Materials Science and Engineering C 58 (2016) 692–699

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Effect of drug precursor in cell uptake and cytotoxicity of redox-responsive camptothecin nanomedicines

Pablo Botella [a,*], Carlos Muniesa [a], Víctor Vicente [b,1], Alejandro Cabrera-García [a]

[a] Instituto de Tecnología Química (UPV-CSIC), Avda. Los Naranjos s/n, 46022 Valencia, Spain
[b] Facultad de Medicina and Instituto Universitario de Investigación "Dr Viña Giner", Universidad Católica de Valencia "San Vicente Mártir", 46001 Valencia, Spain

ARTICLE INFO

Article history:
Received 30 April 2015
Received in revised form 4 August 2015
Accepted 2 September 2015
Available online 9 September 2015

Keywords:
Drug delivery
Controlled release
Nanomedicines
Mesoporous silica
Camptothecin
Redox-responsive

ABSTRACT

Novel redox-responsive nanomedicines have been synthesized by conjugating camptothecin prodrugs ((pyridine-2-yldisulfanil)alkyl carbonate derivatives) to hybrid porous silica nanoparticles through disulfide bond. After disulfide reduction, camptothecin may be released by an intramolecular cyclization mechanism or by carbonate bond hydrolysis. Samples have been characterized by physico-chemical techniques, and stability and drug release in PBS and human serum have been determined. Moreover, cell uptake was studied by fluorescence microscopy and flow cytometry, whilst cytotoxic activity was validated by MTT test. Obtained results indicate that prodrug side chain carbon number ($n = 1,2,3$) determines material hydrophobic properties and, as a consequence, its stability in aqueous medium. When n value increases, the negative surface charge decreases dramatically due to a shielding effect provoked by hydrophobic ligands, which promotes particle aggregation and favors cell internalization. Furthermore, the n value determines the type of products released and, subsequently, the cytotoxic activity. Full disulfide bridge reduction takes place in all cases, but quick delivery of the free drug by intramolecular cyclization is only possible with the shortest linker ($n = 1$), whereas other nanomedicines only present slow discharge of camptothecin by carbonate hydrolysis. Overall, the drug precursor incorporated to the inorganic nanoplatform modulates both cell uptake rate and cytotoxicity according to the different functionalization.

1. Introduction

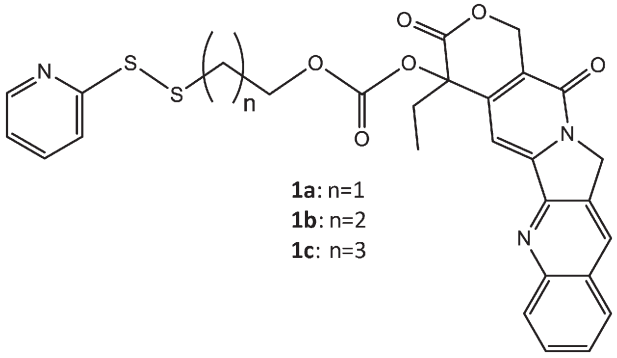
Nanomedicine rating in the pharmaceutical market has risen in recent years due to its advantages over single therapeutic molecules, namely, plasma stability, lower toxicity and controlled release [1–3]. Here, the incorporation of a drug into a nanoparticle can be carried out by simple click-chemistry reactions that provide good stability to the conjugate [4–5]. Accurate intracellular release may be achieved under specific stimuli, avoiding side effects over non-target cells. Most usual possible stimuli able to trigger drug discharge are pH changes, redox reactions, vis/NIR light-induced bond cleavage and the activity of some cytosolic enzymes. Moreover, some external physical issues may be useful to activate a specific release mechanism, e.g., ultrasounds, temperature and magnetic fields [6–9].

However, covalent coupling of antitumor drugs to nanoparticles is circumvented mostly by two issues. Firstly, modified-structure derivatives usually have strongly lower therapeutic activity than initial molecules [10]. To avoid this, smart delivery systems must be developed to transport and release the drug with no molecular modification. Secondly, the variation of the material physicochemical properties, mainly particle size and surface charge, may involve significant changes in cell uptake and intracellular trafficking. Interestingly, very small nanoparticles with positively charged surfaces appear to be the best option to deliver therapeutic molecules inside cells [11–18]. We have recently developed a nanoplatform for the antitumor drug camptothecin (CPT, a topoisomerase I inhibitor) based on a mercapto-functionalized silica hybrid containing a non-porous core and a mesoporous shell. By attaching a CPT prodrug to surface thiol groups, the system is sensitive to reducing compounds as dithiotreitol (DTT) or glutathione (GSH), releasing unmodified CPT at the cytosol [19].

In this work we present novel CPT redox-responsive nanomedicines by conjugating different prodrugs ((pyridine-2-yldisulfanil)alkyl carbonate camptothecin) to hybrid porous silica nanoparticles through disulfide bond. Then, after disulfide reduction, CPT may be released by an intramolecular cyclization or by carbonate bond hydrolysis. Here, we

* Corresponding author.
E-mail address: pbotella@itq.upv.es (P. Botella).
[1] Present address: School of Chemistry, Yachay Tech, Yachay City of Knowledge, 100,119 Urcuqui, Ecuador.

http://dx.doi.org/10.1016/j.msec.2015.09.012
0928-4931/



Scheme 1. Redox-sensitive camptothecin (CPT) prodrugs synthesized in this work (n = 1–3).

show that drug precursor structure determines not only the release mechanism but also hydrophobicity and surface charge. As a consequence, cell uptake and cytotoxicity can be monitored according to the different functionalization.

2. Experimental section

Reagents and solvents were purchased from Sigma-Aldrich (China), unless otherwise indicated, and used without further purification. HPLC grade solvents were provided by Scharlab (Spain) whilst LC/MS grade Optima solvents came from Fisher (UK). Water was deionized to 18.2 $m\Omega \cdot cm^{-1}$ using a miliQ pack system. HeLa cells were originally obtained from the American Type Culture Collection (Rockville, MD), maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS, from Lonza, Verviers, Belgium) at 37 °C under a humidified atmosphere of 95% air and 5–10% CO_2.

(Pyridine-2-yldisulfanil)alkyl carbonate derivatives of CPT (alkyl = ethyl **1a**, propyl **1b**, butyl **1c**), as presented in Scheme 1, were synthesized following procedures reported earlier [19]. The purity was determined by RP-HPLC and ESI-MS and the chemical structure confirmed by ^{1}H NMR, ^{13}C NMR and Q-TOF MS analysis (see Supplementary data).

2.1. Synthesis of CPT nanomedicines

Hybrid silica nanoparticles with a rhodamine-B doped non-porous core and a mercapto-functionalized mesoporous shell (Si@mSi-SH) were prepared as reported elsewhere [19–20]. 100 mg of these thiol derivatized nanoparticles were reacted with a solution of the prodrug **1a**, **1b** or **1c** (3.3 mL in DMSO, 18 μmol mL^{-1}), and methanol (16 mL), and the mixture was stirred for 16 h.

Then the suspension was centrifuged (12,500 *g*, 15 min) and the resulting solid was washed thoroughly with methanol to remove completely non covalently bound prodrug and CPT. This was confirmed by measuring absorbance at 368 nm in the washing solution by using a Nanodrop N1000 spectrophotometer. Finally, the sample was freeze-dried. Nanomedicines were named as Si@mSi-CPTn, where n = 1,2,3.

2.2. Materials characterization

Powder X-ray diffraction patterns (XRD) were collected using a Philips X'Pert diffractometer equipped with a graphite monochromator, operating at 40 kV and 45 mA and using nickel-filtered Cu Kα radiation (λ = 0.1542 nm). Nitrogen gas adsorption isotherms were measured in a Micromeritics Flowsorb apparatus. Surface area calculations were carried out using the Brunauer–Emmett–Teller (BET) method, whereas pore size distribution was calculated according to the Kruk–Jaroniec–Sayari (KJS) estimation [21]. Nanoparticles morphology was studied by transmission electron microscopy (TEM) in a Philips CM-10 microscope operating at 100 kV. Samples were dispersed in methylchloride and transferred to carbon coated copper grids. Particle size and Z-potential measurements were conducted by diffuse light scattering (DLS) at 25 °C and a concentration of 5 μg/mL in a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The mean hydrodynamic diameter was determined by cumulant analysis. Diffuse-reflectance UV–vis spectra were collected on a Cary 5 equipped with a 'Praying Mantis' attachment from Harric.

2.3. CPT stability and release

CPT release from nanomedicines Si@mSi-CPTn was monitored at 37 °C in a Thermomixer® equipment (5 mg mL^{-1}, 1350 rpm). After a 2 h incubation period with no reducing agent, DTT (10 mM) was added and the process continued for another 3 h. We used this concentration to mimic as much as possible the real situation inside cell, with an estimated cytosolic concentration of reducing agents, mostly GSH, of about 10 mM [22]. At the corresponding time points, samples were centrifuged (12,500 *g*, 15 min), the supernatant freeze-dried and further dissolved with methanol/HCl solution (5%), and analyzed by RP-HPLC and ESI-MS. Triplicate samples were run for every experiment.

Stability tests were carried out in human serum (HS) by incubating 5 mg mL^{-1} at 37 °C (Thermomixer®, 1350 rpm) for 3 h. Samples were centrifuged (12,500 rpm, 15 min), the supernatant was diluted with 10 mL of methanol at 0 °C and serum proteins were precipitated using a 5% aqueous solution of trichloroacetic acid at 0 °C prior to freeze-drying. In all cases, the residue was reconstituted with 1 mL of a methanol/HCl (95:5 *v*/*v*) solution, and analyzed by RP-HPLC and ESI-MS. Experiments were done in triplicate.

2.4. Cell internalization assays

Nanoparticle entry in HeLa cells was formerly studied by co-localization experiments at the inverted/fluorescence microscope (Olympus CKX41). 2000 cells/well (96-well plates) were seeded and stabilized for 48 h in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (Streptomycin/Penicillin) at 37 °C in 95% air and 5% CO_2 environment. Then, the growth medium was exchanged and cells with fresh medium were treated with CPT nanomedicines, with a final dose of 0.025 μg mL^{-1} (in CPT equivalents), during 24 h. After incubation, cells were repetitively washed with fresh medium to completely remove non-internalized particles before image acquisition.

Quantitative cell uptake determinations were carried out by flow cytometry. For this purpose, HeLa cells were seeded in 6-well plates at a density of 100,000 cells/well and allowed to attach for 24 h. Then, the

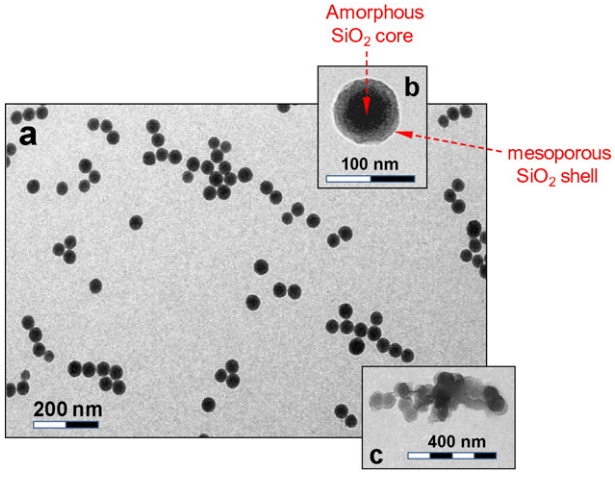


Fig. 1. Transmission electron microscopy (TEM) images of redox-responsive Si@mSi-CPTn nanomedicines. (a) Monodispersed particles of Si@mSi-CPT1 sample. (b) Detail on particle core-shell nature. (c) Large aggregate corresponding to Si@mSi-CPT3 sample.