Supporting Information

Ensemble and single particle analysis of doxorubicin silk nanoparticles

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Experimental Section

Materials. All reagents and solvents used for silk nanoparticle fabrication were purchased from Sigma-Aldrich or Acros Organics (>98% purity) and used without further purification unless otherwise noted. Reagents and chemicals not specifically mentioned in the text for cell culture experiments were obtained from Gibco™ (Thermo Fisher Scientific). *Bombyx mori* silk cocoons were purchased from the Scientific Center on Sericulture, Vratsa, Bulgaria. MDA-MB-231 (HTB-26) cells were obtained from ATCC.

Aqueous Silk Fibroin Preparation. The silk solution was prepared from *B. mori* cocoons, as described elsewhere.[1, 2] *B. mori* cocoons were boiled in Na_2CO_3 for 1 h, and the degummed silk was air-dried overnight. Dissolution was performed in LiBr at 60 °C for 4 h, followed by 48 h of dialysis against deionized water using a 3.5 kDa MWCO dialysis cassette (Slide-A-Lyzer 3.5K Dialysis Cassette G2, Thermo Scientific, Rockford, IL, USA).

Centrifugation (2,885*g*, 40 min at 4 °C) (PK 121R Centrifuge, rotor T515, ACL International Srl, Milan, Italy) was conducted to collect the purified silk solution. The concentration of silk solution (% *w/v*) was determined from the dried weight aliquots incubated at 60 °C for 24 h.

Drug-Loaded Silk Nanoparticle Manufacture. Placebo SNPs were manufactured using the antisolvent precipitation method. Briefly, it was carried out by adding 3% (*w/v*) silk solution (6 mL) dropwise into isopropanol (30 mL). SNP suspensions were centrifuged at 48,400*g* for 2 h at 4 °C. The SNP pellet was washed three times in distilled water by sonication (30% amplitude, 30 s) and centrifugation. Placebo SNPs were mixed with DOX solution to yield SNP mixture (2 mL), containing 5 mg/mL SNPs and 0, 25, 50, and 100 μM DOX. The mixtures were incubated at 4 °C with 30 oscil/min for 18 h (PMR-30 Platform 2D Rocker–Fixed Tilt, Grant Instruments (Cambridge) Ltd, UK), then centrifuged at 48,400 *g* at 4 °C for 2 h to collect the doxorubicin-loaded SNPs (DOX-SNPs). Doxorubicin concentration in the supernatant was quantified using UV-vis spectroscopy (Abs: 480 nm) (Polarstar Omega, BMG Labtech, Ortenberg, Germany). The remaining SNP pellet was resuspended in distilled water, sonicated (30% amplitude, 30 s), and stored at 4 °C for further analyses. The drug encapsulation efficiency and drug loading content were calculated by the following equations.

$$\text{Encapsulation efficiency (\%)} = \frac{Total\ added\ amount\ of\ DOX\ (mg) - Amount\ of\ DOX\ in\ supernatant\ (mg)}{Total\ added\ amount\ of\ DOX\ (mg)} \times 100$$

Equation 5.1

$$\text{Drug loading content (μg/mg)} = \frac{Total\ added\ amount\ of\ DOX\ (\mu g) - Amount\ of\ DOX\ in\ the\ supernatant\ (\mu g)}{Nanosphere\ weight\ (mg)}$$

Equation 5.2

Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS). Particle size and zeta potential were measured using the DLS and ELS, respectively. The SNP suspension was diluted in deionized water at a 1:25 ratio for DLS measurement and a 1:20 ratio for ELS measurement. The measurement methods were set up as described elsewhere.[2]

Field Emission Scanning Electron Microscopy (FE-SEM). An aliquot of doxorubicin-loaded silk nanoparticles (DOX-SNPs) (20 mL of 0.1 mg/mL) suspension was dropped onto a silicon wafer. Next, samples were air-dried at ambient temperature for 24 h, and gold coated using a gold sputter coater; 35 mm height, 40 s, 0.08 mb, and 30 mA (Agar Scientific Manual Sputter Coater, Agar Scientific Ltd., Essex, UK) and imaging *via* FE-SEM (Hitachi SU6600, Hitachi High-Tech Europe GmbH, Krefeld, Germany) with magnifications of 10,000×, 20,000×, and 60,000× at 5 kV.

Fourier-Transform Infrared (FTIR) Spectroscopy. Freeze-dried DOX-SNPs were prepared by pre-freezing a 5 mg/mL DOX-SNP suspension at −20 °C overnight, followed by freeze-drying at −10 °C and 0.14 mbar for 24 hours. The freeze-dried DOX-SNPs were used for subsequent Fourier-Transform Infrared (FTIR) Spectroscopy analysis. Briefly, the secondary structures of silk and silk nanoparticles were analyzed using FTIR spectroscopy.[2] The air-dried silk film and 70% ethanol-treated silk film served as the silk I and silk II references. FTIR spectra were recorded over 400–4000 cm^{-1} (4 cm^{-1} resolution) with an ATR-equipped TENSOR II spectrometer (Bruker Optik GmbH, Ettlingen, Germany), using 32 scans for the background and 128 for samples. Secondary structure content was determined from deconvoluted amide I spectra, assigning peaks to specific structures: antiparallel β-sheet (1697–1710 cm^{-1}), β-turn (1663–1696 cm^{-1}), random coil (1638–1662 cm^{-1}), native β-sheet (1620–1635 cm^{-1}), and intermolecular β-sheet (1605–1625 cm^{-1}).[1] Second-derivative amide I spectra (1600–1700 cm^{-1}) were used for determining correlation coefficient calculations against air-dried silk films.

Frit-Inlet Asymmetric Flow Field-Flow Fractionation (FI-AF4). DOX-SNPs were separated using a Frit-inlet Asymmetric Flow FFF system (AF2000, PostNova Analytics, Landsberg am Lech, Germany). The regenerated cellulose membrane, MWCO of 10 kDa (Postnova AF2000 MF-AF4 Analytic RC Membrane, Z-AF4-MEM-618-10kDa, Postnova Analytics, Landsberg am Lech, Germany) was used as the accumulation wall for all measurements with a spacer thickness of 350 μm. The in-line detectors multiplexed with FI-AF4 included a 21-multi-angle light scattering (MALS) detector (#PN3621, Postnova Analytics), a UV detector (#SPD-M40, Postnova Analytics), a fluorescence detector (Ex/Ex = 480/594 nm) (#FLD, Postnova Analytics), and an online Zetasizer Nano ZS (Malvern Panalytical). The DOX-SNPs (0.5 mg/mL) were dispersed in 0.1 μm-filtered Milli-Q water as the carrier liquid. The separation method (67 min) was adapted with minor modifications from[3] (**Fig. S1a**). The direct injection method (20 min) was carried out for the determination of percent recovery and the doxorubicin calibration curve (0.1–100 μM) (**Fig. S1b**). The separation principle of FFF alongside FI-AF4 separation conditions is highlighted in **Fig. S1c.**

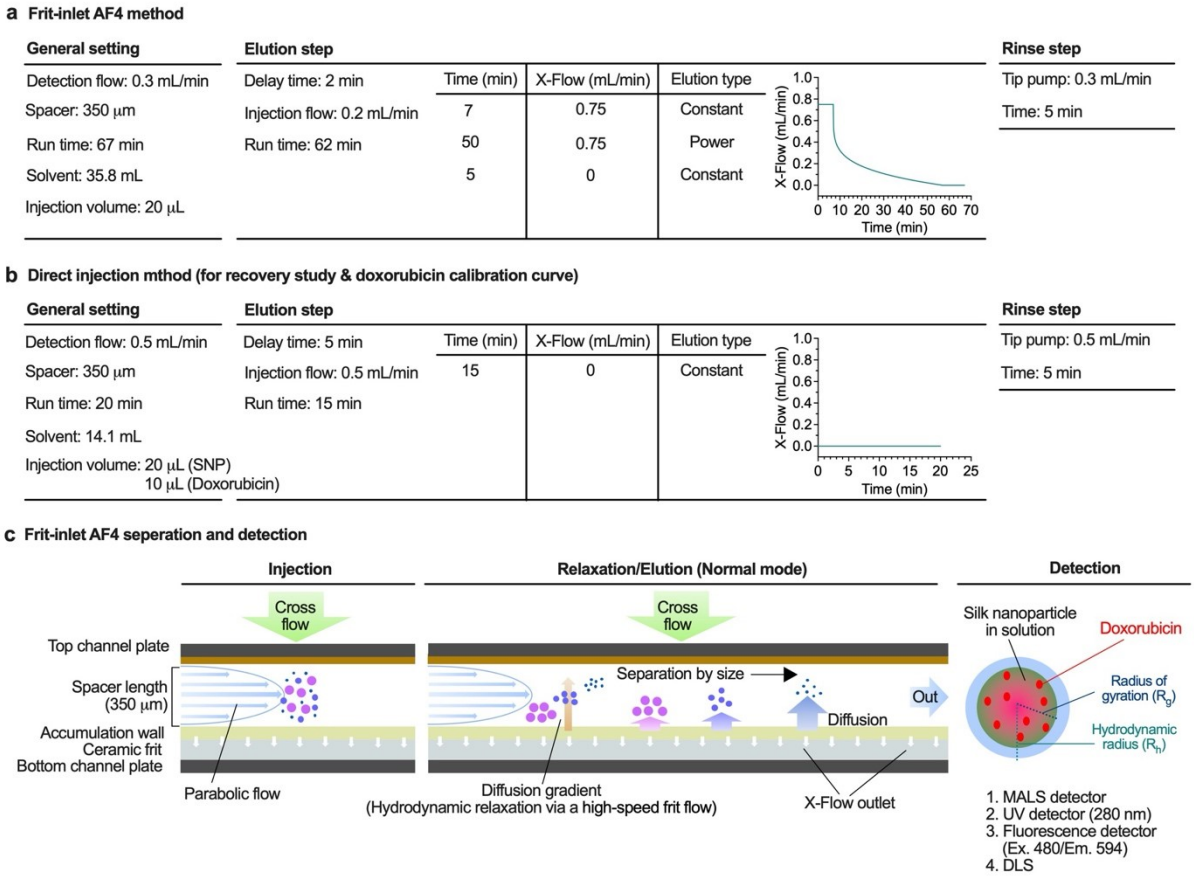

Fig. S1 Schematic of silk nanoparticle characterisation using the Frit-Inlet Asymmetric Flow Field-Flow Fractionation (FI-AF4). The 0.5 mg/mL doxorubicin-loaded silk nanoparticle was dispersed in 0.1 μm-filtered Milli-Q water: (**a**) the FI-AF4 method used for the doxorubicin-loaded silk nanoparticle separation and characterisation, (**b**) the FI-AF4 direct injection method used for the recovery determination and the doxorubicin calibration curve, (**c**) the channel geometry, separation principle, and orthogonal detectors, including a multiangle light scattering (MALS) detector, a UV detector (DAD), a fluorescence detector (FLD), and a dynamic light scattering (DLS).

Single Particle Automated Raman Trapping Analysis (SPARTA) measurements and spectral analysis. Population heterogeneity of doxorubicin-loaded silk nanoparticles compared to empty silk nanoparticles was assessed using Single Particle Automated Raman Trapping Analysis (SPARTA) (SPARTA® AGIS I, SPARTA Biodiscovery Ltd, London, UK).

The SPARTA Raman micro-spectroscopy system has been described elsewhere.[4] Briefly, the setup comprised a Raman micro-spectroscope (alpha300R$^+$, WITec, Ulm, Germany); equipped with a 786 nm laser light source (Toptica XTRA II) and a 63 × /1.0 NA water immersion objective lens (W Plan-Apochromat, Zeiss, Oberkochen, Germany). Scattered light was collected using a UHTS 300 spectrometer (WITec, Ulm, Germany), and spectra were acquired at a spectral resolution of 3 cm^{-1} with an 85 mW laser power (iDus DU401-DD, Andor, Belfast, UK).

Following synthesis *via* the adsorption method, silk nanoparticles were dispersed in 0.1 μM-filtered Milli-Q water to achieve a final concentration of 2.5 mg/mL of SNP. An aliquot of SNP (200 μL) was transferred to a 22 mm diameter coverslip with the blank (0.1 μM-filtered Milli-Q water), and analysed using a water-immersion lens. For each replicate, 300 individual trapped particles were trapped and analysed for 10 s, with a 1 s laser shutter off interval. The average of 50 blank spectra were used to perform background subtraction. Data processing and analysis were performed using the Discovery software (Release 1.2.1) (SPARTA® AGIS I, SPARTA Biodiscovery Ltd, London, UK).

***In Vitro* Drug Release.** Cumulative release of doxorubicin was performed at 37 °C over 15 days. Briefly, 1.5 mg of DOX-SNPs formulated at a doxorubicin concentration of 100 μM were dialyzed against 1×PBS (pH 7.0), HEPES buffer (pH 6.5), and citrate phosphate buffer (pH 4.5) using a dialysis tube (Slide-A-Lyzer™ MINI Dialysis Devices, 3.5K MWCO, Thermo Fisher Scientific, UK). Dialysis was carried out at 37 °C with continuous stirring at 400 rpm (Variomag Multipoint, Thermo Fisher Scientific, UK). At pre-determined timepoints (1, 2, 3, 6, 24, 48, 72, 144, 216, and 360 h), 500 μL aliquots were removed to the dissolution media and transferred to a microtube for absorbance readings (480 nm), while 500 μL of pre-warmed fresh buffer was added to maintain a batch volume of 2 mL. All procedures were conducted under sterile conditions. Placebo SNPs were also processed in parallel as controls, and

cumulative drug release was calculated by subtracting the release from placebo SNPs. Release kinetics of DOX-SNPs were fit to four different kinetic models, including zero-order, first-order, Higuchi, and Korsmeyer-Peppas models (**Table S1**).

Table S1 Kinetic models used for fitting the release kinetics of doxorubicin from silk nanoparticles. F indicates the % cumulative drug release up to time t. The k_0, k_1, k_H, and k are the kinetic constants derived from the mathematical models, which indicate the rate of drug release over time.[5, 6]

Kinetic model	Equation	Plot	Model explanation
Zero-order	$F = k_0 t$	F vs t	Describe a slow released drug with a constant concentration and without disaggregation.
First-order	$\ln(1\text{-}F) = -k_1 t$	ln(1-F) vs t	Describe a drug dissolution that its concentration is a direct correlation to its release rate.
Higuchi	$F = k_H \sqrt{t}$	F vs √t	Describe a controlled drug release from the matrix according to a water-solubility, resulting from a swelling and/or a degradation of the matrix.
Korsmeyer-Peppas	$F = kt^n$	log(F) vs log(t)	Describe a drug release from the polymeric matrix (e.g., spherical nanoparticles, films, tablets), resulting from a water diffusion into matrix, matrix swelling, and matrix dissolution. This model is fitted to the first 60% drug release data. Interpretation of n value: $n \leq 0.45$ is Fickian diffusion (case I diffusion); $0.45 < n < 0.89$ is Anomalous (non-Fickian) diffusion; $n = 0.89$ is Zero order release (Case II diffusion); $n > 0.89$ Super case II transport.

***In vitro* Cytotoxicity of Doxorubicin-Loaded Silk Nanoparticles.** The dose-dependent cytotoxicity of DOX-SNPs was examined in MDA-MB-231 cells (triple-negative breast cancer cell lines). Cells were cultured in complete RPMI 1640 media (10% *v/v* FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM sodium pyruvate, and 1% *v/v* RPMI amino acids). Cells were plated into a 96-well plate to obtain a cell density of 9,375 cells/cm^2 and incubated overnight in a 5% CO_2 incubator at 37 °C before treatment. Test concentrations used ranged between 0.1–1,500 mg/mL for placebo SNPs and 0.001–1,000 mg/mL for DOX-SNPs. A

concentration range of 0.001−100 mM doxorubicin was used to determine the dose-dependent cytotoxicity of the payload in the absence of SNPs. Next, cells were incubated for 48 h with growth media containing test samples, and cell viability was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega UK Ltd., UK). The luminescence signal was measured using an integration time of 0.3 sec/well in a Spark® Multimode Microplate Reader (Tecan Trading AG, CH).

Cell Uptake Assay and Intracellular Doxorubicin Measurement. MDA-MB-231 cells were plated into a 24-well plate at cell density mentioned above. Cells were cultured in complete media for 24 h, before being exposed to fresh serum-free media containing DOX-SNPs at a concentration of 0.5 × EC_{50} and 1 × EC_{50} value. The serum-free media served as a negative control. The 0.5 × EC_{50} doxorubicin served as a free drug control. For fixed cell imaging, the cells were allowed to expose with DOX-SNPs for 1, 4, and 8 h. Cells were fixed with 4% *v/v* paraformaldehyde, stained with 1 μg/mL DAPI (Ref 62248, Thermo Scientific, UK), and imaged using a confocal microscope (Leica SP8, Leica Microsystems Ltd, UK). The mean intensity ratio of DOX/DAPI and the nuclei circularity index were computed by a particle analysis function of Fiji/ImageJ.[7, 8]

Analysis Software and Statistical Analyses. All data were collated using Microsoft Excel version 16.61.1 (Microsoft Office 365 for Mac Software, Redmond, WA, USA). FTIR data were deconvoluted using Origin 2019b (OriginLab, Northampton, MA, USA). The result graphs and statistical analyses were conducted using GraphPad Prism 10 (GraphPad Software, Boston, MA, USA). Statistical analyses are denoted with symbols indicating statistical significance, and the number of experimental repeats (*n*) is specified in each figure legend. All illustrations were created using Microsoft PowerPoint version 16.107.3 (Microsoft Office 365 for Mac Software, Redmond, WA, USA) and GraphPad Prism 10.

Supplementary Figure

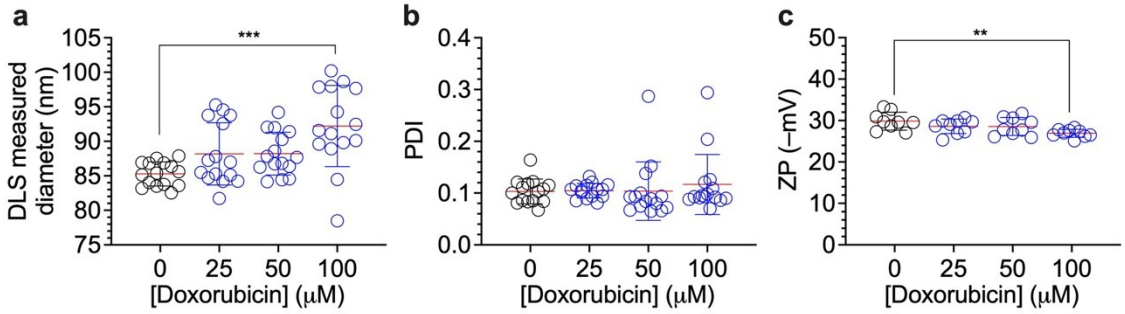

Fig. S2 Physicochemical properties of doxorubicin-loaded silk nanoparticles (DOX-SNPs), manufactured by the antisolvent precipitation followed by the adsorption method with 0–100 μM doxorubicin. (a) Particle diameter (Z-average) *via* Dynamic Light Scattering (DLS). (b) PDI *via* DLS. (c) Zeta potential (ZP) *via* Electrophoretic Light Scattering (ELS). One-way ANOVA and Dunnett's multiple comparisons test were used for statistical analyses and compared to 0 μM doxorubicin (SNPs): $p < 0.1$(*), $p < 0.01$(**), $p < 0.001$(***) ($n = 3$).

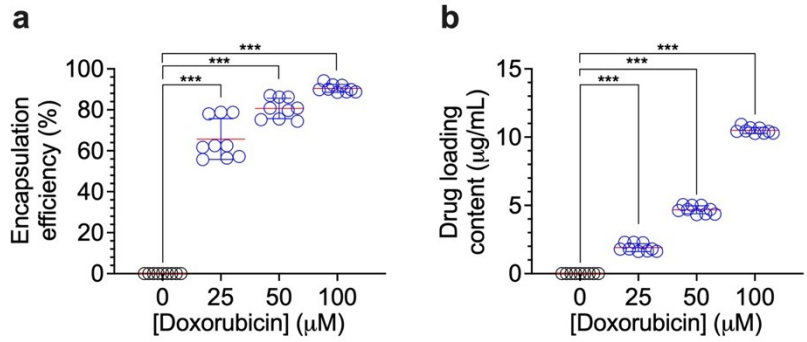

Fig.S3 Encapsulation efficiency and drug loading content of doxorubicin-loaded silk nanoparticles (DOX-SNPs), manufactured by the antisolvent precipitation followed by the adsorption method with 0–100 μM doxorubicin. (**a**) Encapsulation efficiency *via* UV-Vis method (Absorbance of 480 nm). (**b**) drug loading content calculated by % *w/w* after 48-h drying in a 60°C oven. One-way ANOVA and Dunnett's multiple comparisons test were used for statistical analyses and compared to 0 μM doxorubicin (SNPs): $p < 0.1$(*), $p < 0.01$(**), $p < 0.001$(***) ($n = 3$).

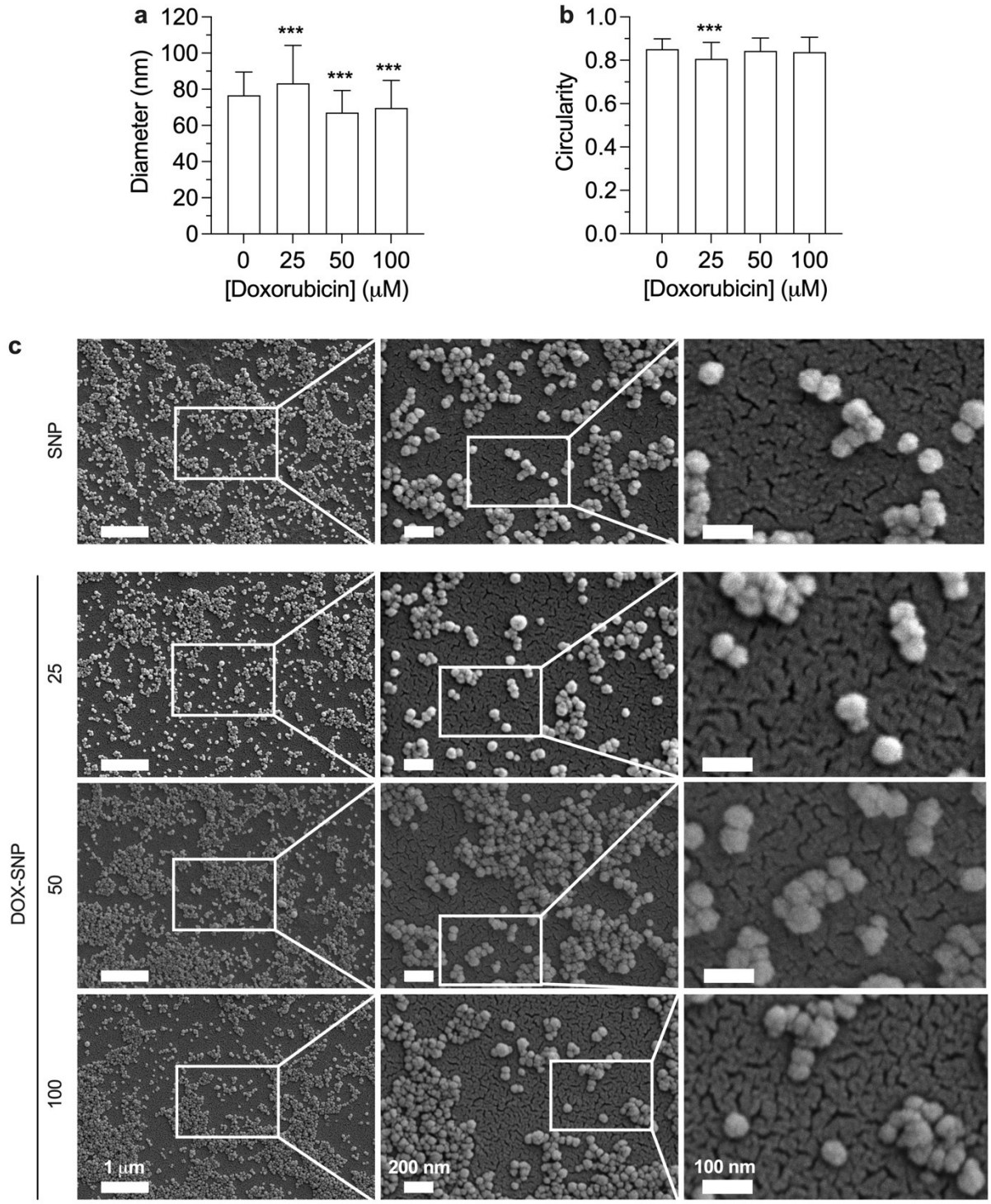

Fig. S4 FE-SEM micrographs and computed particle size and shape of doxorubicin-loaded silk nanoparticles (DOX-SNPs), manufactured by the antisolvent precipitation followed by the adsorption method with 0–100 μM doxorubicin. DOX-SNPs were prepared at 0.1 mg/mL in deionized water, air-dried at ambient temperature, and gold-coated. (**a**) Computed diameter and (**b**) circularity *via* ImageJ software, using 400–500 particles. (**c**) FE-SEM images with multiple magnifications. One-way ANOVA and Dunnett's multiple comparisons test were used for statistical analyses and compared to 0 μM doxorubicin (SNPs): $p < 0.1$(*), $p < 0.01$(**), $p < 0.001$(***) ($n = 3$).

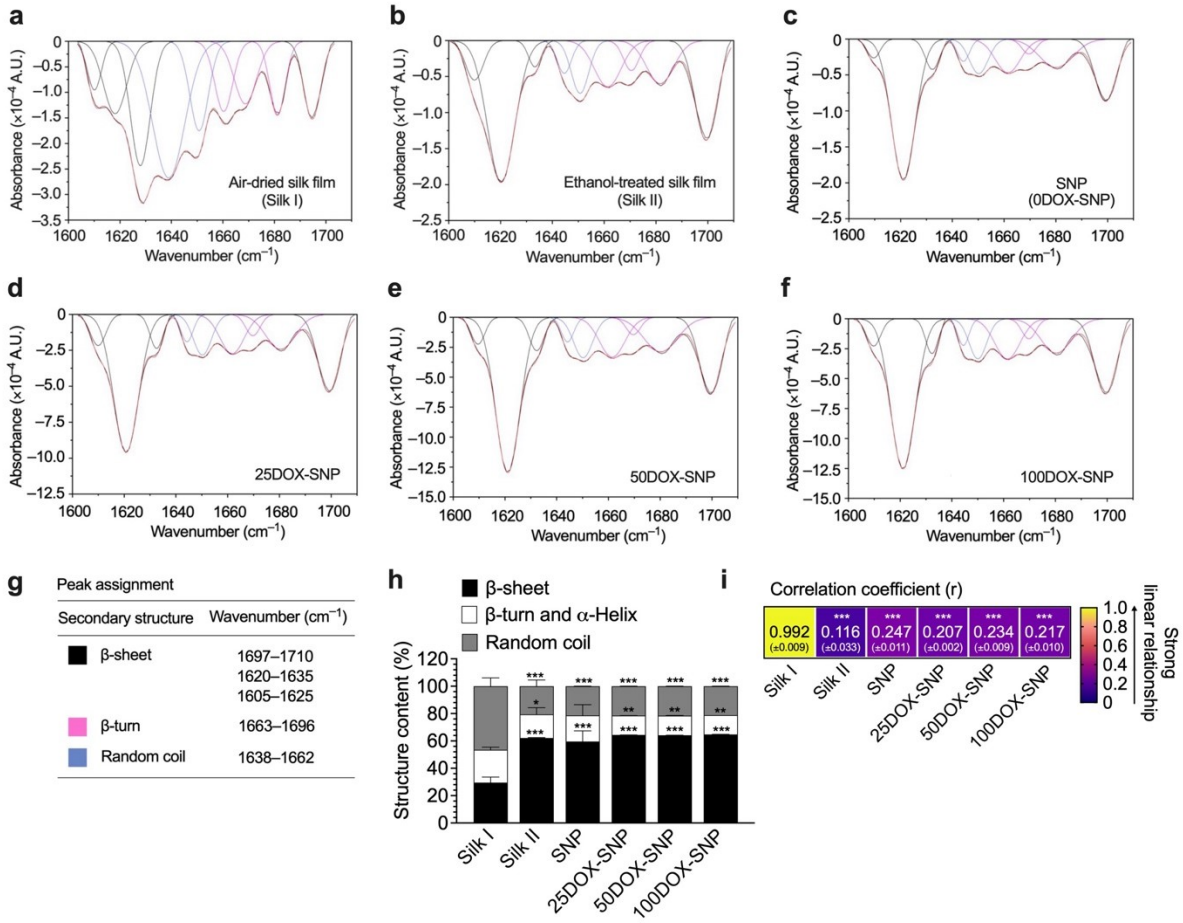

Fig. S5 Exemplar IR fitting in the Amide I region (1700–1600 cm^{-1}). Doxorubicin-loaded silk nanoparticles (DOX-SNPs) were manufactured by the antisolvent precipitation followed by the adsorption method with 0–100 μM doxorubicin. Freeze-dried DOX-SNPs were prepared before Fourier-Transform Infrared Spectroscopy (FTIR). (**a**) Air-dried silk film served as a silk I reference. (**b**) Ethanol-treated silk film served as a silk II reference. (**c-f**) DOX-SNP prepared by 0–100 μM doxorubicin. (**g**) IR peak assignment for secondary structure analysis. (**h**) Secondary structure content. (**i**) Correlation coefficient determining the linear relationship of overall IR adsorption spectra (4000–400 cm^{-1}) using silk I as reference. Two-way ANOVA and Uncorrected Fisher's LSD were used for secondary structure content analysis. One-way ANOVA and Dunnett's multiple comparisons test were used for correlation coefficient analysis: $p < 0.1$(*), $p < 0.01$(**), $p < 0.001$(***) ($n = 3$).

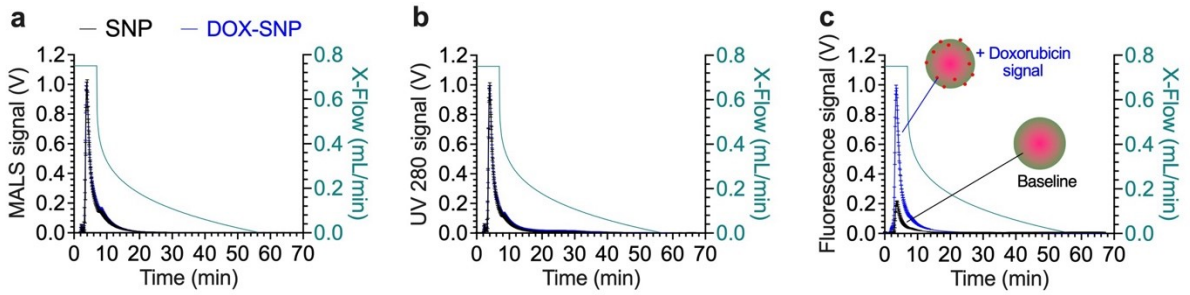

Fig. S6 FI-AF4 fractogram of doxorubicin-loaded silk nanoparticles (DOX-SNP) prepared with 100 μM doxorubicin. Multiple detectors were used to track particles: (**a**) MALS detector; (**b**) UV detector; (**c**) fluorescence detector.

Table S1 Retention time, particle radius, morphology, and recovery analysed by frit-inlet asymmetric flow field-flow fractionation (FI-AF4). An unpaired t-test was used for statistical analyses: $p < 0.1$(*), $p < 0.01$(**), $p < 0.001$(***) ($n = 3$).

Silk nanoparticle	Retention time (min)			Shape factor (R_g/R_h)	% Recovery (*via* UV 280 detector)
	MALS	UV 280	Fluorescence		
SNP	3.88 (±0.13)	3.97 (±0.08)	3.66 (±0.08)	0.796 (±0.008)	83.31 (±1.18)
DOX-SNP	3.88 (±0.00)	3.93 (±0.08)	3.53 (±0.08)	0.777 (±0.014)	89.94 (±0.74)**

Table S2 Raman shift assignment for doxorubicin.

Doxorubicin characteristics[9]	Raman shift (cm^{-1}) (l_0 = 785 nm)			Statistic method
	Reference 1[9]	Reference 2[10]	Experimental	Range (cm^{-1})
C-C-O	440*		440	437-446
		450		
C-O	462*	465	463	460-467
C-C of ring	989*			
C-O	1082*			
C-O-H	1209*	1205	1209	1200-1215
		1232		
C-H	1241*		1240	1233-1254
		1247	1250	
		1291	1292	1288-1296
Vibration in-plane of C-O and C-O-H; C-H bending	1299			
			1411	1408-1430
		1426	1423	
C=O and C-C stretching of the aromatic hydrocarbons	1448			
		1460		
Ring stretching	1578*	1577	1576	1570-1580
C=O	1637*	1644		

* indicates the crucial characteristic peaks of doxorubicin, as evidenced elsewhere.[9]

Table S3 Raman shift assignment for proteins.

Protein Characteristic (SPARTA and[11])	Raman shift (cm^{-1})
Protein primary peaks	1000, 1210-1275, 1425-1480, 1635-1695
Amide stretches	1210-1275, 1635-1695
Amide III (C–N) stretches	1200-1300
Amide I (C=O) stretches	1660
Phenylalanine/Tyrosine	1609
C–N stretches	1080
Phenylalanine	1000, 1003
C–C backbone stretches α-helix	937
Tyrosine	850
Tyrosine	825

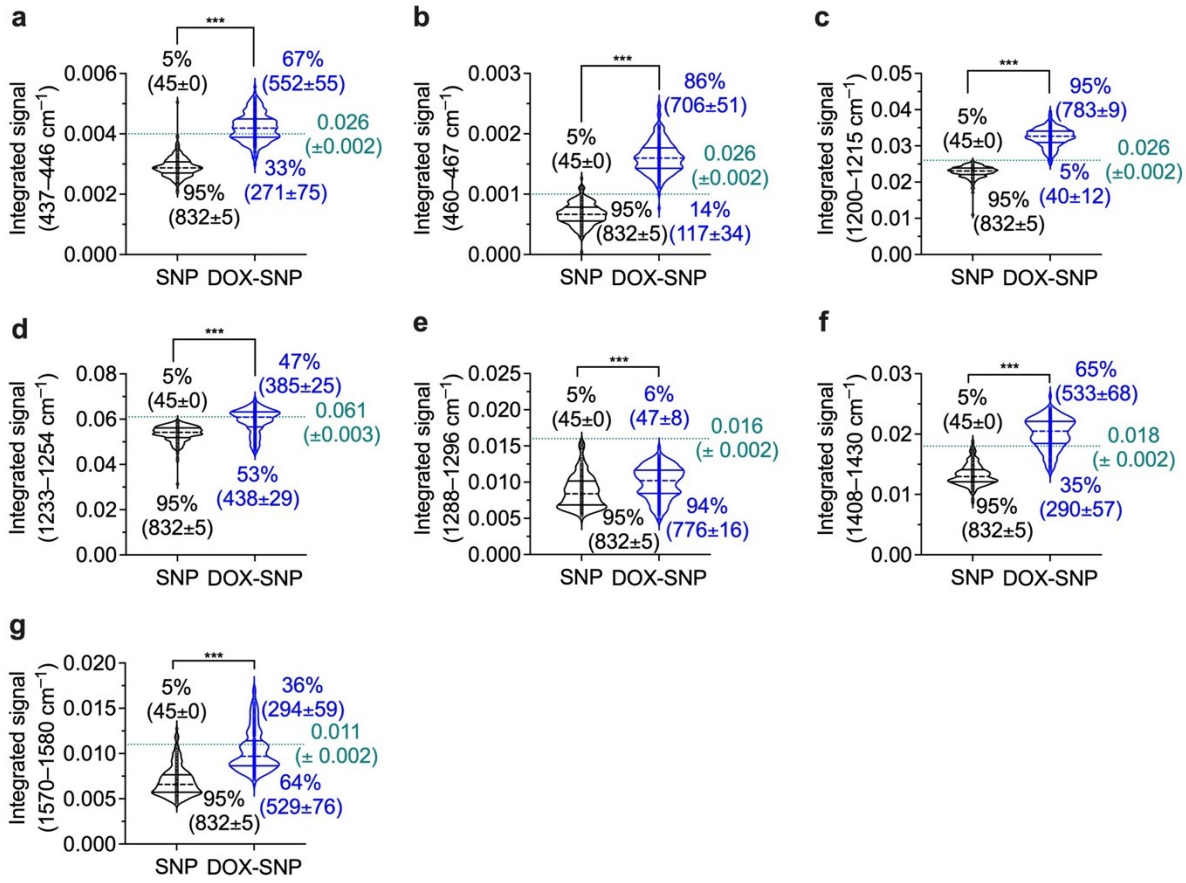

Fig. S7 Single Particle Automated Trapping Analysis (SPARTA) of doxorubicin-loaded silk nanoparticles (DOX-SNPs) prepared by the antisolvent precipitation followed by the adsorption method with 100 µM doxorubicin. An aliquot of 100 µL silk nanoparticle suspension (0.25 mg/mL) was prepared in 0.1 µm-filtered Milli-Q water, which also served as a blank. The measurement was performed in triplicate: 877 and 823 nanoparticles for Placebo SNPs and DOX-SNPs, respectively. The integrated Raman signal analysed by the univariate analysis mode was determined in a range covering the identified peaks for doxorubicin (421–1800 cm^{-1}): (**a**) 437–446 cm^{-1}; (**b**) 460–4667 cm^{-1}; (**c**) 1200–1215 cm^{-1}; (**d**) 1233–1254 cm^{-1}; (**e**) 1288–1296 cm^{-1}; (**f**) 1408–1430 cm^{-1}; (**g**) 1570–1580 cm^{-1}. An unpaired t-test was used for statistical analysis to compare between two groups: $p < 0.1$(*), $p < 0.01$(**), $p < 0.001$(***) ($n = 3$).

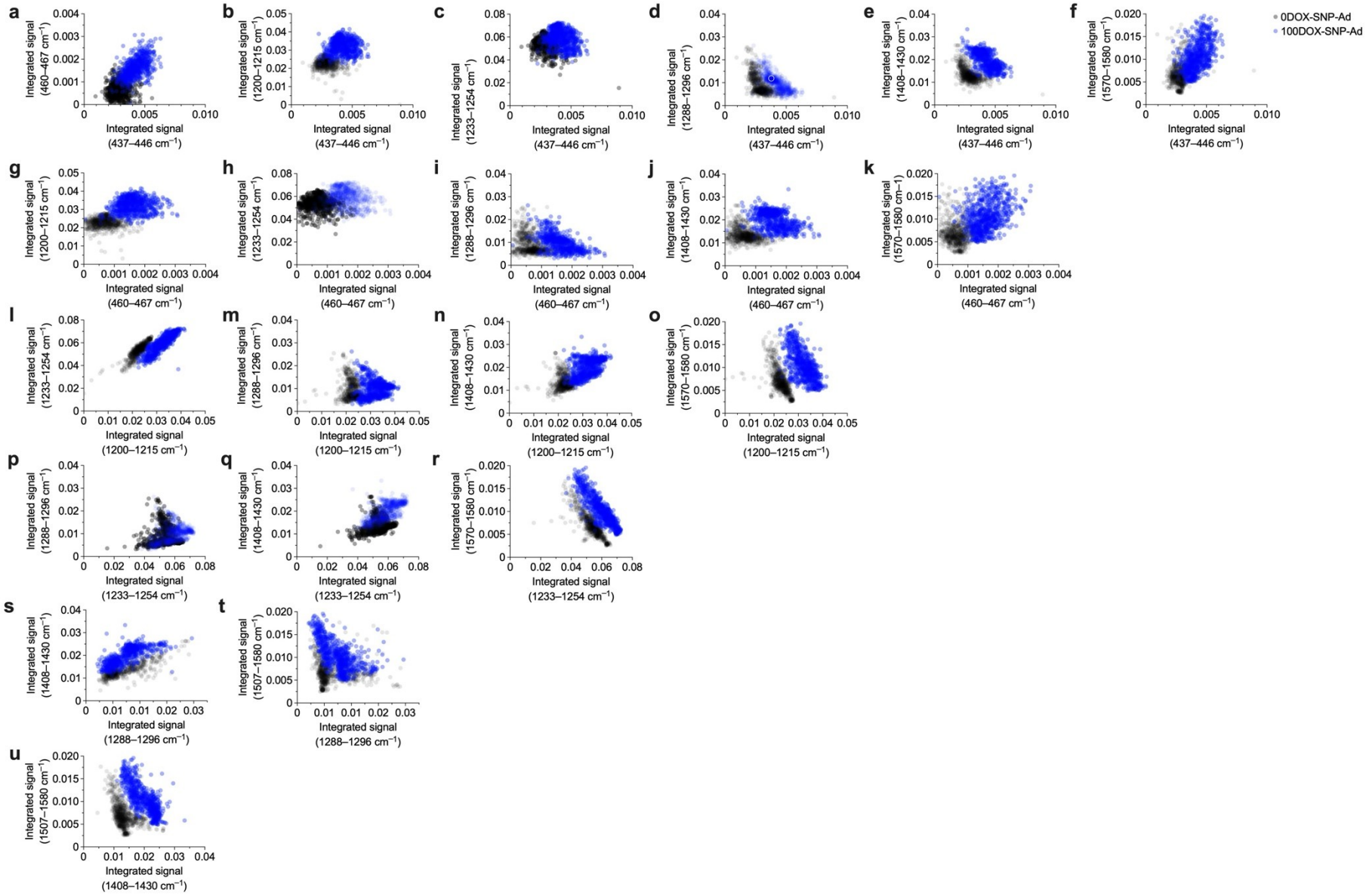

Fig. S8 Bivariate analysis using Single Particle Automated Trapping Analysis (SPARTA). The doxorubicin-loaded silk nanoparticles (DOX-SNPs) were prepared by the antisolvent precipitation followed by the adsorption method with 100 µM doxorubicin. An aliquot of 100 µL silk nanoparticle suspension (0.25 mg/mL) was prepared in 0.1 µm-filtered Milli-Q water, which also served as a blank. The measurement was performed in triplicate: 877 and 823 nanoparticles for Placebo SNPs and DOX-SNPs, respectively. Seven Raman shift ranges were integrated before bivariate analysis, showing a correlation with (**a-f**) 437–446 cm^{-1}, (**g-k**) 460–467 cm^{-1}, (l-**o**) 1200–1215 cm^{-1}, (**p-r**) 1233–1254 cm^{-1}, (**s, t**) 1288–1296 cm^{-1}, and (**u**) 1408–1430 cm^{-1}on the X-axis

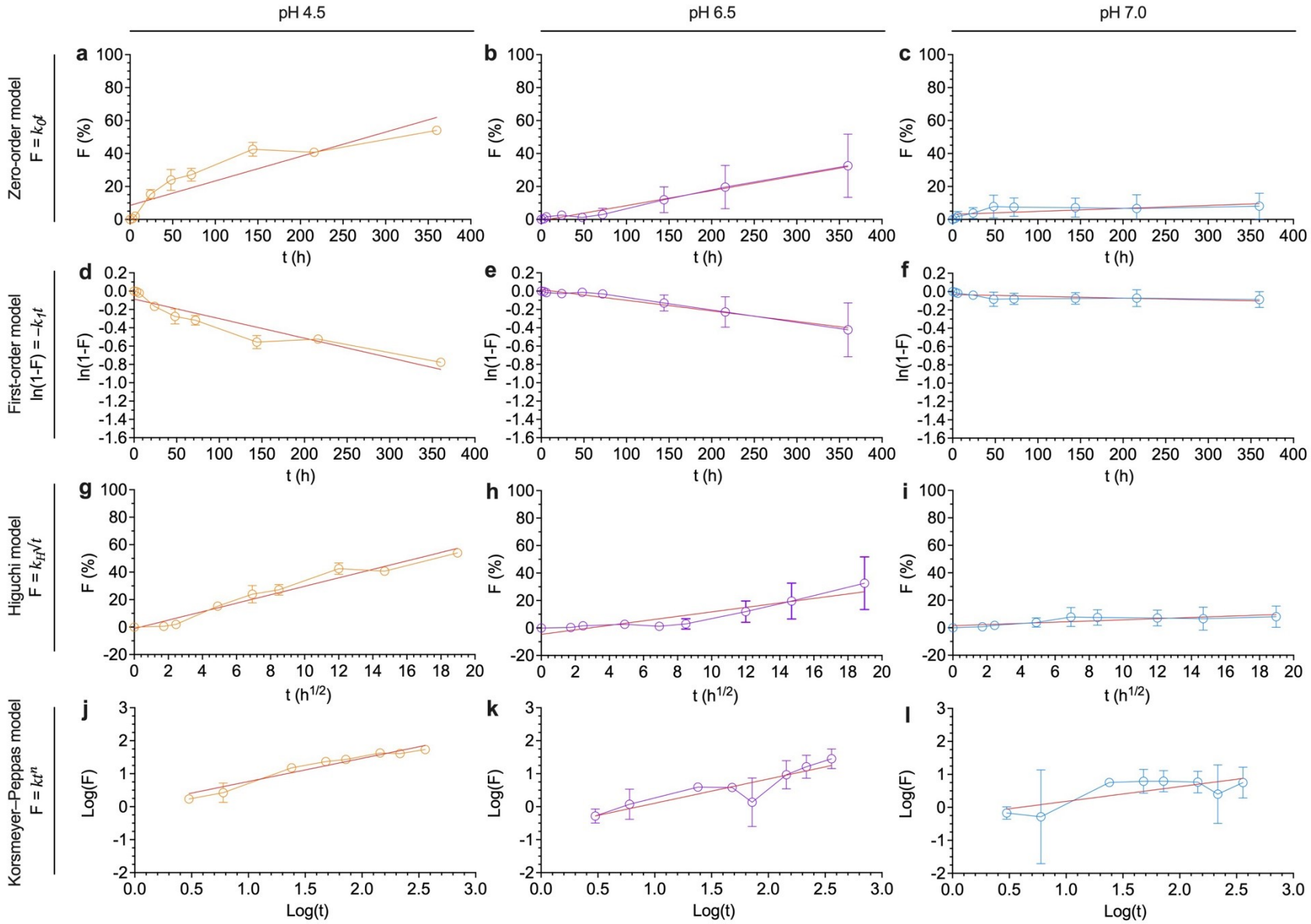

Fig. S9 Release kinetics study of doxorubicin-loaded silk nanoparticles (DOX-SNPs), manufactured by the antisolvent precipitation followed by the adsorption method with 100 μM doxorubicin. The drug release profiles were studied at pH 7.0, pH 6.5, and pH 4.5 for 15 days. The kinetic models, including the zero-order model, first-order model, Higuchi model, and Korsmeyer-Peppas model, were used to describe the drug release mechanism. All models were calculated using a linear regression.[12] F indicates the % cumulative drug release up to time t. The k_0, k_1, k_H, and k are the kinetic constants obtained from the mathematical models, which indicate the rate of drug release over time (n = 3).

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