COs-alkynyl dissolution. The solution was stirred at 40 °C for 36 h. The reaction mixture was poured into 80 mL of water containing 200 μL of acetic acid to precipitate unreacted PCL-N_3 and centrifuged at 8000 rpm during 20 min at 4 °C. The



Scheme 1. Synthesis of amphiphilic polycaprolactone-*graft*-chitosan (PCL-*g*-COs) by CuAAC coupling.

solid was removed and the supernatant was ultrafiltered through a 10,000 MWCO cellulose acetate membrane and washed with 2 L of deionized water to remove salts and unreacted COs-alkynyl. Finally, purified PCL-*g*-COs was lyophilized to give a white solid with a yield in the range of 62–79%.

2.3.2. Preparation of PCL-g-COs micelles

The graft copolymer-based micelles were prepared by direct dissolution in water, a selective solvent for oligosaccharides. Briefly, 5 mg of PCL-*g*-COs, was directly added into 2.5 mL of water. The solution was stirred at 300 rpm at 40 °C for 12 h.

2.3.3. Cross-linking of the PCL-g-COs micelles

50 mg of freshly synthesized graft copolymer was dissolved in 25 mL of DMF at r.t. and stirred 4 h. Then 2 molar equivalents for each residual azide groups of cross-linking agent Cys-alkynyl (5.7 mg), and CuI·P(OEt)$_3$ (6.4 mg) were added. The solution was stirred for 48 h at 40 °C. The reaction mixture was poured into 100 mL of water containing 200 μL of acetic acid and the final solution was ultrafiltered through a 10,000 MWCO cellulose acetate membrane and washed with 2 L of deionized water to remove organic solvent, salts and cross-linker. Finally, cross-linked PCL-*g*-COs was lyophilized to yield 87% of a white solid.

2.3.4. Preparation of DOX-loaded micelles

10 mg of freshly synthetized grafted copolymers, PCL-*g*-COs, was dissolved with 1 mg of DOX·HCl into 20 mL of DMF. The solution was mixed with 0.5 μL of triethylamine and stirred for 4 h resulting in the regeneration of the DOX free base. Then 2 molar equivalents for each residual azide groups of cross-linking agent Cys-alkynyl (1.2 mg), and CuI·P(OEt)$_3$ (1.3 mg) were added. The reaction mixture was stirred during 48 h at 40 °C then poured into 100 mL of water containing 200 μL of acetic acid. Free DOX was separated by ultrafiltration through a 10,000 MWCO cellulose acetate membrane. The UV-absorbance at 480 nm of the outer aqueous phase allowed the determination of the drug loading content and loading efficiency. Empty PCL-*g*-COs nanoparticles were used as a blank test and standard DOX solutions at varied concentrations (1–100 μg mL^{-1}) were prepared by dilution from a stock solution. The following equations were used to determine drug content and loading efficiency: Drug content = [(drug weight in the copolymer nanoparticles)/(weight of copolymer nanoparticles)] × 100. Loading efficiency = [(residual drug in the copolymer nanoparticles)/(initial feeding amount of DOX)] × 100.

2.3.5. Reduction-triggered release of DOX

A solution of DOX-loaded micelles at 2 mg mL^{-1} into PBS buffer (pH 7.4, 10 mM) was put in 1000 MWCO membrane dialysis tubing and exchanged against PBS buffer. The reductive environment were brought by reaching 10 mM or 2 μM GSH. At pre-determined time intervals, an aliquot of the outer buffer phase was removed and replaced with fresh PBS buffer, and then the concentration of released DOX was calculated by fluorescence spectroscopy. Standard DOX solutions (1–100 μg mL^{-1}) were prepared by dilution from a stock solution.

3. Results and discussion

The PCL-*g*-COs copolymers were prepared by copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) coupling between polycaprolactone backbones with α-azide pendent groups and alkynyl reducing-end functionalized COs (COs-alkynyl, DP = 4) (Scheme 1). These commercially available COs are of particular interest since they exhibit various biological activities (www.kittolife.co.kr).

3.1. Synthesis of well-defined polycaprolactone with α-azide pendent groups

As a first step toward the preparation of PCL-*g*-COs graft copolymers (Scheme 1), we prepared, following the procedure reported by Riva et al., a series of polycaprolactones (Table 1) with tunable and controlled degree of polymerization (50, 100 and 200) and azide content (5 and 20% of molar fraction), abbreviated as PCL$^{50,\ 100\ or\ 200}$-N$_3{}^{5\ or\ 20\%}$.

Table 1
Main characteristics of PCL-N$_3$ copolymers.

Copolymers	PCL-N$_3{}^{5\%}$			PCL-N$_3{}^{20\%}$		
	PCL^{50}-N$_3{}^{5\%}$	PCL^{100}-N$_3{}^{5\%}$	PCL^{200}-N$_3{}^{5\%}$	PCL^{50}-N$_3{}^{20\%}$	PCL^{100}-N$_3{}^{20\%}$	PCL^{200}-N$_3{}^{20\%}$
$M_{n\ NMR}$ (g mol^{-1})	5800	11,600	23,100	6000	12,100	24,100
$M_{n\ SEC}$ (g mol^{-1})	5300	11,000	19,700	5100	10,100	19,700
$Đ$[a]	1.4	1.2	1.3	1.4	1.5	1.4
$F_{\alpha N3\varepsilon CL}$[b]	0.07	0.04	0.04	0.21	0.22	0.21

[a] Determined by SEC analyses using polystyrene standards calibration.
[b] Calculated from ^{1}H NMR spectra.

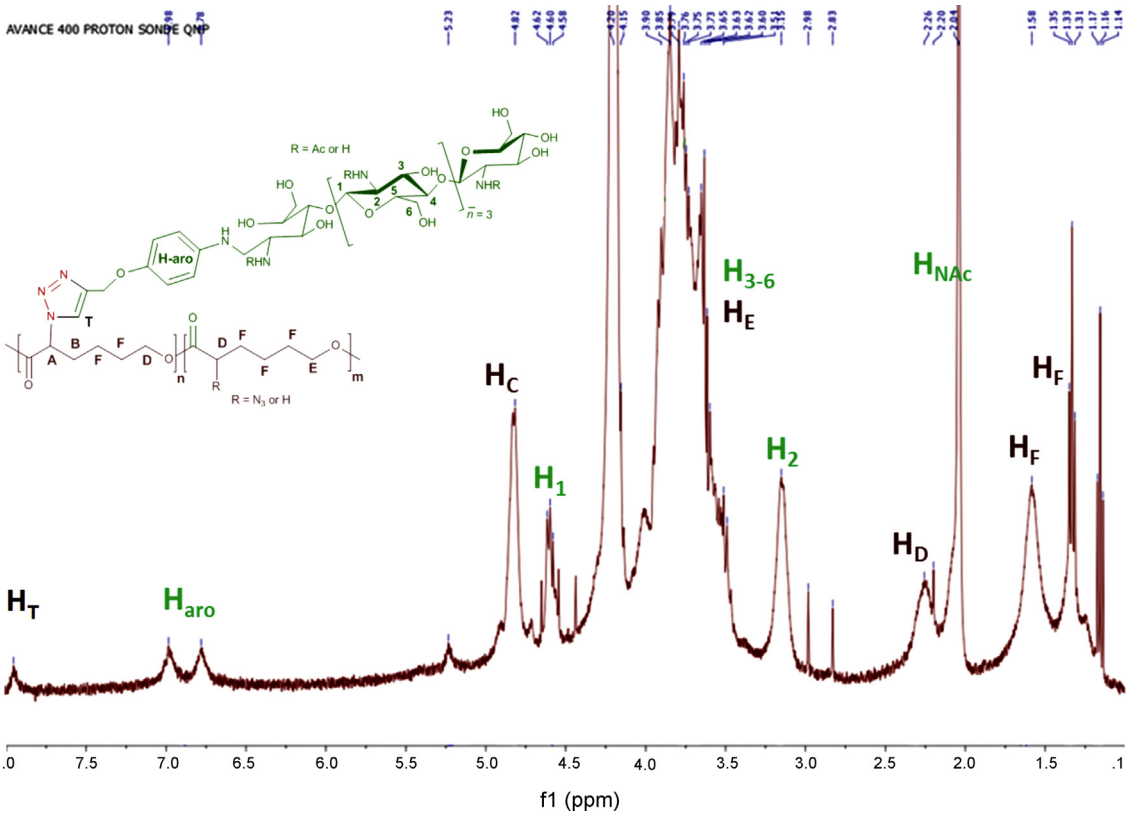


Fig. 1. ^{1}H NMR spectrum (400 MHz, DMF-d_7/D_2O (3:1, v/v) + AcOH-d_4, 353 K) of PCL^{200}-*g*-COs$^{17\%}$.

Briefly, copolymerizations of α-chloro-ε-caprolactone (αClεCL) generated by Baeyer-Villiger oxidation of α-chlorocyclohexanone, and ε-caprolactone (ε-CL) were performed with different αClεCL/ε-CL ratios in toluene using DSDOP as initiator. After polymerization (99% conversion in 4 h at 40 °C), the pendent chloride units (PCL-Cl) were quantitatively substituted by azide groups (PCL-N_3) as confirmed by the appearance of the typical azide band at 2100 cm^{-1} (Fig. S1 in Supporting Information) and the full shift of the proton in α-position of the carbonyl group from 4.3 to 3.8 ppm (Figs. S2–S3 in Supporting Information). Consistent with the nearly full consumption of the monomers and the efficiency of the azidation, good agreement between monomer feed ratio and final polymer chemical composition was observed.

3.2. Synthesis of chitosan oligosaccharides-grafted polycaprolactone (PCL-g-COs)

After performing CuAAC ligation between COs-alkynyl and PCL$^{50,\ 100\ or\ 200}$-$N_3{}^{5\ or\ 20\%}$, we observed that PCL-*g*-COs series obtained from PCL with around 5% of azide (PCL-$N_3{}^{5\%}$) are insoluble in water owing to their low content of hydrophilic COs grafts. Thus, we further focused the study on the PCL series having the highest content in azide groups (PCL^{50}-$N_3{}^{20\%}$, PCL^{100}-$N_3{}^{20\%}$ and PCL^{200}-$N_3{}^{20\%}$). The graft copolymers obtained therefrom were purified by precipitation and subsequent ultrafiltration on MWCO 10,000 membranes under acidic conditions to remove unreacted COs-alkynyl, organic co-solvents and copper salts.

The coupling between PCL-$N_3{}^{20\%}$ backbones and COs-alkynyl oligosaccharides was first assessed by SEC analysis in DMF containing 0.01 mol L^{-1} LiCl. Purified PCL-*g*-COs revealed a single monomodal symmetrical peak at higher molecular weight than the peak of PCL-$N_3{}^{20\%}$ precursor and no degradation products or unreacted chitosan oligosaccharides were observed (Fig. S4 in Supporting Information). Further evidence of the formation of PCL-*g*-COs copolymers was given by ^{1}H NMR analyses (Fig. 1 and Figs. S5 and S6 in Supporting Information). The CuAAC ligation was indeed confirmed by ^{1}H NMR with the emergence of signals for aniline and triazole groups between 6.70 and 7 ppm and around 8.10 ppm, respectively. The typical peaks of the COs at 3.17 ppm (H_2 protons), between 3.40 and 4.00 ppm (H_3, H_4, H_5, H_6 protons) and at 4.60 ppm (H_1 protons) were also observed. The molar fraction of COs on the PCL backbone (F_{COs}) was calculated from integration of the methylene protons of PCL (protons D) at 2.27 ppm and H-2 of COs derivatives at 3.17 ppm according to equation $F_{COs} = (I_{H\text{-}2}/4)/(I_{H\text{-}D}/2)$. Whatever the degree of polymerization of PCL^x-$N_3{}^{20\%}$ (x = 50, 100 or 200), F_{COs} values were around 16% indicating that the yield of COs grafting was around 80% and that 20% of the azide groups (whose presence was also confirmed by FTIR with a characteristic band at 2100 cm^{-1}; see Fig. S7 in Supporting Information) are still available for further post-modifications (cross-clicking reactions). The covalent attachment of oligosaccharide grafts to PCL backbones was further confirmed by DOSY NMR experiments which pointed out the presence of one single diffusion coefficient (see Fig. S8 in Supporting Information).

Aqueous solutions of graft copolymers (2 mg mL^{-1}) were then characterized by a combination of DLS and TEM analyses. DLS measurements highlighted the presence of two populations for all graft copolymer solutions. The major population (in number) with diameters ranging from 9 to 18 nm corresponds to micelles which are prone to aggregate into larger particles of around 100 nm (Table 2). However, this second population represents 1% or less of the sample. Consistent with these DLS results, TEM pictures from graft copolymers solutions confirmed the presence of a major population of spherical nano-objects with a radius of about 10 nm (Figs. S9–S10 in Supporting Information). These observations are in agreement with the assumption that amphiphilic PCL-*g*-COs micelles consist of a hydrophobic PCL core surrounded by a hydrophilic COs shell.

Table 2
Isolated yields of PCL-*g*-COs and their molecular characteristics.

Graft copolymer	F_{COs}[a] (% molar)	Coupling yield (%)	R_h of micelles (nm)[b]	R_h of aggregates (nm)[b]	Aggregation ratio (%)[b]
PCL^{50}-*g*-$COs^{16\%}$	16	80	18	87	1.1
PCL^{100}-*g*-$COs^{15\%}$	15	75	13	137	0.5
PCL^{200}-*g*-$COs^{17\%}$	17	85	9	88	0.4

[a] Molar content of COs in the graft copolymer determined by ^{1}H NMR.
[b] Determined by DLS in water (aggregation ratio = $(N_{agg}/(N_{micelles} + N_{agg}))$ with N_{agg} and $N_{micelles}$ corresponding to the population observed at higher and smaller size, respectively).

3.3. Core cross-"clicked" PCL-g-COs nanoparticles

To maintain the integrity of the nanoparticles, the graft copolymer micelles were subsequently cross-linked at the core using CuAAC reaction thanks to the presence of unreacted azide groups along PCL backbones (Fig. 2). With the objective of conferring a redox-sensitive and bio-reducible character to the nano-objects, the cross-linking reaction was performed on PCL^{100}-*g*-$COs^{15\%}$ using a disulfide-containing dialkyne cross-linker (Cys-alkynyl). This step was performed at low concentration (2 mg mL^{-1}) in order to avoid inter-particle coupling reactions. The self-assembled nanoparticles were purified by ultrafiltration using a filtration membrane with

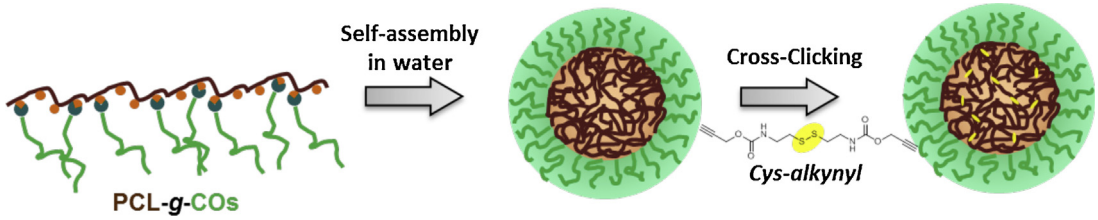


Fig. 2. Graft copolymer self-assembly in aqueous solution (2 mg mL^{-1}) and core cross-clicking of PCL-*g*-COs with the disulfide Cys-alkynyl.

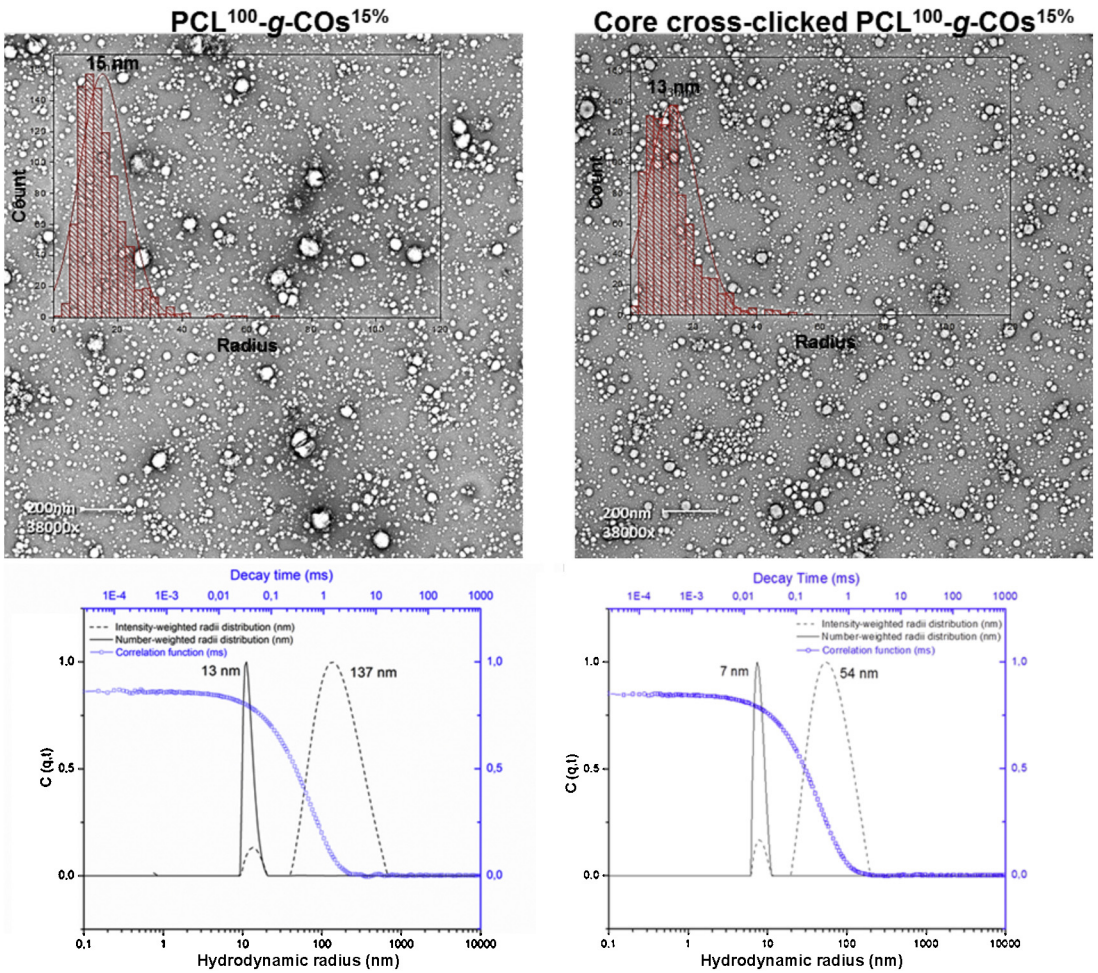


Fig. 3. Transmission electron micrograph with size distribution and dynamic light scattering autocorrelation function (scattering angle = 90°, temperature = 25 °C), intensity-weighted (- - -) and number-weighted radii distribution (—), of PCL^{100}-*g*-$COs^{15\%}$ (left) and core cross-clicked PCL^{100}-*g*-$COs^{15\%}$ (right) micelles in water.

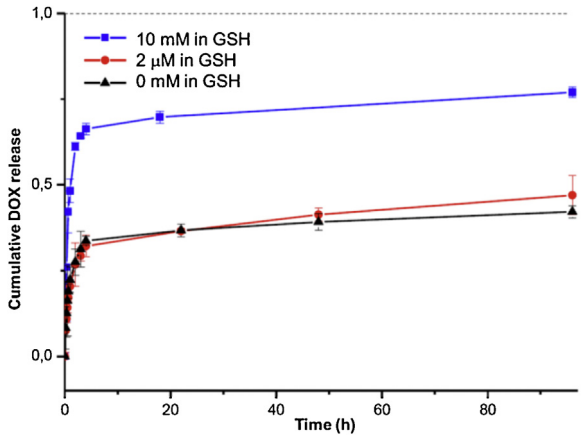


Fig. 4. *In vitro* release of DOX from PCL^{100}-*g*-COs$^{15\%}$ in PBS (pH 7.4, 10 mM) buffer at 37 °C in the absence and presence of GSH (2 μM and 10 mM). The error bars in the graph represent standard deviations ($n = 3$).

a molecular weight cut-off of 10,000 Da. The occurrence of the crosslinking reaction was first assessed by IR analyses with a significant decrease of the azide absorption band at 2100 cm^{-1} (Fig. S11 in Supporting Information).

The cross-linked nanoparticles were characterized by DLS and TEM as shown in Fig. 3. After CuAAC reaction with Cys-alkynyl, the diameter of PCL^{100}-*g*-COs$^{15\%}$ nanoparticles determined by DLS dropped to 7 nm (*vs* 13 nm for PCL^{100}-*g*-COs$^{15\%}$ micelles) consistent with the efficient cross-linking of the polyester core of the nanoparticles and the absence of undesirable inter-nanoparticle coupling reaction. TEM analysis confirmed the spherical shape of the nanoparticles and highlighted a tendency of the nanoparticles to aggregate in the dry state (number average diameter around 13 nm *vs* 15 nm before cross-linking).

3.4. Loading and reduction-triggered release of DOX

We subsequently investigated the loading of DOX, a model hydrophobic drug that is commonly used in the treatment of different types of solid malignant tumors, into self-assembled PCL^{100}-*g*-COs$^{15\%}$. The commercial DOX hydrochloride was first converted to the free base by the addition of 2 equivalents of triethylamine in DMF. The DOX solution was added to the copolymer PCL^{100}-*g*-COs$^{15\%}$ one hour before the cross-linking procedure. After cross-linking, the system was purified by ultrafiltration and the amount of residual free DOX was estimated by UV–vis absorbance at 480 nm. The results showed that PCL^{100}-*g*-COs$^{15\%}$ nanoparticles effectively encapsulated DOX, affording a drug loading efficiency and content of about 86% and 8.6%, respectively. These high values indicate a strong interaction between DOX and the hydrophobic core of PCL^{100}-*g*-COs$^{15\%}$ nanoparticles.

We anticipated that the presence of disulfide bridges linking the PCL blocks of PCL^{100}-*g*-COs$^{15\%}$ nanoparticles should allow a reductive glutathione-responsive DOX release. To demonstrate the responsiveness of our drug delivery system, GSH was incorporated (at different concentrations) to a solution of DOX-loaded cross-linked PCL^{100}-*g*-COs$^{15\%}$ micelles in PBS buffer (pH 7.4, 10 mM) and the release of DOX over time was measured by fluorescence spectroscopy (Fig. 4). At 2 μM GSH, which physiologically corresponds to extracellular GSH concentrations (*e.g.*, plasma), less than 30% of the DOX was released throughout a period of 50 h. Comparable release profiles were observed for a control experiment performed in the absence of GSH, suggesting a burst effect rather than redox-triggered disassembly of core-nanoparticle as dominant drug release mechanism under such conditions. More importantly, DOX release was significantly accelerated upon addition of 10 mM GSH (66% of DOX released after 4 h), which is comparable to intracellular levels. These results suggest that the core-cross-linked PCL^{100}-*g*-COs$^{15\%}$ micelles may be able to circulate in extracellular environments over a prolonged period of time with limited drug release and liberate drugs within target cells in response to intracellular GSH.

4. Conclusions

Original well-controlled amphiphilic graft copolymers composed of poly(ε-caprolactone) backbone and chitosan oligosaccharide grafts were synthesized by click reaction between the reducing-end alkynyl-functionalized chitosan oligosaccharides and the preformed poly(ε-caprolactone) with pendent azides. Their self-assembly in water, followed by core-cross-clicking with bis-alkynyl cystamine, led to redox-sensitive drug release nanoparticles. These drug-loaded cross-linked nanoparticles were effectively decross-linked in reductive environments (owing to the presence of GSH) mimicking cytoplasm and cell nucleus ones to release doxorubicin. The next step of this project will consist to perform *in vitro* cytotoxic and drug release studies. Drug delivery systems associating biodegradable polyester core and mucoadhesive chitosan corona with GSH-triggered drug release may have a great promise for cancer chemotherapy of epithelial cell adenocarcinomas, among others, by maximizing intracellular delivery efficiency (Zhang, Wang, Zhang, & Sun, 2013). Through this work, we have proved that reducing-end "clickable' COs open new perspectives for the design of advanced chitosan oligosaccharide-based materials.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.06.052.

References

Artusso, P., Lindmark, T., Davis, S. S., & Illum, L. (1994). Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). *Pharmaceutical Research*, *11*, 1358–1361.

Feng, H., & Dong, C.-M. (2006). Preparation, characterization, and self-assembled properties of biodegradable chitosan–poly(L-lactide) hybrid amphiphiles. *Biomacromolecules*, *7*, 3069–3075.

Gao, K.-J., Li, G., Lu, X., Wu, B.-Q., & Fuhrhop, J.-H. (2008). Giant vesicle formation through self-assembly of chitooligosaccharide-based graft copolymers. *Chemical Communications*, *12*, 1449–1451.

Gref, R., Rodrigues, J., & Couvreur, P. (2002). Polysaccharides grafted with polyesters: Novel amphiphilic copolymers for biomedical applications. *Macromolecules*, *35*, 9861–9867.

Guerry, A., Bernard, J., Samain, E., Fleury, E., Cottaz, S., & Halila, S. (2013). Aniline-catalyzed reductive amination as a powerful method for the preparation of reducing end-"clickable" chitooligosaccharides. *Bioconjugate Chemistry*, *24*, 544–549.

Haijun, Y., Wenshou, W., Xuesi, C., Chao, D., & Xiabin, J. (2006). Synthesis and characterization of the biodegradable polycaprolactone-*graft*-chitosan amphiphilic copolymers. *Biopolymers*, *83*, 233–242.

Haradaa, A., & Kataoka, K. (2006). Supramolecular assemblies of block copolymers in aqueous media as nanocontainers relevant to biological applications. *Progress in Polymer Science*, *31*, 949–982.

Huanli, S., Bingnan, G., Xiaoqing, L., Ru, C., Fenghua, M., Haiyan, L., et al. (2010). Shell-sheddable micelles based on dextran-SS-poly(ε-caprolactone) diblock copolymer for efficient intracellular release of doxorubicin. *Biomacromolecules*, *11*, 848–854.

Kaditi, E., Mountrichas, G., Pispas, S., & Demetzos, C. (2012). Block copolymers for drug delivery nano-systems. *Current Medicinal Chemistry*, *19*, 5088–5100.

Khorsand, B., Lapointe, G., Brett, C., & Oh, J.-K. (2013). Intracellular drug delivery nanocarriers of glutathione-responsive degradable block copolymers having p disulfide linkages. *Biomacromolecules*, *14*, 2103–2111.

Kricheldorf, H. R., & Eggerstedt, S. (1998). Macrocycles 2. Living macrocyclic polymerization of ε-caprolactone with 2,2-dibutyl-2-stanna-1,3-dioxepane as initiator. *Macromolecular Chemistry and Physics*, *199*, 283–290.

Lee, M. H., Yang, Z., Lim, C. W., Lee, Y. H., Dongbang, S., Kang, C., et al. (2013). Disulfide-cleavage-triggered chemosensors and their biological applications. *Chemical Reviews*, *113*, 5071–5109.

Liu, J., Jiang, X., Xu, L., Wang, X., Hennink, W. E., & Zhuo, R. (2010). Novel reduction-responsive cross-linked polyethylenimine derivatives by click chemistry for nonviral gene delivery. *Bioconjugate Chemistry*, *21*, 1827–1835.

Manickam, D. S., Li, J., Putt, D. A., Zhou, Q. H., Wu, C., Lash, L. H., et al. (2010). Effect of innate glutathione levels on activity of redox-responsive gene delivery vectors. *Journal of Controlled Release*, *141*, 77–84.

McCarley, R. L. (2012). Redox-responsive delivery systems. *Annual Review of Analytical Chemistry*, *5*, 391–411.

Meng, L., Huang, W., Wang, D., Huang, X., Zhu, X., & Yan, D. (2013). Chitosan-based nanocarriers with pH and light dual response for anticancer drug delivery. *Biomacromolecules*, *14*, 2601–2610.

Minghui, X., Junmin, Q., Aili, S., Hongjie, W., Xueqing, Y., Xuefeng, L., et al. (2013). Reduction/pH dual-sensitive PEGylated hyaluronan nanoparticles for targeted doxorubicin delivery. *Carbohydrate Polymers*, *98*, 181–188.

Moyuan, C., Haixia, J., Weijuan, Y., Peng, L., Liqun, W., & Hongliang, J. (2012). A convenient scheme for synthesizing reduction-sensitive chitosan-based amphiphilic copolymers for drug delivery. *Journal of Applied Polymer Science*, *123*, 3137–3144.

Mura, S., Nicolas, J., & Couvreur, P. (2013). Stimuli-responsive nanocarriers for drug delivery. *Nature Materials*, *12*, 991–1003.

Novoa-Carballal, R., Riguera, R., & Fernandez-Megia, E. (2012). Chitosan-based systems for biopharmaceuticals: delivery, targeting and polymer therapeutics. In B. Sarmento, & J. das Neves (Eds.), *Chapter 19: Chitosan copolymers for biopharmaceuticals*. John Wiley & Sons, Ltd.

Qu, X., Wirsen, A., & Albertsson, A. C. (1999). Synthesis and characterization of pH-sensitive hydrogels based on chitosan and D,L-lactic acid. *Journal of Applied Polymer Science*, *74*, 3193–3202.

Richard, I., Thibault, M., De Crescenzo, G., Bushmann, M. D., & Lavertu, M. (2013). Ionization behavior of chitosan and chitosan–DNA polyplexes indicate that chitosan has a similar capability to induce a proton-sponge effect as PEI. *Biomacromolecules*, *14*, 1732–1740.

Riva, R., Schmeits, S., Jérôme, C., Jérôme, R., & Lecomte, P. (2007). Combination of ring-opening polymerization and "click chemistry": Toward functionalization and grafting of poly(ε-caprolactone). *Macromolecules*, *40*, 796–803.

Sanyakamdhorn, S., Agudelo, D., & Tajmir-Riahi, H.-A. (2013). Encapsulation of antitumor drug doxorubicin and its analogue by chitosan nanoparticles. *Biomacromolecules*, *14*, 557–563.

Shukla, S. K., Mishra, A. K., Arotiba, O. A., & Mamba, B. B. (2013). Chitosan-based nanomaterials: A state-of-the-art review. *International Journal of Biological Macromolecules*, *59*, 46–58.

Sinha, S., Ilankumaran, P., & Chandrasekaran, S. (1999). The prop-2-ynyloxy carbonyl function (POC): A new amino-protecting group removable from sulfur-containing peptides by ultrasonic irradiation with tetrathiomolybdate under mild and neutral conditions. *Tetrahedron Letters*, *40*, 771–774.

Sogias, I. A., Williams, A. C., & Khutoryanskiy, V. V. (2008). Why is chitosan mucoadhesive? *Biomacromolecules*, *9*, 1837–1842.

Wang, Y.-C., Wang, F., Sun, T.-M., & Wang, J. (2011). Redox-responsive nanoparticles from the single disulfide bond-bridged block copolymer as drug carriers for overcoming multidrugr in cancer cells. *Bioconjugate Chemistry*, *22*, 1939–1945.

Zhang, L., Wang, S., Zhang, M., & Sun, J. (2013). Nanocarriers for oral drug delivery. *Journal of Drug Targeting*, *21*, 515–527.

Zhang, Q., Koa, N. R., & Oh, J. K. (2012). Recent advances in stimuli-responsive degradable block copolymer micelles: Synthesis and controlled drug delivery applications. *Chemical Communications*, *48*, 7542–7552.

Ziegler, F. E., Fowler, K. W., Rodgers, W. B., & Wester, R. T. (1987). Ambient-temperature Ullman reaction: 4,5,4′,5′-Tetramethoxy-1,1′-biphenyl-2,2′-dicarboxaldehyde. *Organic Syntheses*, *65*, 108–118.

Zonghua, L., Ziyong, Z., Changren, Z., & Yanpeng, J. (2010). Hydrophobic modifications of cationic polymers for gene delivery. *Progress in Polymer Science*, *35*, 1144–1162.