

Electronic Supplementary Material

CD44 and CD221 directed magnetic cubosomes for the targeted delivery of helenalin to rhabdomyosarcoma cells

Hakmin Mun¹, Yuriy Chaban², Tanveer A. Tabish³, Nanasaheb Thorat¹, Nathan Cowieson⁴, C. David Owen², and Helen E. Townley^{1,5} (✉)

¹ Nuffield Department of Women's and Reproductive health, University of Oxford, Oxford OX3 9DU, UK

² Electron Bio-Imaging Centre, Diamond Light Source, Didcot OX11 0DE, UK

³ Radcliffe Department of Medicine, University of Oxford, Oxford OX3 7BN, UK

⁴ B21, Diamond Light Source, Didcot OX11 0DE, UK

⁵ Department of Engineering Science, University of Oxford, Oxford OX1 3PJ, UK

Supporting information to <https://doi.org/10.1007/s12274-022-5037-4>

Additional experimental methods

Synthesis of cubosomes

Unfunctional cubosomes (Cub^{unfun}; empty cubosome comprising GMO, Nile Red and F127) and blank cubosomes (Cub^{blank}; PEGylated cationic cubosome with no functionalization consisting of GMO, DSPE-PEG-Mal, DOTAP, Nile Red and F127) were prepared using a method previously published with some modifications [1]. GMO, DSPE-PEG-Mal, DOTAP, Nile Red, helenalin, SPIONs were dissolved in ethanol and vortex-mixed thoroughly (Table S1). The organic solvent was evaporated in a heat block using a vacuum at 70 °C and further dried under a stream of N₂ gas. The lipid mixture was freeze-dried overnight. Two microgram per millilitre of Pluronic F127 in PBS was then added to the dry lipid followed by sonication at a frequency of 20 kHz with a 5 s on and 5 s off mode for 5 min. In order to separate unencapsulated compounds such as helenalin and Nile Red from the cubic phase dispersion, the solution was dialyzed using a 10 kDa MWCO Slide-A-Lyzer MINI Dialysis unit (Fisher Scientific Ltd, Loughborough, UK) for 2 h. For the antibody conjugation, 5 µg of anti-CD221 Abs were thiolated by reaction with 50 ng of Traut's reagent (Sigma Aldrich, Gillingham, UK) in a phosphate buffer (0.1 M, 2 mM EDTA, pH 8.0) for 1 h at room temperature (RT), resulting in attachment of -SH groups into intact Abs [2]. Alternatively, anti-CD221 Abs were cleaved at the hinge region through a reaction with 10 mM DTT at RT for 2 h. After the reaction, the residual chemicals were removed from the thiolated Abs or half Abs by 10 kDa MWCO dialysis for 2 h [3]. The purified thiolated Abs or half Abs were conjugated into Cub^{blank} through the thiol-maleimide Michael reactions between -SH groups of Abs and maleimide groups present on cubosomes overnight, leading to formation of Cub^{wh-Abs} or Cub^{ha-Abs}. For the hyaluronic acid (HA) conjugation, different volumes of 1 mg/mL of hyaluronic acid were incubated with Cub^{blank} at RT for 4 h, resulting in production of Cub^{1-5%HA}. We incorporated various amount of SPIONs into the lipid mixture before the solvent evaporation and generated Cub^{1-5%ION} by sonication. The triple functional cubosomes (Cub^{fun}) were synthesized by conjugating half Abs with Cub^{1%ION} followed by the HA attachment. The encapsulation efficiencies (EEs) of helenalin in cubosomes were calculated by dissolving helenalin-laden cubosomes with ethanol after 10 kDa MWCO dialysis and quantifying helenalin entrapped in NPs through liquid chromatography (LC) followed by dividing the amounts of encapsulated helenalin by total amount of helenalin and multiplying it by 100. The release rate of helenalin was assessed by subtracting the EE from 100.

Dynamic Light Scattering

The hydrodynamic diameter along with the polydispersity index (PDI) and the zeta potential (ζ) of cubic phase dispersions were determined using a Zetasizer Nano ZS (Malvern, UK) at 25 °C. Diluted samples (1:50 in water) were transferred into disposable plastic cuvettes and backscattered by a 4 mW He-Ne laser (λ=633 nm) at a scattering angle of 173 °. Each measurement was equilibrated for 2 min, and the values were evaluated as averages from 5 measurements of each sample.

SDS-PAGE and Ab quantification

Ab conjugated cubosomes were subjected to 300 kDa MWCO ultrafiltration centrifugation at 12,000 g for 10 min using Vivaspin 500 centrifugal filter units (Fisher Scientific Ltd, Loughborough, UK). Thirty microliters of non-flow-through or flow-through solutions were mixed with 10 µL of 4× Pierce LDS loading buffer (Life Technologies, Paisley, UK) followed by loading into the wells of Novex WedgeWell Tris-Glycine gels (Life Technologies, Paisley, UK). SDS-PAGE was performed at 180 V and 400 mV for 1 h. Then the gel was taken out from cassettes and stained for 1 h with Imperial Protein Stain (Life Technologies, Paisley, UK). After destaining the gels with water overnight, the protein bands from the gel were imaged. The conjugation rate of Abs in the cubosomes was estimated by quantifying Ab proteins in the 300 kDa MWCO flow-through solutions using a Bio-Rad Protein Assay kit (Watford, UK) followed by dividing the protein amount subtracted that in the flow-through solution from total amount by total amount of protein and multiplying it by 100.

Liquid chromatography

An Agilent LC 1120 Compact LC equipped with an Agilent Eclipse Plus C18 column (4.6 × 150 mm, 5 µm particle size) was used to quantify helenalin and hyaluronic acid. For the helenalin analysis, 10 µL of samples were eluted with a mobile phase constituted of water and methanol (40:60 v/v) with a flow rate of 0.5 mL/min. Detection was performed at 225 nm. The retention time for helenalin was 5.7 min. The LC condition for the hyaluronic acid analysis was as follows: an injection volume-10 µL, a mobile phase- 0.05 M K₂HPO₄ (pH=7.0 adjusted using KOH), the detection wavelength- 205 nm and the detection temperature- 25 °C.

Drug release assay

The kinetics of the drug release from cubosomes were calculated using the Korsmeyer-Peppas Model, which have been used to interpret the drug release from the cubic phase nanoparticles as well as polymeric systems [4, 5]. The model can be described as the following equation:

$$M_t/M_\infty = k \cdot t^n$$

where M_t is the amount of drug released at time t , M_∞ is the total amount of drug, k is a kinetic constant, t is the release time, and n is the diffusion exponent. The drug release triggered by alternating magnetic field (AMF) was performed using a BENPAO mini magnetic induction heater kit. The cubosome solution (2 mg/mL) diluted in PBS was transferred in Eppendorf tubes and subjected to AMF in a 5 cm inner diameter coil at 60 A and 96 kHz. During the AMF treatment, the coil was placed on ice to prevent excessive heat from the inductive heater. The cumulative drug release with or without AMF was evaluated by LC following the aforementioned centrifugal filtration of unbound drug.

Cell culture

An RMS cell line, RD cells (ATCC no. CRL-7763) and a normal control cell line, the fibroblast cells, which was kindly provided by Dr Jo Poulton from Nuffield Department of Women's and Reproductive Health, University of Oxford, were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 100 U/mL penicillin/0.1 mg/mL streptomycin at 37 °C in a 5% CO₂ atmosphere.

Flow cytometry

To estimate the expression levels of CD44, CD334 and CD221 on the cell surface, cells were incubated with PE coupled primary Abs and analysed by flow cytometry. Cells were splitted from flasks and washed with PBS and FACS staining buffer containing 5% FBS and 0.05% sodium azide. One million cells were incubated with 1:500 dilution of isotype or primary Abs conjugated PE for 30 min on ice. After washing with PBS several times, cells were subjected to flow cytometry using a BD FACS Calibur flow cytometer by measuring PE fluorescence using a 488 nm laser for excitation and a bandpass filter at 585/42 nm for emission (FL2-H channel). For the cellular uptake of cubosomes, RMS cells were seeded at 6-well plates at 3×10^5 cells/well and allowed to adhere overnight. The cell medium was replaced by fresh medium containing different concentration of NPs tagged with Nile Red and incubated for 0.5–1 h. After detaching cells and washing them with PBS several times, the Nile Red fluorescence (FL2-H) intensities were evaluated using a BD FACSCalibur flow cytometer.

To analyse the intracellular levels of reactive oxygen species (ROS) and mitochondrial membrane potential (MMP), the cells that were treated with drugs were incubated with phenol-free DMEM containing 2 μ M CM-H₂DCFDA or 500 nM TMRM at 37 °C for 30 min. The stained cells were washed with PBS twice and 500 μ L of cell suspension were subjected to flow cytometry by evaluating them in the FL1-H (a 488 nm laser for excitation and a bandpass filter at 530/30 nm for emission) or FL2-H channel (a 488 nm laser for excitation and a bandpass filter at 585/42 nm for emission) for the ROS and MMP analysis, respectively.

Cellular uptake assay by confocal microscopy

Cells were seeded in 35 mm μ -Dishes (ibidi GmbH, Gräfelfing, Germany) at 4×10^5 cells/well and allowed to adhere overnight. Then the cell media were changed into those containing 20 μ g/mL cubosomes and incubated for 2 h at 37 °C in a 5% CO₂ atmosphere followed by cell fixation in 4% paraformaldehyde for 10 min at RT. Cells were washed with PBS three times and incubated with 5 μ M Hoechst 33342 staining solution (Abcam, Cambridge, UK) for 15 min at RT. Images were acquired using a Zeiss LSM880 confocal microscope and ZEN software.

Colony formation evaluation

The clonogenic assay was carried out based on the colony formation ability of single cells in vitro. Cells were seeded in 24-well plates at 8,000 cells/well and left overnight in the incubator to adhere. Then cells were incubated with drugs for 24 h. Then the growth medium containing drugs was replaced with fresh medium and incubated for another 5-day-period, followed by crystal violet staining. Cell colonies were then counted.

Cell viability assay

Cell proliferation in the 2D cell culture was evaluated using the MTT assay [6]. Cell cycle analysis and apoptosis/necrosis detection were performed using flow cytometry [7]. To create 3D tumour spheroids, 25 μ L of heated 1% agarose in PBS were added to each well followed by seeding 3000 cells/well and growing them in a 96-well plate for 72 h. Cell media in the wells containing spheroids were changed into fresh media including helenalin or helenalin-laden cubosomes, and the spheroids were cultured for another 5 or 10 days. At the end of drug treatment, the cells were stained with 1 μ M calcein AM and 10 μ g/mL PI for 30 min followed by 15 min incubation with 5 μ M Hoechst 33342 at 37 °C. The stained 3D spheroids were observed using a Zeiss LSM880 confocal microscope.

Table S1 Composition and characteristics of cubosomes

Cubosomes	Composition									Particle Characteristics						
	GMO	DSPE-PEG-Mal	DOTAP	SPION	F127	HA	Ab	Hele	Nile Red	Size (nm)	ζ (mV)	PDI	EE (%)	LPs	R_w	Phase
Cub ^{unfun}	1 mg	-	-	-	200 μ g	-	-	-	1 μ g	190 \pm 5	-6.4 \pm 0.5	0.35 \pm 0.02	-	145 \AA	27 \AA	Im3m
Cub ^{unfun} _{hele}	1 mg	-	-	-	200 μ g	-	-	32 μ g	1 μ g	178 \pm 4	-9.1 \pm 0.5	0.17 \pm 0.02	70.8 \pm 2.4	142 \AA	26 \AA	Im3m
Cub ^{blank}	1 mg	10 μ g	100 μ g	-	200 μ g	-	-	-	1 μ g	147 \pm 1	15.6 \pm 0.3	0.31 \pm 0.04	-	137 \AA	25 \AA	Im3m
Cub ^{1%HA}	1 mg	10 μ g	100 μ g	-	200 μ g	10 μ g	-	-	1 μ g	177 \pm 2	10.7 \pm 0.5	0.18 \pm 0.03	-	152 \AA	29 \AA	Im3m
Cub ^{2%HA}	1 mg	10 μ g	100 μ g	-	200 μ g	20 μ g	-	-	1 μ g	173 \pm 2	4.6 \pm 0.5	0.17 \pm 0.01	-	154 \AA	30 \AA	Im3m
Cub ^{3%HA}	1 mg	10 μ g	100 μ g	-	200 μ g	30 μ g	-	-	1 μ g	164 \pm 1	-2.8 \pm 0.3	0.18 \pm 0.01	-	155 \AA	30 \AA	Im3m
Cub ^{4%HA}	1 mg	10 μ g	100 μ g	-	200 μ g	40 μ g	-	-	1 μ g	160 \pm 1	-5.0 \pm 0.5	0.18 \pm 0.04	-	154 \AA	30 \AA	Im3m
Cub ^{5%HA}	1 mg	10 μ g	100 μ g	-	200 μ g	50 μ g	-	-	1 μ g	159 \pm 1	-7.5 \pm 0.6	0.21 \pm 0.01	-	153 \AA	30 \AA	Im3m
Cub ^{wh-Ab}	1 mg	10 μ g	100 μ g	-	200 μ g	-	5 μ g*	-	1 μ g	166 \pm 3	22.3 \pm 0.4	0.28 \pm 0.04	-	158 \AA	32 \AA	Im3m
Cub ^{ha-Ab}	1 mg	10 μ g	100 μ g	-	200 μ g	-	5 μ g**	-	1 μ g	175 \pm 3	19.9 \pm 0.8	0.27 \pm 0.01	-	143 \AA	27 \AA	Im3m
Cub ^{0.5%ION}	1 mg	10 μ g	100 μ g	5 μ g	200 μ g	-	-	-	1 μ g	159 \pm 1	11.8 \pm 0.6	0.24 \pm 0.02	-	137 \AA	25 \AA	Im3m
Cub ^{1%ION}	1 mg	10 μ g	100 μ g	10 μ g	200 μ g	-	-	-	1 μ g	166 \pm 2	10.1 \pm 0.2	0.19 \pm 0.02	-	132 \AA	24 \AA	Im3m
Cub ^{2%ION}	1 mg	10 μ g	100 μ g	20 μ g	200 μ g	-	-	-	1 μ g	178 \pm 2	9.2 \pm 0.7	0.16 \pm 0.03	-	140 \AA	26 \AA	Im3m
Cub ^{3%ION}	1 mg	10 μ g	100 μ g	30 μ g	200 μ g	-	-	-	1 μ g	169 \pm 2	7.7 \pm 0.8	0.13 \pm 0.02	-	-	-	-
Cub ^{4%ION}	1 mg	10 μ g	100 μ g	40 μ g	200 μ g	-	-	-	1 μ g	164 \pm 1	3.8 \pm 0.5	0.12 \pm 0.03	-	-	-	-
Cub ^{fun}	1 mg	10 μ g	100 μ g	10 μ g	200 μ g	20 μ g	5 μ g**	-	1 μ g	178 \pm 3	2.7 \pm 0.2	0.17 \pm 0.06	-	126 \AA	22 \AA	Im3m
Cub ^{blank} _{hele}	1 mg	10 μ g	100 μ g	-	200 μ g	-	-	32 μ g	1 μ g	147 \pm 1	15.7 \pm 1.6	0.29 \pm 0.02	83.6 \pm 1.4	138 \AA	25 \AA	Im3m
Cub ^{2%HA} _{hele}	1 mg	10 μ g	100 μ g	-	200 μ g		-	32 μ g	1 μ g	174 \pm 1	2.5 \pm 0.3	0.29 \pm 0.02	76.4 \pm 1.9	142 \AA	26 \AA	Im3m
Cub ^{ha-Ab} _{hele}	1 mg	10 μ g	100 μ g	-	200 μ g		5 μ g**	32 μ g	1 μ g	177 \pm 6	17.1 \pm 1.1	0.25 \pm 0.01	80.3 \pm 1.1	139 \AA	25 \AA	Im3m
Cub ^{1%ION} _{hele}	1 mg	10 μ g	100 μ g	10 μ g	200 μ g		-	32 μ g	1 μ g	157 \pm 1	8.5 \pm 1.6	0.23 \pm 0.02	64.5 \pm 2.2	137 \AA	25 \AA	Im3m
Cub ^{fun} _{hele}	1 mg	10 μ g	100 μ g	10 μ g	200 μ g		5 μ g**	32 μ g	1 μ g	169 \pm 4	2.4 \pm 0.3	0.19 \pm 0.02	63.0 \pm 2.1	131 \AA	23 \AA	Im3m

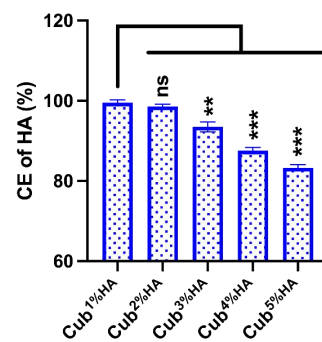


Figure S1 Conjugation efficiency (CE) of HA in Cub^{1-5%HA}. n=3, ns p>0.05, **p<0.01, ***p<0.001 (one-way ANOVA and Dunnett post hoc test).

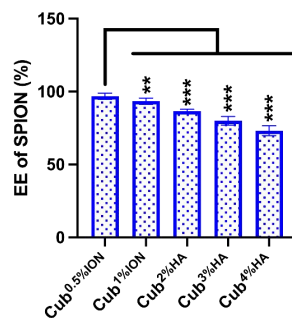


Figure S2 Encapsulation efficiency (EE) of SPIONs in Cub^{0.5-4%HA}. n=3, **p<0.01, ***p<0.001 (one-way ANOVA and Dunnett post hoc test).

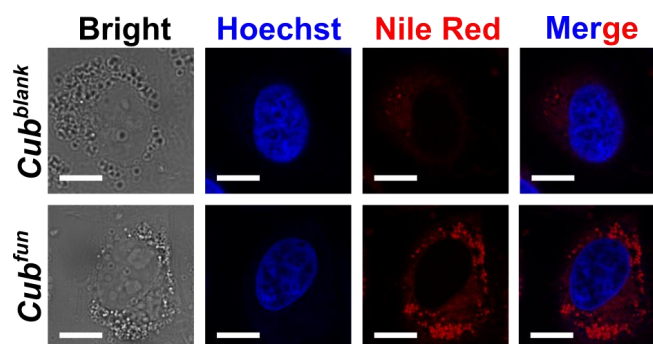


Figure S3 Confocal images of RMS cells incubated with Cub^{blank} and Cub^{fun} for 1h. Scale bar = 10 μm.

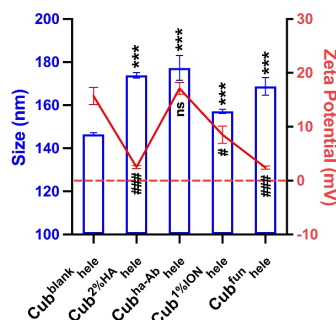


Figure S4 Characterization of cubosomes carrying helenalin by DLS and ζ. n=3. ns p>0.05, #p<0.05, ***, ###p<0.001 (one-way ANOVA and Dunnett post hoc test).

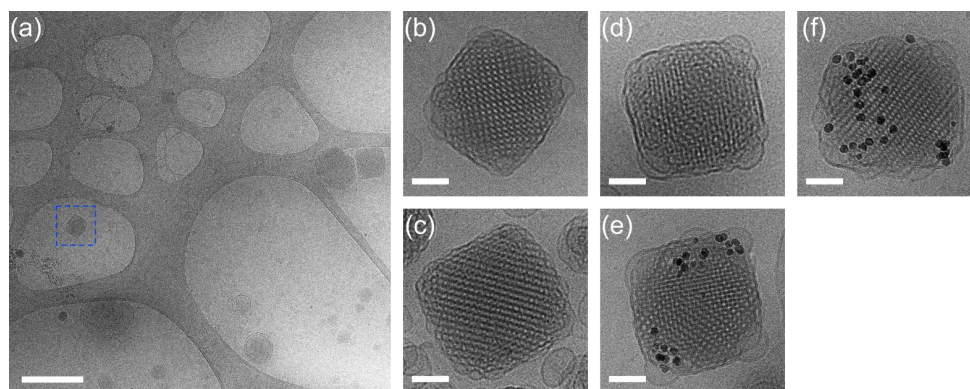


Figure S5 Representative cryo-EM images of (a) Cub^{blank}_{hele} (an expanded image, scale bar = 500 nm), (b) Cub^{blank}_{hele} (a magnified image of the denoted NP in (a)), (c) $Cub^{2\%HA}_{hele}$, (d) Cub^{ha-Ab}_{hele} , (e) $Cub^{1\%ION}_{hele}$ and (f) Cub^{fun}_{hele} . Scale bar = 50 nm (b-f).

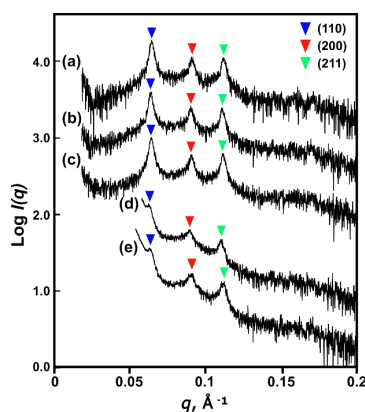


Figure S6 Representative SAXS profiles of (a) Cub^{blank}_{hele} , (b) $Cub^{2\%HA}_{hele}$, (c) Cub^{ha-Ab}_{hele} , (d) $Cub^{1\%ION}_{hele}$, and (e) Cub^{fun}_{hele} .

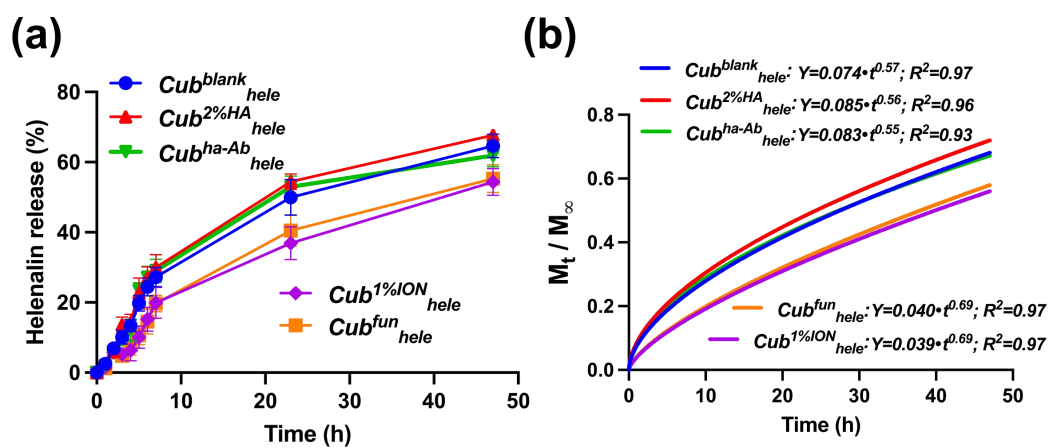


Figure S7 (a) Hele release profiles from helenalin-laden cubosomes at 37 °C for 48 h, (b) The fitted graphs of hele release from helenalin-laden cubosomes at 37 °C for 48 h according to the Korsmeyer-Peppas model.

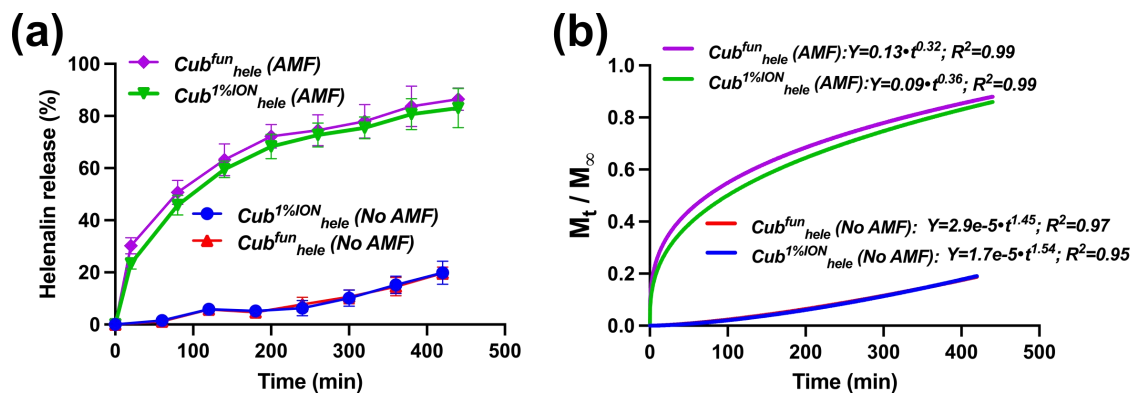


Figure S8 (a) Hele release profiles from Cub^{1%ION}hele and Cub^{fun}hele with or without AMF at 37 °C for 7 h, (b) The fitted graph of hele release from Cub^{1%ION}hele and Cub^{fun}hele with or without AMF at 37 °C for 7 h according to the Korsmeyer-Peppas model.

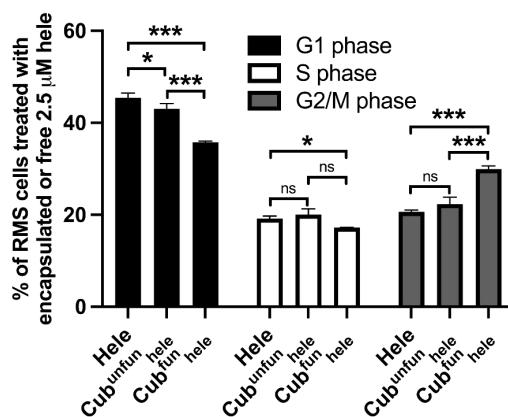


Figure S9 Cell cycle analysis of RMS cells treated with pure 2.5 μM helenalin or cubosomes (Cub^{unfun}hele & Cub^{fun}hele) carrying 2.5 μM helenalin by flow cytometry and comparison of the cell population in the G1, S and G2/M phase (n=3, ns p>0.05, *p<0.05, ***p<0.001; one-way ANOVA and Tukey post hoc test).

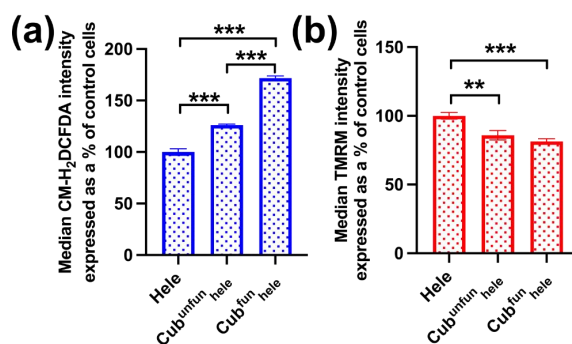


Figure S10 Mechanism of cell death of RMS cells induced by helenalin or nanodrugs. Comparison of cellular (a) ROS levels and (b) MMP levels after treating with pure 2.5 μM helenalin or cubosomes (Cub^{unfun}hele & Cub^{fun}hele) carrying 2.5 μM helenalin (n=3, **p<0.01, ***p<0.001; one-way ANOVA and Tukey post hoc test).

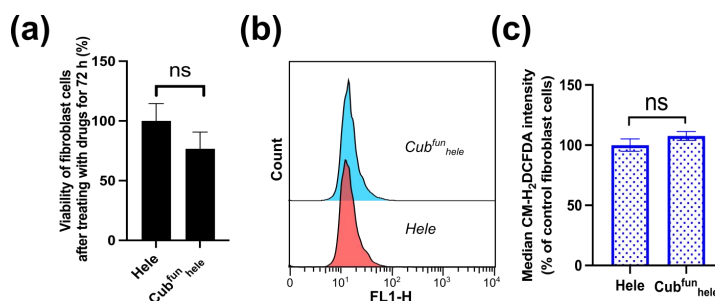


Figure S11 Off-target cytotoxicity and cellular functional changes of fibroblast cells induced by helenalin or nanodrugs. (a) Cytotoxicity of fibroblast upon treatment with pure 2.5 μ M helenalin or triple functional cubosomes carrying 2.5 μ M helenalin for 72 h, (b) & (c) Flow cytometry analysis and comparison of ROS levels of fibroblast cells treated with pure 2.5 μ M helenalin or triple functional cubosomes carrying 2.5 μ M helenalin for 72 h. (n=3, ns p>0.05, a two-tailed t test).

References

- [1] Zhai, J. L.; Tan, F. H.; Luwor, R. B.; Reddy, T. S.; Ahmed, N.; Drummond, C. J.; Tran, N. In Vitro and In Vivo Toxicity and Biodistribution of Paclitaxel-Loaded Cubosomes as a Drug Delivery Nanocarrier: A Case Study Using an A431 Skin Cancer Xenograft Model. *Acs Appl Bio Mater* **2020**, *3*, 4198-4207.
- [2] Eloy, J. O.; Petrilli, R.; Chesca, D. L.; Saggiaro, F. P.; Lee, R. J.; Marchetti, J. M. Anti-HER2 immunoliposomes for co-delivery of paclitaxel and rapamycin for breast cancer therapy. *Eur J Pharm Biopharm* **2017**, *115*, 159-167.
- [3] Wong, B. C. K.; Zhang, H. Q.; Qin, L.; Chen, H. B.; Fang, C.; Lu, A. P.; Yang, Z. J. Carbonic anhydrase IX-directed immunoliposomes for targeted drug delivery to human lung cancer cells in vitro. *Drug Des Dev Ther* **2014**, *8*, 993-1001.
- [4] Kulkarni, C. V.; Vishwapathi, V. K.; Quarshie, A.; Moinuddin, Z.; Page, J.; Kendrekar, P.; Mashele, S. S. Self-Assembled Lipid Cubic Phase and Cubosomes for the Delivery of Aspirin as a Model Drug. *Langmuir* **2017**, *33*, 9907-9915.
- [5] Mierzwa, M.; Cytryniak, A.; Krysinski, P.; Bilewicz, R. Lipidic Liquid Crystalline Cubic Phases and Magnetocubosomes as Methotrexate Carriers. *Nanomaterials-Basel* **2019**, *9*.
- [6] Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **1983**, *65*, 55-63.
- [7] Mun, H.; Townley, H. E. Mechanism of Action of the Sesquiterpene Compound Helenalin in Rhabdomyosarcoma Cells. *Pharmaceuticals (Basel)* **2021**, *14*.