Supplementary Material (ESI) for Chemical Communications

Supplemental Information

SCKs as nanoparticle carriers of doxorubicin: Investigation of core composition on the loading, release and cytotoxicity profiles

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Instrumentation:

UV-Vis spectra were acquired on a Varian Cary 1E UV-Vis system (Varian, Inc., Palo Alto, CA) using PMMA cuvettes.

Gel permeation chromatography (GPC) was conducted on a Waters 1515 HPLC (Waters Chromatography, Inc.) equipped with a Waters 2414 differential refractometer, a PD2026 dual-angle (15 and 90°) light scattering detector (Precision detectors, Inc.), and a three-column series PL_{gel} 5 μm Mixed C, 500 Å, and 10^4 Å, 300 x 7.5 mm columns (Polymer Laboratories, Inc.). The system was equilibrated at 35 °C in THF, which served as the polymer solvent and eluent with a flow rate of 1.0 mL min^{-1}. Polymer solutions were prepared at a known concentration (*ca.* 1-3 mg mL^{-1}) and an injection volume of 100 μL was used. Data collection and analysis were performed, respectively, with Precision Acquire software and Discovery 32 software (Precision Detectors, Inc.).

Differential scanning calorimetric (DSC) studies were performed on a Mettler Toledo DSC822 (Mettler Toledo, Gmbh.) calibrated according to standard procedures. The heating and cooling rates were 10 °C min^{-1} with a temperature range of -120 °C to 150 °C. The crystalline melting point (T_m) and crystallization temperature (T_c) were determined during the second heating or cooling run, respectively. For analysis of the hydrated nanoparticle samples, a temperature range of -40 °C to 80 °C was used with heating and cooling rates of 10 °C min^{-1}.

Samples for transmission electron microscopy (TEM) measurements were diluted with a 1 % phosphotungstic acid (PTA) stain (v/v, 1:1). Carbon grids were exposed to oxygen plasma treatment to increase the surface hydrophilicity. Micrographs were collected at 50,000 and 100,000× magnification and calibrated using a 41 nm polyacrylamide bead from NIST. The number average particle diameters (D_{av}) and standard deviations were generated from the analysis of a minimum of 150 particles from at least two different micrographs.

Hydrodynamic diameters (D_h) and size distributions for the SCKs in aqueous solutions were determined by dynamic light scattering (DLS) and are reported for the number-average D_h values. The DLS instrumentation consisted of a dynamic light scattering detector (90 Plus Particle Size Analyzer, Brookhaven Instruments Company) equipped with a 24 mW laser operating at 678 nm. The instrument was calibrated using 96 nm latex microspheres in water (Duke Scientific, Inc.). Measurements were made at 20 ± 1 °C. The analysis of the temperature-dependent size changes were performed at 6 different temperatures. Prior to analysis, solutions were filtered through a 0.45 μm Millex®-GV PVDF membrane filter (Millipore Corp.). The initial delay was set to 5 μs, and the final delay to 1 s. Only measurements in which the measured and calculated baselines of the intensity autocorrelation function agreed to within 0.1 % were used to calculate particle size. The calculations of the particle size distributions and distribution averages were performed with the ISDA software package (Brookhaven Instruments Company), which employed single-exponential fitting, cumulants analysis, non-negatively constrained least-squares (NNLS) and CONTIN particle size distribution analysis routines. All determinations were made in quadruplicate.

Materials: SCK **1**, **2** and SCK **3**, **4** were synthesized as previously reported.[1,2] Doxorubicin was obtained from Sigma-Aldrich (St. Louis, MO.) and was used as received.

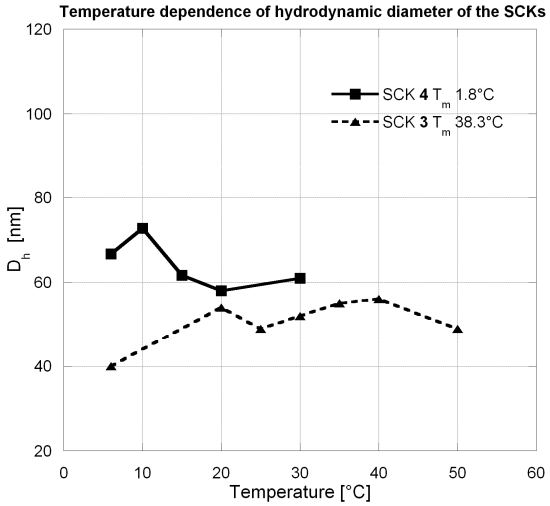
General procedure for the preparation of SCK-doxorubicin nanoparticles: A solution of doxorubicin (2.7 mg mL^{-1} in CH_2Cl_2 and 3 eq of triethylamine, 30% w/w with respect to the SCK) was added to a vial containing a magnetic stir bar and the SCK solution (4 mL, polymer concentration ~0.25 mg mL^{-1}). The solution was protected from light and stirred without cap in a well-ventilated fume hood for 16 h in order evaporate the CH_2Cl_2. The solution was then transferred to a centrifugal filter device (Amicon Ultra 4, 30 kDa MWCO, Millipore Corp., Billerica MA, USA)[3] and washed extensively with 5 mM PBS pH 7.4 buffer at 37°C to remove all unincorporated dox. After several washing cycles the filtrate was analyzed by UV-vis (488 nm) to confirm the successful removal of the free dox. The dox-nanoparticle solution was then reconstituted to a final volume of 4 mL. The amount of incorporated DOX was determined by UV-vis (480 nm, ε = 13050 $M^{-1}cm^{-1}$ determined by a calibration curve in DMF/PBS 4:1) in a 4:1 v/v mixture of DMF and dox-nanoparticle solution.

General procedure for the release experiments: 2 mL of the dox-nanoparticle solution was transferred to a presoaked dialysis cassette (Slide-A-Lyzer, 10 kDa MWCO, Pierce Biotechnology, Rockford IL, USA). The cassette was then stirred in a beaker containing 4 L of 5 mM PBS at pH 7.4 and 37° C for a period of 48 h. Samples (~100 µL) were removed from the cassette at 0, 1, 2, 3, 4, 8, 16, 24, and 48 h and analyzed by UV-vis (488 nm, ε = 12500 $M^{-1}cm^{-1}$ determined by a calibration curve in PBS). All release experiments were conducted in duplicate.

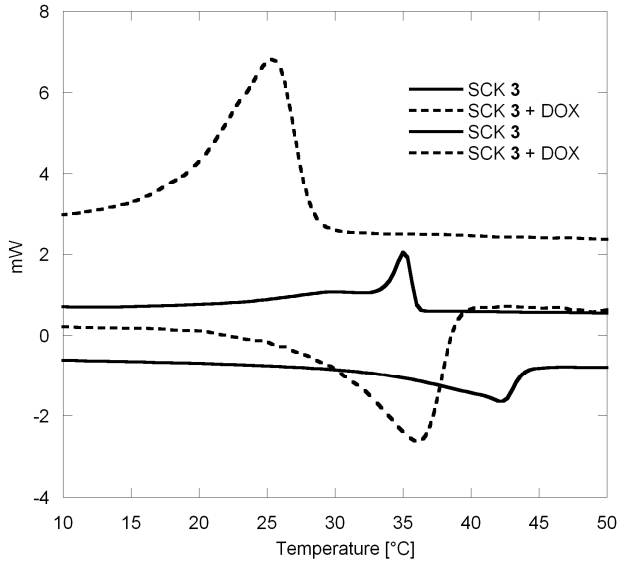
Cell cytotoxicity assay on U87MG-EGFR-v-III cells: A human glioblastoma cell line that expresses a mutant variant of the EGFR receptor-v-III that was modified to express click-beetle red luciferase and (Sheila Stewart, unpublished data) and used to test cytotoxicity of dox and dox-SCKs. Briefly, 5000 cells/well were plated in triplicate in 96-well plate 24 h before treatment. Cells were then washed thrice with sterile PBS and exposed to different dose of either free dox or dox-SCKs at 37 °C for 2 h in 10% FBS α MEM. After 2 h of incubation, the cells were washed again thrice with sterile PBS and re-fed with fresh media and further incubated at 37 °C for 70 h before MTT assay. To asses cell viability assay, 10 µL of MTT (5mg ml^{-1}) solution was added into each well and incubated at 37 °C for 4 h. The reaction was terminated with 10% SDS stop solution. Absorbance was read at 570 nm using µQuant plate reader (Bio-Tek Instruments, Inc.) and viability was calculated as the percentage of control (cells receiving no treatment).

In vitro fluorescent microscopy study: Cells were prepared as described for the MTT assay above. Cells were treated for 24 h with either 1 µg ml^{-1} of free-doxorubicin or Dox-loaded NP (SCK **1**). Afterwards, cells were fixed with 4% paraformaldehyde at room temperature for 10 min and the nuclei were counterstained with 1 µg ml^{-1} of DAPI. The fluorescence was observed with an inverted fluorescence microscope (Olympus) with appropriate filter sets.

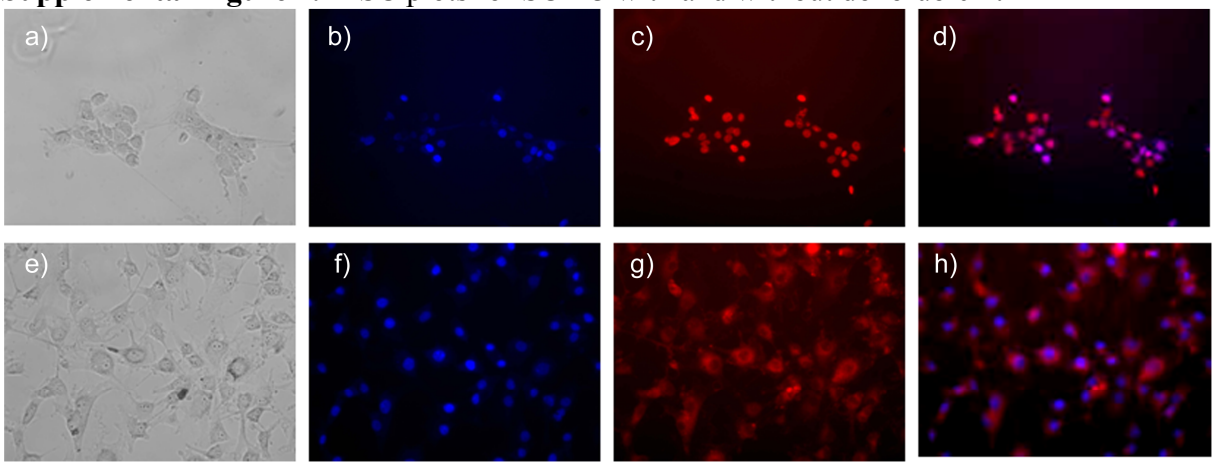
Apoptosis study of Dox-loaded NPs by flow cytometry: U87MG-EGFRvIII-CBR cells ($1x10^6$) were plated on 12-well plates 24 h before treated with different doses of Dox-loaded NPs and free-dox. After 2 h incubation, cells were washed twice with PBS and re-fed with fresh media containing 10% FBS (fetal bovine serum). At 72 h post treatment, cells were typsinized and washed twice with PBS and then re-suspended into Annexin-V binding buffer. Apoptosis labeling with Annexin-V-FITC were carried out per the manufacture's instructions (Apo-Alert Annexin-V-FITC apoptosis kit, Clontech, Mountain view, CA). The percentages of apoptotic cells were then measured with BD FACS scan and analyzed with CellQuest software (BD Biosciences, San Jose, CA).



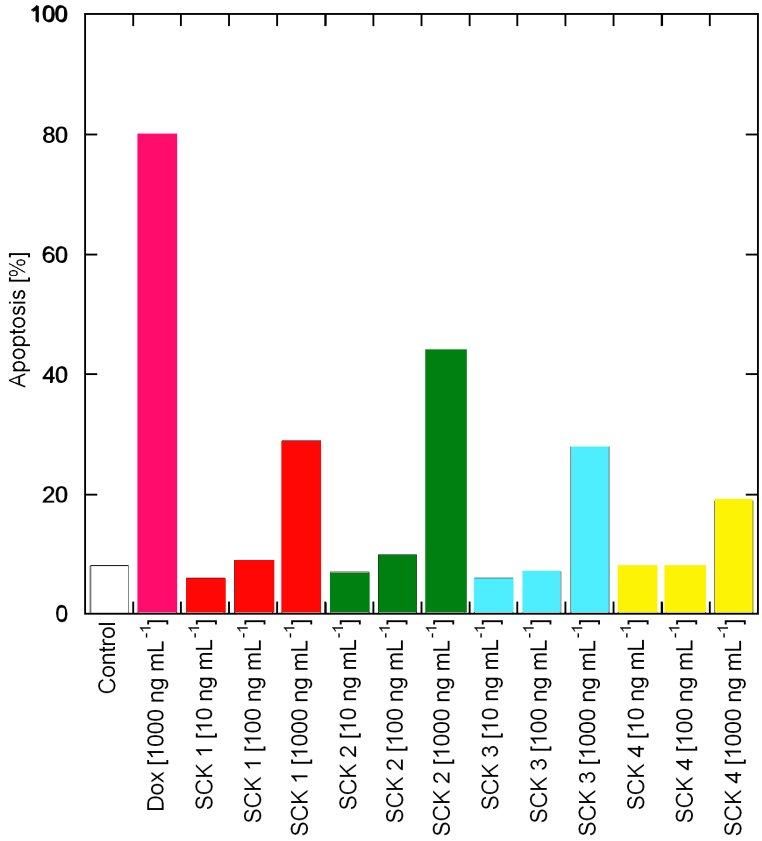
Supplemental Figure 1. Dynamic light scattering results for SCK **3** and **4.**[1]



Supplemental Figure 2. DSC plots for SCK **3** with and without doxorubicin**.**



Supplemental Figure 3. U87MG-EGFRvIII-CBR cells incubated for 2 h and assayed after 24 h, with free doxorubicin (a) (phase), red channel of **a** (b), DAPI stain of **a** (c), overlay of **b** and **c** (d), SCK **1** (e) (phase), red channel of **e** (f), DAPI stain of **e** (g), overlay of **f** and **g** (h).



Supplemental Figure 4. FACS results from the apoptosis study for U87MG-EGFRvIII-CBR cells incubated for 2 h in PBS (control) and with the addition of free Dox and SCK nanoparticles 1-4 loaded with Dox.

Clearly, the FACS data are consistent with previous MTT results. At higher doses of each SCK sample, there was about 20-30% apoptotic cells, while free dox showed 80% apoptosis at 1000 ng ml^{-1}. At lower doses (100 ng ml^{-1}), the percentages of apoptotic cells were still higher than observed for the untreated control. However, at 10 ng ml^{-1}, there were no apoptotic effects among all 4 nanoparticles tested; untreated cells also showed some spontaneous apoptosis during the experiment period (72 h).

References:

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2. Sun, G.; Xu, J.; Hagooly, A.; Rossin, R.; Li, Z.; Moore, D. A.; Hawker, C. J.; Welch, M. J.; Wooley, K. L. *Adv. Mater.* **2007,** *19*, 3157-3162.
3. Gillies, E. R.; Fréchet, J. M. J. *Bioconjugate Chem.* **2005,** *16*, 361-368.