Supporting Information

Unprecedented behavior of (*9R*)-9-hydroxystearic acid loaded keratin nanoparticles on cancer cell cycle

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Table of content

Keratin nanoparticles synthesis by <u>self-assembly</u> procedure (@ker)

Keratin powder (15 mg) was dissolved in $NaHCO_3$ buffer, pH = 9.2, (1 mL) and stirred at room temperature in the dark for 2 h. To increase the protein self-assembling by cross-linking, 6 μL of glutaraldehyde (8%) was added into the above solution, which was maintained under shaking (in the dark) for additional 24 h. The suspension of keratin nanoparticles was obtained eliminating the excess of glutaraldehyde and free keratin by 3 cycles of centrifugation (12000 rpm) with filter devices (MWCO: 100 kDa). At each cycle, particles were redispersed in 1 mL of H_2O milliQ.

FITC loaded keratin nanoparticles synthesis by self-assembling procedure (@FITC:ker).

Keratin was conjugated with FITC as follows: FITC (1 mg) was dispersed in anhydrous dimethyl sulfoxide (DMSO, 1 mL). The resulting FITC was then added to a solution of keratin (20 mg/mL) dissolved in $NaHCO_3$ buffer (0.1M, pH = 9.2) using an initial Ce6/keratin molar ratio of 10 eq. The reaction mixture was then stirred in the dark at room temperature for 48 h. The FITC conjugated proteins were then purified by 8 cycles of centrifugation (10000 rpm) with filter devices (MWCO: 10 kDa). At each cycle, particles were redispersed in 1 mL of PBS. Therefore, the purified functionalized protein solution was freeze-dried into powder.

Ker-FITC (15 mg) was dissolved in H_2O milliQ (1 mL) and stirred at room temperature in the dark for 2 h. To increase the protein self-assembling by cross-linking, 6 μL of glutaraldehyde (8%) was added into the above solution, which was maintained under shaking (in the dark) for additional 24 h. The suspension of keratin nanoparticles was obtained eliminating the excess of glutaraldehyde and free keratin by 3 cycles of centrifugation (12000 rpm) with filter devices (MWCO: 100 kDa). At each cycle, particles were redispersed in 1 mL of H_2O milliQ. Therefore, the purified functionalized protein solution was freeze-dried into powder.

FITC loaded (*9R*)-9-HSA keratin nanoparticles (9R@FITC:ker)

Ker-FITC (1 mg) was dissolved in H_2O milliQ (1 mL) and stirred at room temperature in the dark for 2 h. A solution of (*9R*)-HSA in ethanol (5 mg/mL) were slowly added to the keratin solution under vigorous magnetic stirring (700 rpm), in order to achieve a (*9R*)-HSA final concentration of 6% (w/w). Twenty minutes after (*9R*)-HSA addition, NPs were formed and the protein solution was freeze-dried into powder (yield = 89.6% (w/w)).

Albumin nanoparticles synthesis by desolvation procedure (@HSAr)

Albumin recombinant, HSAr, (10 mg) was dissolved in H_2O milliQ, (1 mL) and stirred at room temperature for 2 h. Nanoparticles were formed by continuous addition of ethanol (4xH_2O milliQ volume) under

permanent stirring at room temperature. After protein desolvation, glutaraldehyde, 8% (0.4 μL x mg of albumin) was added for particles stabilization by cross-linking. Particles were stirred for 24 h and then purified by 3 cycles of centrifugation (12000) with filter devices (MWCO: 100 kDa) to remove ethanol, excess of glutaraldehyde and free albumin. At each cycle, particles were redispersed in 1 mL of water. Therefore, the purified functionalized protein solution was freeze-dried with sucrose (0.5 mg/mL solution) into powder.

(*9R*)-9-HSA or d9-HSA loaded albumin nanoparticles 9R@HSAr, *d*9@HSAr

HSAr was dissolved in H_2O milliQ (1mg/mL) and stirred at room temperature for 2 h. A solution of (*9R*)-9-HSA (or deuterated d9-HSA) in ethanol (5 mg/mL) were slowly added to the albumin solution under vigorous magnetic stirring (700 rpm), in order to achieve a (*9R*)-9-HSA (or d9-HSA) final concentration of 6% wt. Twenty minutes after (*9R*)-9-HSA addition (or d9-HSA), NPs were formed and the protein solution was freeze-dried with sucrose (0.5 mg/mL solution) into powder.

(*12R*)-12-HSA or d9-HSA loaded keratin nanoparticles 12R@ker, *d*9@ker

Keratin powder (9 mg) was dissolved in H_2O milliQ (9 mL) at room and the solution was then sonicated for 30 minutes and filtered (0.45 µm) in order to remove possible aggregates. 0.1 mL of (*12R*)-12-HSA (or deuterated, d9-HSA) in ethanol (5 mg/mL) were slowly added to the keratin solution under vigorous magnetic stirring (700 rpm). Twenty minutes after (*12R*)-12-HSA (or d9-HSA) addition, NPs were formed, preliminarily analyzed by dynamic light scattering (DLS), followed by lyophilization.

Table S1. Hydrodynamic diameter, polydispersity index (P.I.) and ζ-potential of 9R@ker at different pH values.

pH	*Diameter (nm)*	*P.I.*	*ζ (mV)*
7.4	273 ± 2	0.218 ± 0.014	-42.17 ± 1.2
5.5	247 ± 5	0.213 ± 0.006	-25.80 ± 0.32
4.5	205 ± 11	0.182 ± 0.008	-26.90 ± 0.31

Table S2. Kinetic parameters of 9R release from 9R@ker nanoparticles.

Sample	**Zero-Order** $Q_t = K_0 t + C_0$			**First-Order** $Q_t = Q_0 \exp(-k_f^t)$		**Higuchi** $Q_t = k_H \sqrt[2]{t} + C_H$	
	K_0	C_0	R^2	K_f	R^2	K_H	R^2
9R@ker (pH 7.4)	1.2±0.1	11±2	0.537	0.05±0.01	0.311	4.1±0.6	0.766
9R@ker (pH 5.5)	0.4±0.1	10±2	0.874	0.03±0.01	0.480	9.9±0.3	0.986

Sample	**Korsmeyer-Peppas** $Q_t = K_{KP} t^n$			**Peppas-Sahlin** $Q_t = k_1 t^{0.45} + k_2 t^{0.90}$			
	K_{KP}	n	R^2	K_1	K_2	m	R^2
9R@ker (pH 7.4)	8.8±0.6	0.53±0.02	0.980	8.5±0.8	0.5±0.2	0.45	0.974
9R@ker (pH 5.5)	8±1	0.36±0.05	0.838	8.1±0.7	0.5±0.2	0.45	0.887

Table S3. Hydrodynamic diameter, polydispersity index (P.I.) and drug loading of the synthesized nanoparticles.

Entries	*NPs*	*Diameter (nm)*	*P.I.*	*Notes*
1	@ker	199 ± 8 nm	0.15 ± 0.045	
2	FITC@ker	120 ± 9 nm	0.20 ± 0.05	17 $\mu g_{FITC}/mg_{ker}$
3	FITC:(*9R*)-9-HSA@ker	131 ± 1 nm	0.12 ± 0.01	17 $\mu g_{FITC}/mg_{ker}$ 6% w/w of (*9R*)-9-HSA
4	d9-HSA@ker	302 ± 7 nm	0.22 ± 0.01	10% w/w of (*9R*)-9-HSA
5	(*12R*)-12-HSA@ker	235 ± 1 nm	0.19 ± 0.01	10% w/w of (*12R*)-12-HSA
6	@HSA_r	208 ± 1 nm	0.14 ± 0.004	
7	(*9R*)-9-HSA@HSA_r	201 ± 7 nm	0.24 ± 0.03	10% w/w of (*9R*)-9-HSA
8	d9-HSA@HSA_r	190 ± 3 nm	0.22 ± 0.007	5.7% w/w of d9-HSA

LC-MS/MS analysis. The HT29 cells treated with *d*9@ker and *d*9@HSAr for 2 h and were extracted by using the Folch method[47] and analysed by liquid chromatography tandem mass spectrometry. Stock standard solution was prepared by dissolving, *d*9R and at a concentration of 2 mg/mL in methanol. Further dilutions for the calibration curve were prepared daily in methanol:0.05% acetic acid in water (70:30), (v/v). Samples were solubilized with 500 μl of methanol:0.05% acetic acid in water (70:30), (v/v), sonicated and filtered with a 0.45 μm syringe filter. Calibration (concentration range 0.22-22 μg/mL) was done by linear regression with a 1/x weighting; and analyte concentration in each sample was back calculated by the interpolation on the regression curve.

LC-MS/MS analysis was carried out using a Series200 modular HPLC (PerkinElmer) interfaced with an API 4000-QTrap triple quadrupole (AB-Sciex Framingham, MA, USA) with an electrospray ionization (ESI) source. Chromatographic analysis was performed with a Sunfire C18 column (2.1x100 mm, 3.5μm; Waters) using the following mobile phases: water with 0.05% acetic acid (A) and methanol with 0.05% acetic acid (B). The gradient elution started with 70% of solvent B, reached 90% of solvent B in 15 minutes, remained at 90% for 5 minutes and then returned to 70% of solvent B in ten minutes. The column temperature was set at 30°C. The injection volume was 2 μL and the flow rate was 0.2 mL/min.

MS parameters were optimized by infusing standard solutions at a concentration of 2.2 μg/ml into the TurboIonSpray1 source operating in negative ion mode by a syringe infusion pump.

The optimized parameters of the ion source were: 600°C probe temperature, 30 psi curtain gas, 40 psi nebulizing gas, 40 psi heating gas, -4000 kV ion spray voltage. For quantitative analysis mass chromatograms were acquired in multiple reaction monitoring (MRM) in double. Parent ion / daughter ion mass-to-charge quantitative and qualitative transition for *d*9R were 300.1/282.3 and 300.1/156.1 *m/z*, de-clustering potential, entrance potential, collision energy and cell exit potential for quantitative and qualitative transitions were: 70V, -10V, -35eV, -10V and -70V, -10V, -35eV, -8V, respectively. Data processing and quantitation were performed by Analyst software (AB-Sciex).

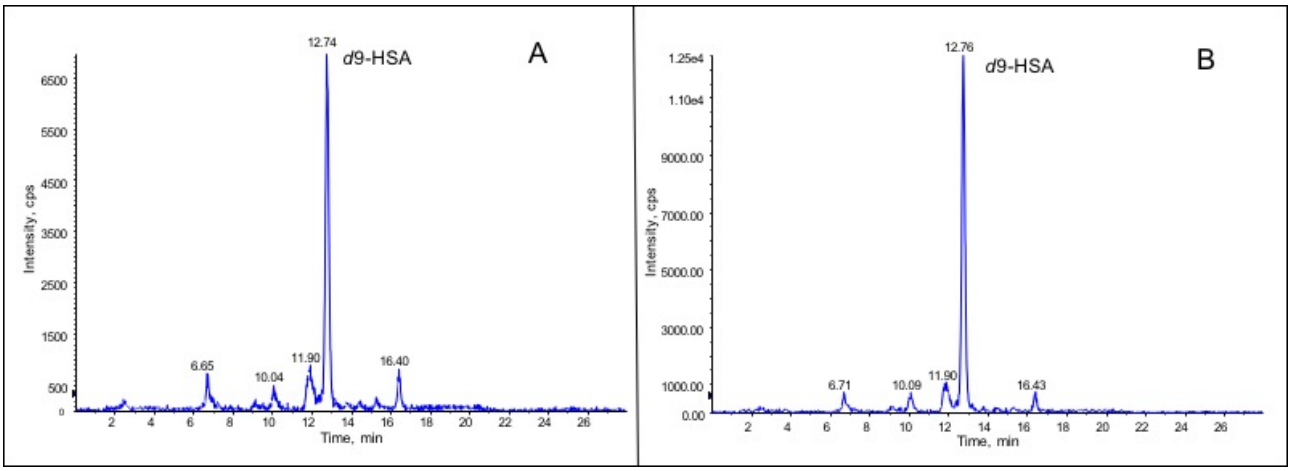

Figure S1 LC-MS chromatograms of HT29 cell extracts, acquired in MRM mode (transition 300.1/282.3). (A) *d*9-HSA from 9*d*@HSAr; (B) *d*9-HSA-d from 9*d*@ker.

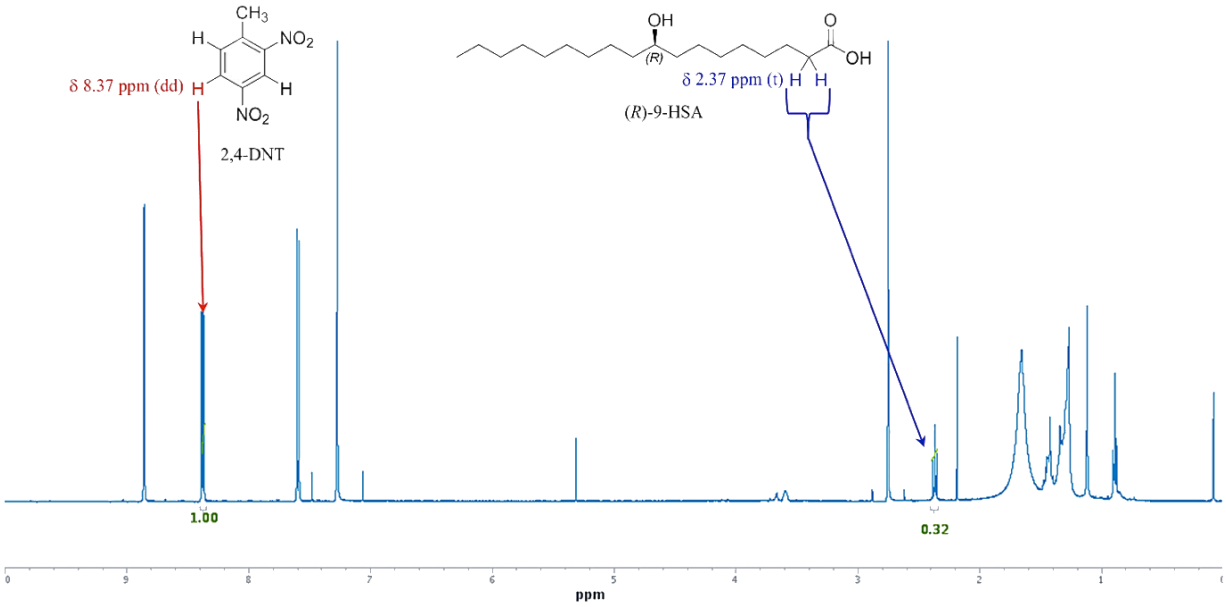

Figure S2. ^{1}H-NMR spectra of the mixture of 9R and 2,4-DNT in $CDCl_3$