pharmaceutics

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Supplementary Materials: Two Beats One: Osteosarcoma Therapy with Light-Activated and Chemo-Releasing Keratin Nanoformulation in a Preclinical Mouse Model

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Supplementary figures

Supplementary tables

Supplementary figures

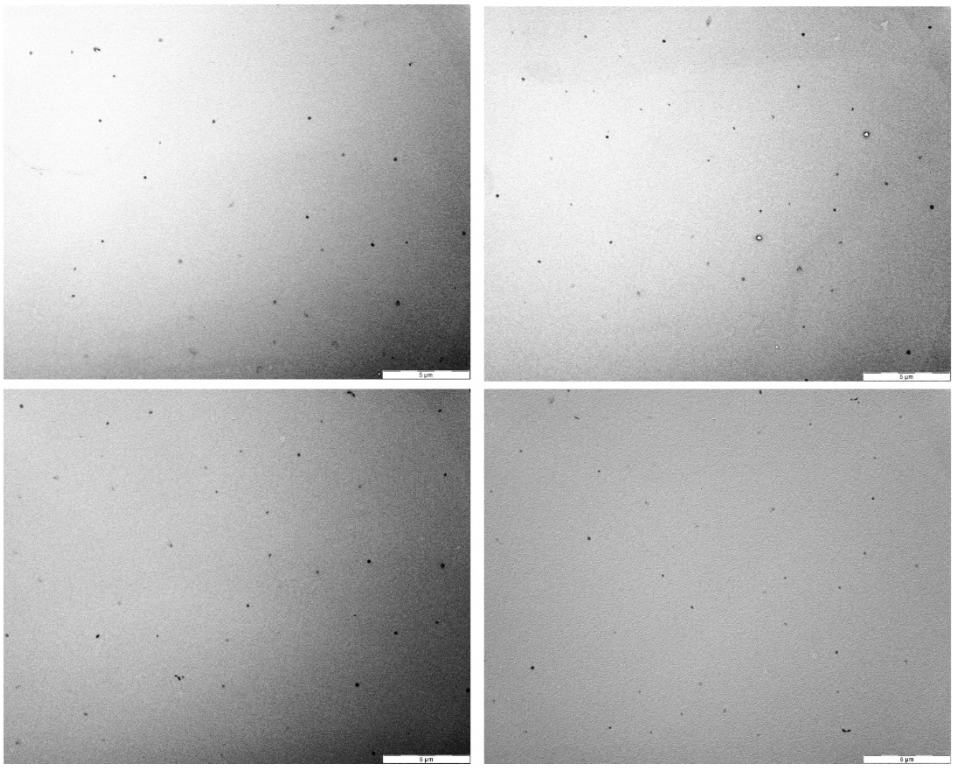

Figure S1. Transmission Electron microscopy images of PTX-Ce6@ker nanoparticles.
Four different representative Transmission electron microscopy (TEM) micrographs of PTX-Ce6@ker nanoparticles performed at a final concentration of 0.1 mg/mL; scale bar: 5µm.

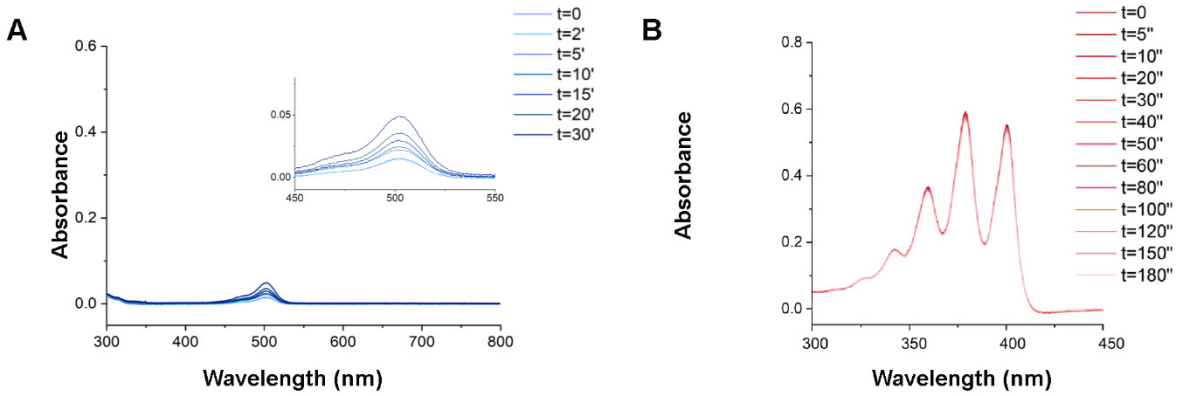

Figure S2. Chemical characterizations of PTX-Ce6@ker nanoformulation: control base line assays.
A-B The graphs show the base line profile of the dichlorofluorescein (DCF) (blue line in **A**) and the 9,10-dimethylanthracene (DMA) (red line in **B**) probes absorbance measured within the wavelength range reported in the x axes and at different irradiation times, indicated as minutes for ROS (′ in A) and seconds (″ in B) for $^1O_2$.

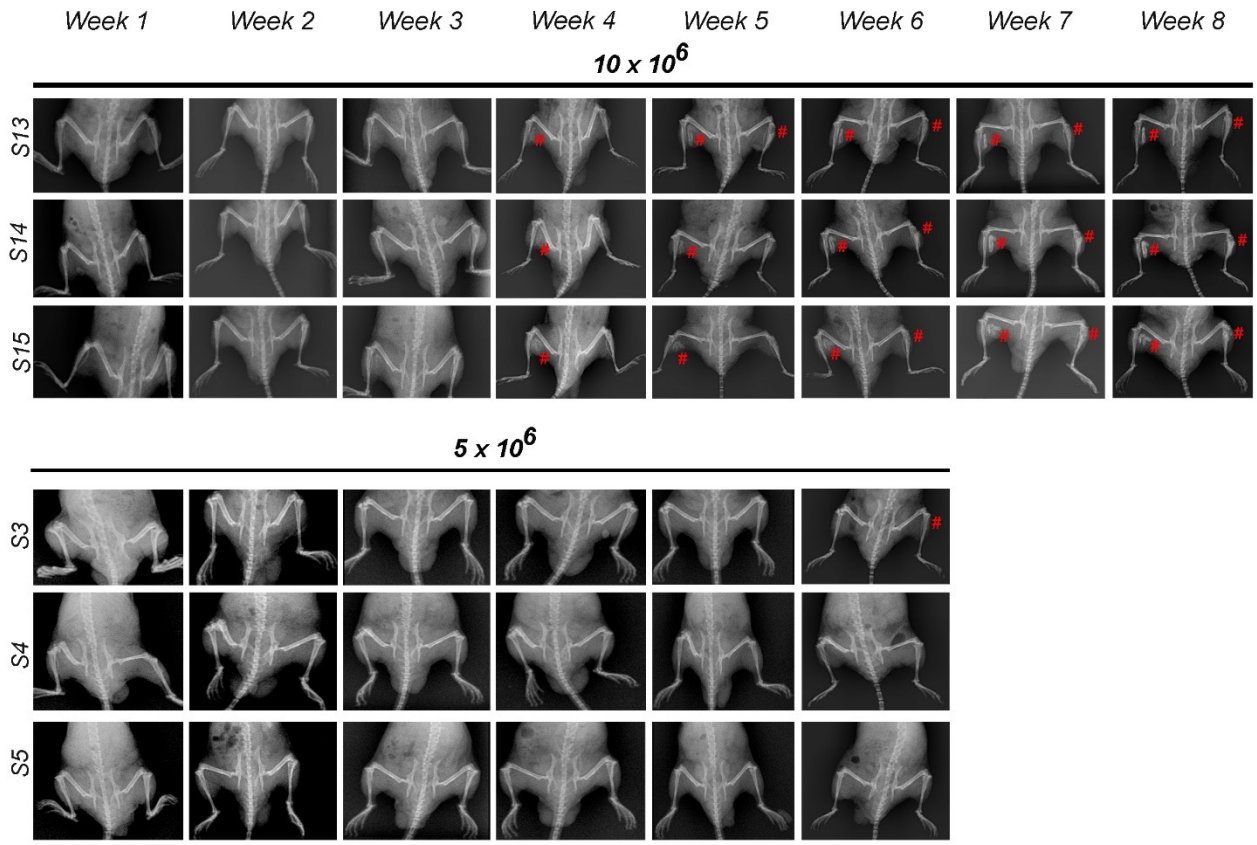

Figure S3. Preclinical Osteosarcoma mouse model set up.
Representative X-ray imaging of 6 animals (shown in the 6 parallel rows) acquired weekly up to week 8 and 6 from initial inoculation of respectively $10x10^6$ and 5×10^6 Saos-2 groups, as indicated. The red hash marks point to the detectable tumor mass.

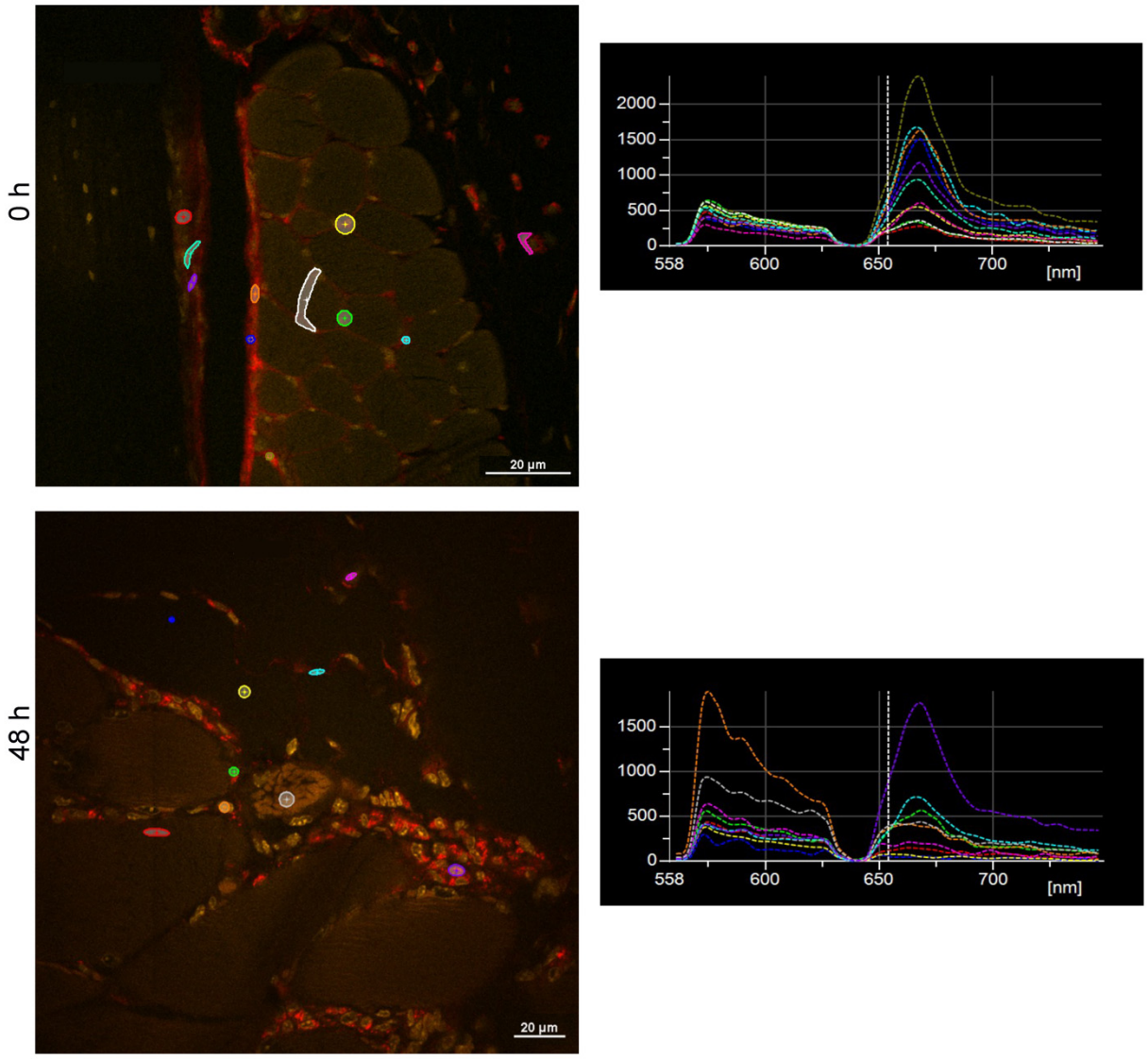

Figure S4. Nanoformulation dosage and treatment's schedule: Spectral analysis of Ce6 fluorescence in mice tissues. Representative confocal spectral images of PTX-Ce6@ker treated tissues at 0 and 48 h after nanoformulation injection. The spectral profiles of the indicated ROIs are reported in the lateral panels. Samples were excited at 405 nm. Zero-intensity at 560 nm ans 640 nm is due to dichroic mirror choice.

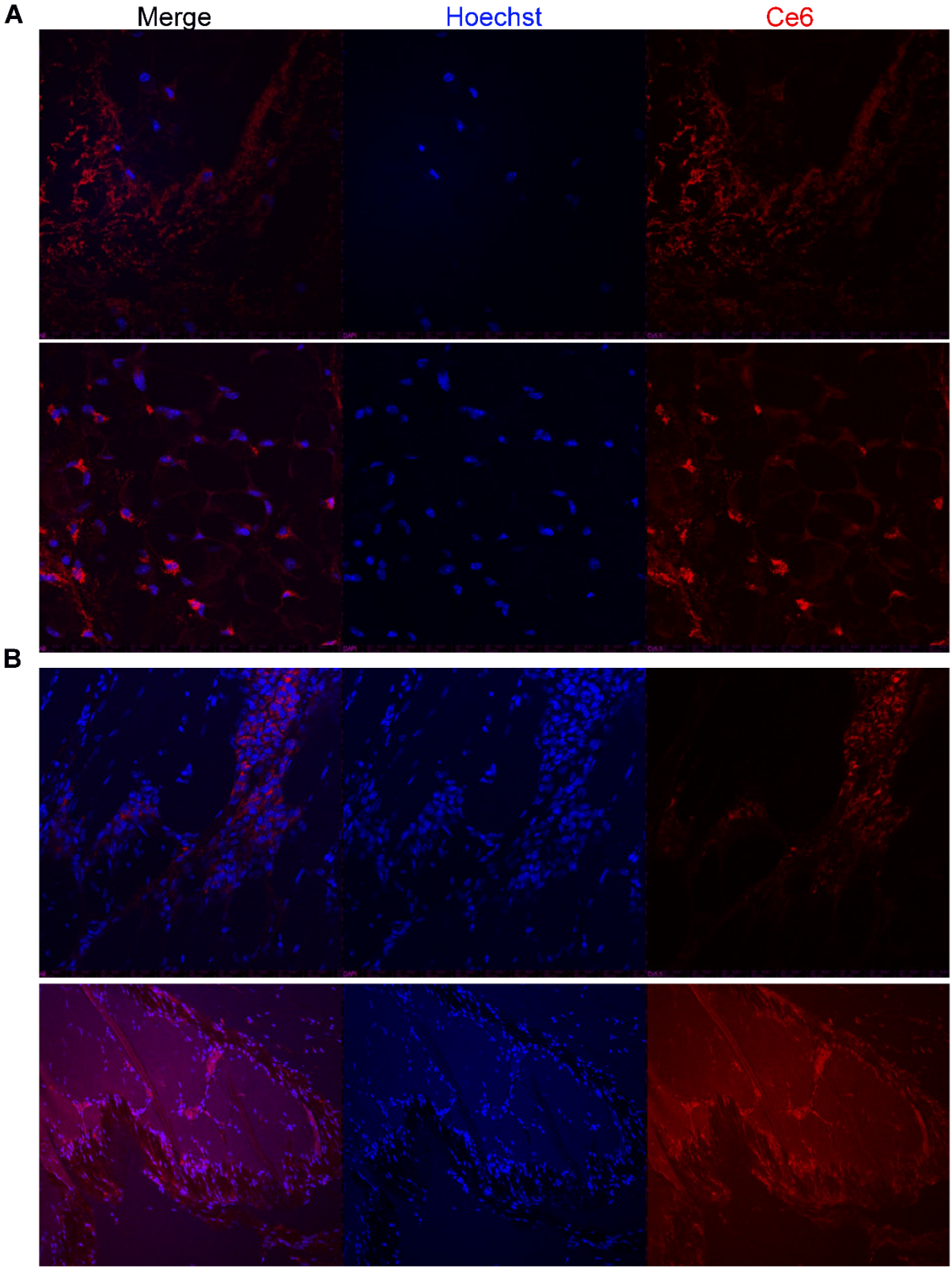

Figure S5. Nanoformulation dosage and treatment's schedule: confocal analysis of Ce6 distribution.
Representative confocal fluorescence intensity images of PTX-Ce6@ker treated tissues at 0 h (A) and 48 h (B) after nanoformulation injection. Hoechst staining in shown in blue, Ce6 signal in red.

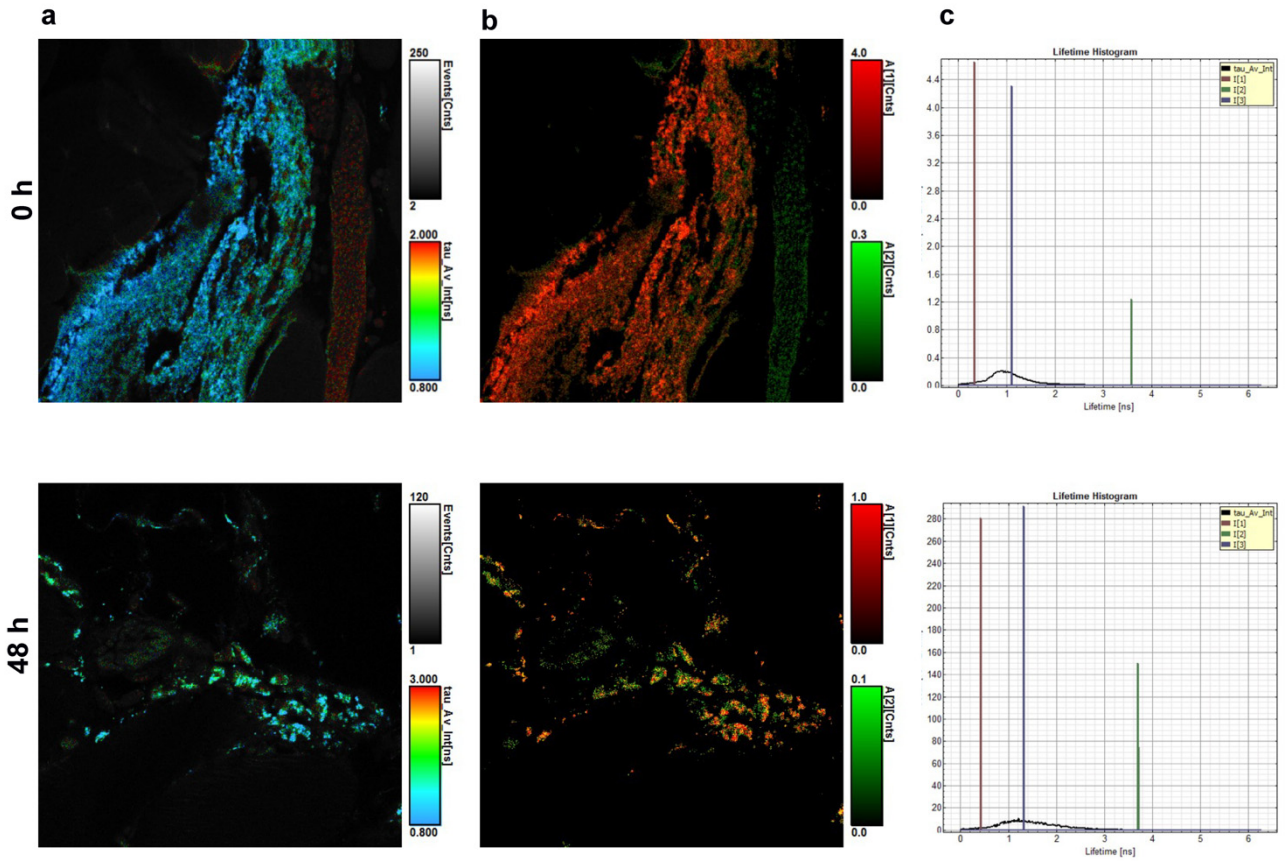

Figure S6. Nanoformulation dosage and treatment's schedule: FLIM analysis of Ce6.
Represenatitive FLIM images Ce6 signal in the samples at 0 and 48 h after nanoformulation injection. In panel **a** color scale indicates the average lifetime; panel **b** shows the pre-exponentail values of a1 and a2, the short and long lifetime respectively; panel **c** represents the lifetime histogram for the fitted area of the image.

Supplementary tables

Table S1. Transmission Electron Microscopy analysis.
Statistical details for the particle size analysis performed on PTX-Ce6@ker nanoparticles from TEM images.

Log-normal distribution function	$$f(x) = \frac{1}{bx\sqrt{2\pi}} e^{-\frac{(\ln x - a)^2}{2b^2}}$$
Fitting parameters	$a = 4.761162$ $b = 0.3565304$
Mean diameter	$\bar{x} = e^{a+\frac{b^2}{2}} = 124.55$ nm
Standard deviation	$\sigma_x = \sqrt{(e^{b^2} - 1)e^{2a+b^2}} = 45.86$ nm

Table S2. Chemical characterizations of PTX-Ce6@ker nanoformulation: PTX release kinetics.
Statistical details for the calibration curve of PTX obtained by HPLC-UV analysis.

Calibration curve	$f(x) = ax + b$
Variables	HPLC peak areas at 228 nm (μV·s) vs. PTX concentrations (μg/mL)

Fitting parameters	$a = 107712.5 \pm 354.5$ $b = -672.8 \pm 3982.0$ $R^2 = 0.99983$
Limit of quantification	$\text{LOQ} = 10\frac{\sigma_b}{a} = 0.370$ μg/mL

Table S3. Chemical characterizations of PTX-Ce6@ker nanoformulation: PTX release kinetics.
PTX release data determined by HPLC-UV analysis during the dialysis of PTX-Ce6@ker nanoparticles against [PBS, pH 7.4]/ethanol 75:25, v/v.

Dialysis time (h)	Released PTX (w/w)
0.5	10.29 ± 0.07%
1.0	14.49 ± 0.14%
1.5	18.54 ± 0.03%
2.0	21.95 ± 0.11%
2.5	26.09 ± 0.18%
3.0	29.46 ± 0.14%
4.5	38.77 ± 0.04%
6.0	46.83 ± 0.07%
7.5	53.41 ± 0.06%
9.0	58.98 ± 0.17%
21.5	80.57 ± 0.30%
24.0	81.24 ± 0.08%
26.5	82.32 ± 0.27%
29.0	82.96 ± 0.19%

Table S4. Chemical characterizations of PTX-Ce6@ker nanoformulation: PTX release kinetics.
Statistical details for the PTX release kinetics analysis on PTX-Ce6@ker nanoparticles

Cumulative Weibull distribution function	$\boldsymbol{f_{\text{PTX}} = 1 - e^{-\alpha t^{\beta}}}$
Fitting parameters	$\alpha = 0.1621 \pm 0.0067$ $\beta = 0.7398 \pm 0.0178$ $R^2 = 0.99628$
Release half-time	$t_{½} = \left(\frac{\ln 2}{\alpha}\right)^{\frac{1}{\beta}} = 7.128$ h
Korsmeyer-Peppas model	$f_{\text{PTX}} = k_P t^{n_P}$
Time range	$0.5 \text{ h} \leq t \leq 9.0$ h ($f_{PTX} < 60\%$, w/w)
Fitting parameters	$k_P = 0.1461 \pm 0.0025$ $n_P = 0.6406 \pm 0.0097$ $R^2 = 0.99875$

Table S5. Summary of the Chlorin e6 lifetimes in all conditions tested.

	τ_1 / ns	τ_2 / ns	τ_3 / ns	τ_{av} / ns
Solution Ce6 in PBS	3.8			
Solution PTX-Ce6@ker	1.8	4.3		4.1
Tissue PTX-Ce6@ker				
0 h cytoplasm	0.3	1.1	3.5	0.9
0 h intercellular	0.3	1.1	3.6	0.8
48 h cytoplasm	0.4	1.3	3.7	1.1