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Ensemble and single particle analysis of doxorubicin silk nanoparticles

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Adsorption-loaded silk nanoparticles exploit protein electrostatics and β-sheet-rich architecture to achieve high doxorubicin loading and pH-responsive release, while preserving silk structure. Ensemble and single-particle analyses reveal exceptional formulation uniformity, while *in vitro* studies confirm preserved cytotoxicity against MDA-MB-231 human breast cancer cells.

Doxorubicin is a widely used chemotherapeutic agent, yet its clinical utility is limited by cumulative cardiotoxicity and chemoresistance, prompting the development of nanocarriers that have partially mitigated some of these limitations. [1-4] Formulation complexity, heterogenous drug loading, and adverse effects of these formulations, continue to constrain translational performance of doxorubicin nanoparticles.[5] Silk fibroin nanoparticles, distinguished by their β-sheet-rich structure and tuneable surface chemistry, offer favourable physicochemical characteristics,[6-8] including controlled release,[9, 10] enhanced internalization and lysosomotropic delivery,[11] and improved solubility.[9, 12] Despite growing interest in silk nanoparticles as nanoscale delivery vehicles, the impact of drug loading strategies exploiting these features to govern drug release behaviour remains underexplored.[13-16] Here, we investigate adsorption-based loading of doxorubicin onto pre-formed silk nanoparticles as a chemically defined strategy to spatially confine doxorubicin without disrupting β-sheet-rich protein architecture. We hypothesised that adsorption would preserve silk secondary structure while directly linking protein electrostatics to pH-responsive release behaviour.

Silk nanoparticles prepared by semi-batch antisolvent precipitation formed monodisperse, spherical assemblies with negative surface charge, consistent with β-sheet-rich structure. Adsorption of doxorubicin resulted in high encapsulation efficiency (~90%) without measurable changes in particle size or morphology (**Fig. 1a-e**, **Fig. S2, Fig. S3**, and **Fig. S4**). A modest shift in surface charge toward less negative values was observed, consistent with electrostatic interactions between protonated amines on doxorubicin and anionic silk residues, while retaining colloidal stability. Fourier-transform infrared spectroscopy (FTIR) analysis confirmed retention of β-sheet-dominated secondary structure of the nanoparticles following doxorubicin adsorption. The preservation of silk-like features indicates that doxorubicin remains localised at or near the nanoparticle surface rather than penetrating the β-sheet-rich core (**Fig. 1f, g** and **Fig. S5**).[17, 18]

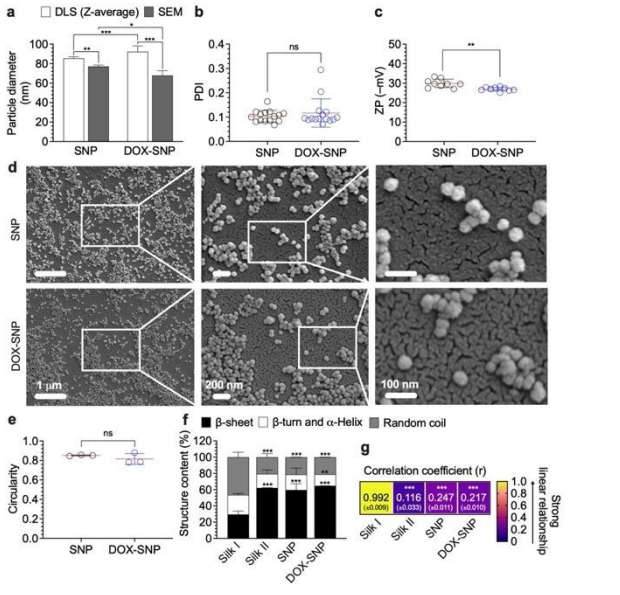

Fig. 1 Physicochemical properties of doxorubicin-loaded silk nanoparticles (DOX-SNPs; 100 μM DOX): (**a**) Particle diameter; (**b**) PDI; (**c**) Zeta potential (ZP); (**d**) FE-SEM micrographs; (**e**) Circularity; (**f**) Secondary structure content; (**g**) Correlation coefficient (FTIR), determining the linear relationship of IR adsorption spectra using silk I structure as reference. $p < 0.1$(*), $p < 0.01$(**), $p < 0.001$(***), $n = 3$.

This partial constraint is chemically significant, as β-sheet-rich domains regulate particle stability, chain mobility, and diffusion pathways, as β-sheet domains regulate silk chain mobility and diffusion pathways that govern drug retention and release. Formulation integrity and population heterogeneity were assessed using FI-AF4 coupled with MALS, DLS, and

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spectroscopic detection (**Fig. S6**). Doxorubicin loading slightly alters nanoparticle size, shape factor, or aggregation behaviour; however, confirming that adsorption effectively preserves nanoparticle morphology (**Fig. 2a, b,** and **Table S1**).[19, 20]

Single particle automated Raman trapping analysis provided direct evidence of formulation homogeneity. Characteristic doxorubicin Raman signatures were detected in ~95% of individual particles, with strong correlation across multiple diagnostic vibrational bands (**Fig. 2c** and **Table S2**-**S3**). Raman spectra of silk nanoparticles (421 to 1800 cm^{-1}) were dominated by protein features, including amide stretches, and phenylalanine, whereas, doxorubicin features slightly shifted relative to reported values (**Fig. 2c** and **Table S2**).[21, 22] These changes likely reflect differences in excitation conditions and the local chemical environment.[21] Despite partial overlap between doxorubicin and protein bands, doxorubicin-loaded nanoparticles showed a clear and consistent increase in drug-associated Raman signals across multiple diagnostic peaks (**Fig. 2c**).

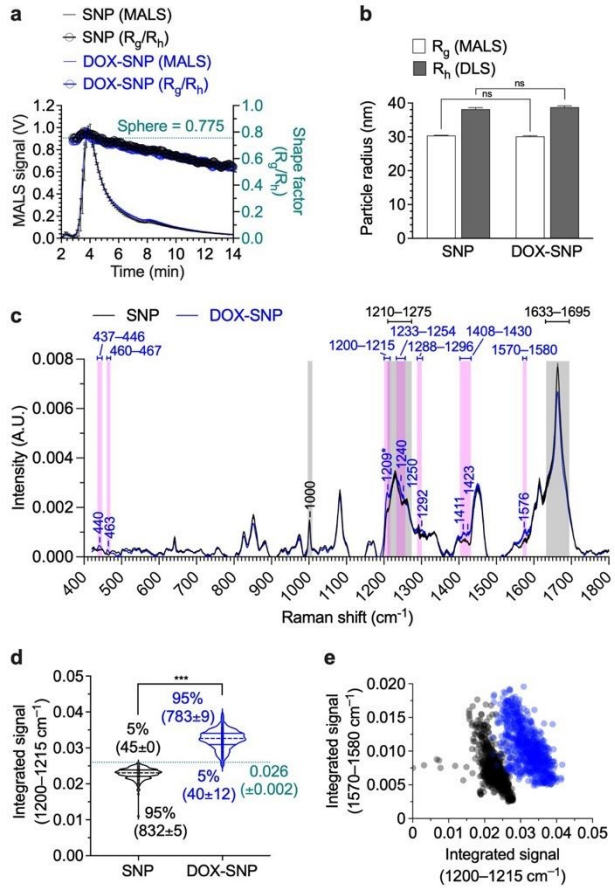

Fig. 2 FI-AF4 fractogram and SPARTA. (**a**) FI-AF4-MALS aligned with computed shape factor (R_g/R_h). Dotted line indicates ideal spherical shape factor of 0.775. (**b**) R_g and R_h were obtained from MALS and DLS detector measurements. (**c**) Overlay of Raman spectra (421–1800 cm^{-1}) for protein (black) and doxorubicin (pink). (**d**) Integrated Raman signal analysed by the univariate analysis mode. (**e**) Bivariate analysis, showing a signal intensity correlation of 1200–1215 and 1570–1580 cm^{-1}. $p < 0.1$(*), $p < 0.01$(**), $p < 0.001$(***), $n = 3$.

Univariate and bivariate analyses confirmed a distinct separation between empty and drug-loaded particle populations, with particularly strong discrimination at 1209 cm^{-1} (**Fig. 2d, e**), indicating highly homogeneous drug incorporation (**Fig. 2d, e, Fig. S7,** and **Fig. S8**).[23] A concomitant reduction in Raman intensity at 1660, 850, and 825 cm^{-1} corresponding to the Amide I (C=O) stretch at 1660 cm^{-1} and tyrosine residues at 850 and 825 cm^{-1},[23] suggests localised perturbation of the silk fibroin structure. These changes are consistent with hydrogen bonding between protonated doxorubicin and silk carbonyl groups, together with hydrophobic interactions involving tyrosine residues, supporting the formation of a surface-associated silk-doxorubicin complex (**Fig. 2c**). This single-particle insight confirms uniform drug distribution, overcoming a key limitation of ensemble-averaged measurements that obscure population heterogeneity. Subtle attenuation of specific protein Raman bands suggests local perturbation of the silk microenvironment upon drug binding, consistent with protein-drug complex formation at the particle surface.[24]

Adsorption-loaded silk nanoparticles exhibited pronounced pH-dependent doxorubicin release under physiologically relevant conditions. Drug release increased systematically with decreasing pH, following the trend pH 7.0 < 6.5 < 4.5, reflecting protonation-driven weakening of electrostatic interactions and increased silk chain mobility below the isoelectric point of silk fibroin (**Fig. 3a**).[10, 25] We speculated that both the physical stability of doxorubicin-loaded silk nanoparticle and dialysis may have influenced the release kinetics, resulting in incomplete doxorubicin release. The physical stability of SNP formulations is pH-dependent.[26] Therefore, doxorubicin encapsulation may further alter formulation stability under dialysis conditions, reducing the release rate and prolonging the release period.

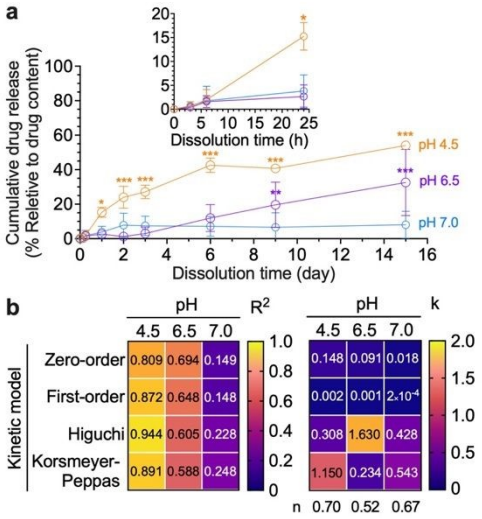

Fig. 3 *In vitro* drug release. Doxorubicin-loaded silk nanoparticles (DOX-SNPs; 100 μM DOX) were prepared at 1.5 mg protein, followed by dialysis against 2 mL of buffer (1×PBS pH 7.0, HEPES buffer pH 6.5, and citrate phosphate buffer pH 4.5) for 15 days; (**a**) Cumulative drug release; (**b**) Release kinetics. $p < 0.1$(*), $p < 0.01$(**), $p < 0.001$(***), $n = 3$.

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Release kinetics were best described by diffusion-dominated models incorporating polymer relaxation (Higuchi and Korsmeyer-Peppas models), indicating diffusion-controlled release coupled with polymer relaxation (**Fig. 3b** and **Fig. S9**).[27, 28]

As proof-of-concept evaluation of biological performance, silk nanoparticles were evaluated in MDA-MB-231 triple-negative breast cancer cells. Placebo nanoparticles exhibited negligible cytotoxicity, confirming biocompatibility. In contrast, doxorubicin-loaded nanoparticles demonstrated dose-dependent cytotoxicity comparable to free doxorubicin (**Fig. 4a**). Confocal imaging revealed time- and concentration-dependent uptake, with intracellular doxorubicin localising in perinuclear regions, consistent with its known mechanism of action. These results confirm that adsorption-mediated loading preserves pharmacological efficacy while enabling pH-responsive delivery (**Fig. 4b-d**).

The pH of the cell culture medium was maintained at approximately 7.4 by bicarbonate buffering. Based on the release profile at pH 7.0, only limited doxorubicin release from doxorubicin-loaded silk nanoparticles (~10%) was expected within 48 h of incubation. Together with the proposed cellular uptake mechanism, we hypothesized that doxorubicin-loaded silk nanoparticles were internalized *via* endocytosis and subsequently sequestered to lysosomes. Under these conditions, the acidic lysosomal environment (pH ~4.5) may enhance doxorubicin release, potentially increasing cumulative release to ~15% within 48 h (**Fig. 3a**). Nevertheless, *in vivo* DOX release should be further investigated, as current *in vitro* release methods may not fully reflect the release behaviour of doxorubicin-loaded silk nanoparticles under physiological conditions.

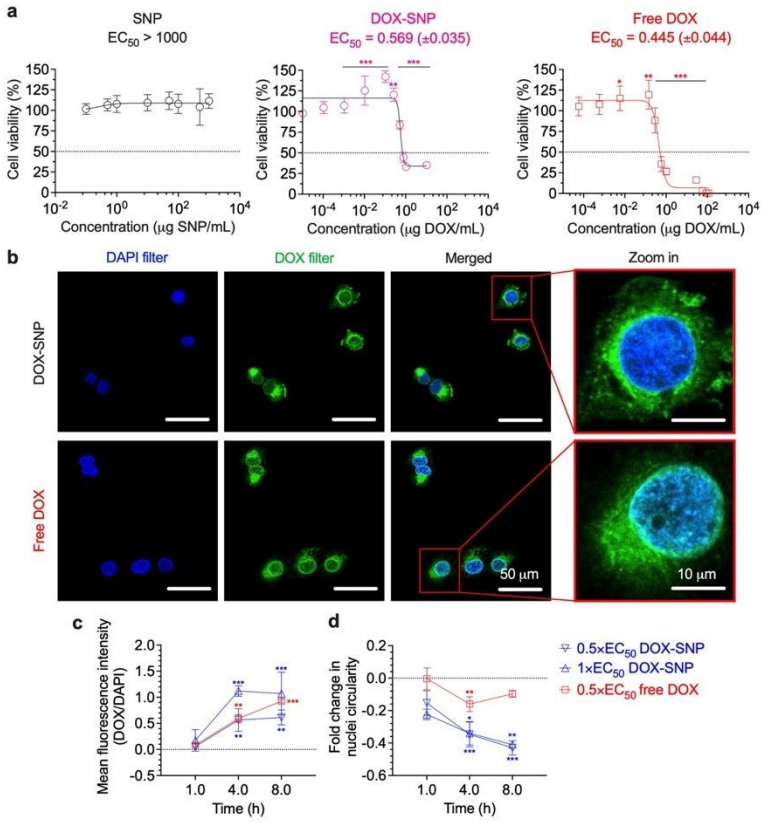

Fig. 4 Cellular responses in MDA-MB-231 cells. Cells were incubated with doxorubicin-loaded silk nanoparticles (DOX-SNPs; 100 μM DOX) for 48 h, followed by the CellTiter-Glo assay; (**a**) Dose-dependent cytotoxicity of placebo SNPs, DOX-SNPs, and doxorubicin; (**b**) Confocal images (63×) after 1 h exposure (0.5×EC_{50}); (**c**) DOX/DAPI intensity; (**d**) Fold-change in nuclear circularity after 1–8 h exposure. $p < 0.1$(*), $p < 0.01$(**), $p < 0.001$(***), $n = 3$.

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In summary, this study established adsorption as a chemically tractable strategy for loading doxorubicin onto silk nanoparticles, coupling protein electrostatics and β-sheet structure to pH-responsive release while demonstrating high biocompatibility, retention of therapeutic efficacy, and potential selectivity toward cancer cells. Together, these findings support the rational design of tunable silk-based nanocarrier systems for targeted doxorubicin delivery.

Conflicts of interest

There are no conflicts to declare.

Author Contributions

NR wrote the manuscript, designed and conducted the experiments, and analysed the data. HG assisted with the use of the confocal microscope. PB assisted with the use of FI-AF4. FPS conceptualized and designed the study and edited the manuscript. ZR conceptualised and designed the study, analysed data, edited the manuscript, and acquired funding.

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Data availability

All data associated with this work are included in the supplementary information or can be accessed at https://doi.org/10.15129/ed1bb0a8-6eff-434f-8355-ac5ede92b5b9.

Abbreviations

B. mori, *Bombyx mori*; DAPI, 4′,6-diamidino-2-phenylindole; DLS, Dynamic Light Scattering; DOX, Doxorubicin; DOX-SNP, Doxorubicin-loaded silk nanoparticle; EC_{50}, half-maximal effective concentration; ELS, Electrophoretic Light Scattering; FE-SEM, Field-Emission Scanning Electron Microscopy; FI-AF4, Frit-Inlet Asymmetric Flow Field-Flow Fractionation; FTIR, Fourier-Transform Infrared; IPA, Isopropanol; LiBr, Lithium bromide; MALS, Multiangle Light Scattering; Na_2CO_3, Sodium carbonate; PDI, Polydispersity index; R_g, Radius of gyration; R_h, Hydrodynamic radius; SNP, Silk nanoparticle; SPARTA, Single Particle Automated Trapping Analysis; UV, Ultra-Violet; X-Flow, Cross-Flow; ZP, Zeta potential

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