

1 **Supplementary Information**

2 3 **Tunable self-assembled nanogels composed of well-defined thermoresponsive hyaluronic** 4 **acid - polymer conjugates**

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7 8 **Materials.**

9 The sample of bacterial sodium hyaluronate (HA) with a weight-average molecular weight of 200 kg/mol was
10 supplied by ARD (Pomacle, France). The molecular weight distribution and the weight-average molecular
11 weight of this HA sample were determined by size exclusion chromatography using a Waters GPC Alliance
12 chromatograph (USA) equipped with a differential refractometer and a light scattering detector (MALLS) from
13 Wyatt (USA); the solution was injected at a concentration of 5×10^{-4} g/mL in 0.1 M NaNO₃. The polydispersity
14 index (PDI) of the sample is $M_w/M_n \sim 1.5$. 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid, di(ethylene
15 glycol) methyl ether methacrylate (DEGMA), oligo(ethylene glycol) methyl ether methacrylate (OEGMA, $M_n =$
16 475 g mol^{-1}), 2,2'-azobis(2-methylpropionitrile) (AIBN), *N*-(2-aminoethyl)maleimide trifluoroacetate salt (AEM),
17 3-mercaptopropionic acid (MPA) and, all other chemicals were purchased from Sigma-Aldrich-Fluka and were
18 used without further purification. The water used in all experiments was purified by a Elga Purelab purification
19 system, with a resistivity of 18.2 MΩ cm.

20 **Methods.**

21 ¹H NMR spectra were recorded on a Bruker spectrometer operating at 400 MHz with deuterium oxide (D₂O) or
22 deuterated chloroform (CDCl₃) (SDS, Vitry, France) as a solvent. Two-dimensional HSQC experiments were
23 acquired using 2K data points and 256 time increments. The phase sensitive (TPPI) sequence was used and
24 processing resulted in a 1K×1K (real-real) matrix. Chemical shifts are given relative to external tetramethylsilane
25 (TMS = 0 ppm) and calibration was performed using the signal of the residual protons or carbons of the solvent
26 as a secondary reference. Fourier transform infrared spectroscopy (FT-IR) measurements were performed on a
27 RX1 spectrometer (Perkin Elmer, UK) with horizontal ATR accessory. For each sample, 32 scans were recorded
28 between 4000 and 400 cm⁻¹ with a resolution of 2 cm⁻¹ using Spectrum software V 5.0.0. The spectrum analysis
29 was performed by using Origin 7.0 software. The molecular weight and polydispersity index of the block
30 copolymer was measured by size-exclusion chromatography (tetrahydrofuran, flow rate: 1 mL/min) at 40 °C
31 with a Waters 600 liquid chromatograph equipped with a differential refractometer and a light scattering
32 detector (MALLS) from Wyatt. The transmittance of the polymer solutions at 500 nm was measured on a Varian
33 Cary 50 UV/Vis Spectrophotometer equipped with a temperature controller. Pyrene emission spectra were
34 measured on a Perkin Elmer luminescence LS 50B spectrometer between 360 and 500 nm. Pyrene solubilized in
35 ethanol was added up to a concentration of 10⁻⁷ M in the polymer solution and excited at 334 nm. The I_1/I_3 ratio
36 of the intensities of the first and the third peaks of fluorescence spectrum of pyrene was used to study the
37 formation of hydrophobic domains resulting from the association of the copolymer chains. Dynamic light
38 scattering (DLS) experiments were carried out using an ALV laser goniometer, which consists of a 22 mW
39 HeNe linear polarized laser operating at a wavelength of 632.8 nm, an ALV-5000/EPP multiple τ digital
40 correlator with 125 ns initial sampling time, and a temperature controller. The accessible scattering angles range
41 from 50° to 140°. The aqueous solutions of the HA-poly(DEGMA-OEGMA) conjugates (0.5 g/L in PBS) were

1 filtered directly into the glass cells through 0.22 μm MILLIPORE Millex LCR filter. Data were collected using
2 digital ALV Correlator Control software and the counting time for measuring the elastic or the quasi-elastic
3 scattering intensities varied for each sample from 180 to 300 s. The relaxation time distributions, $A(t)$, were in
4 the sequence obtained using CONTIN analysis of the autocorrelation function, $C(q,t)$. Diffusion coefficients D
5 were calculated from following equation:

$$6 \quad \frac{\Gamma}{q^2} \Big|_{q \rightarrow 0} = D \quad (1)$$

7 where Γ is relaxation frequency ($\Gamma = \tau^{-1}$), and q is the wave vector defined as following equation:

$$8 \quad q = \frac{4\pi n}{\lambda} \sin\left(\frac{\theta}{2}\right) \quad (2)$$

9 where λ is the wavelength of the incident laser beam (632.8 nm), θ is the scattering angle, and n is the refractive
10 index of the media. Consequently, the hydrodynamic radius (R_h) was calculated from the Stokes-Einstein
11 relation as follows:

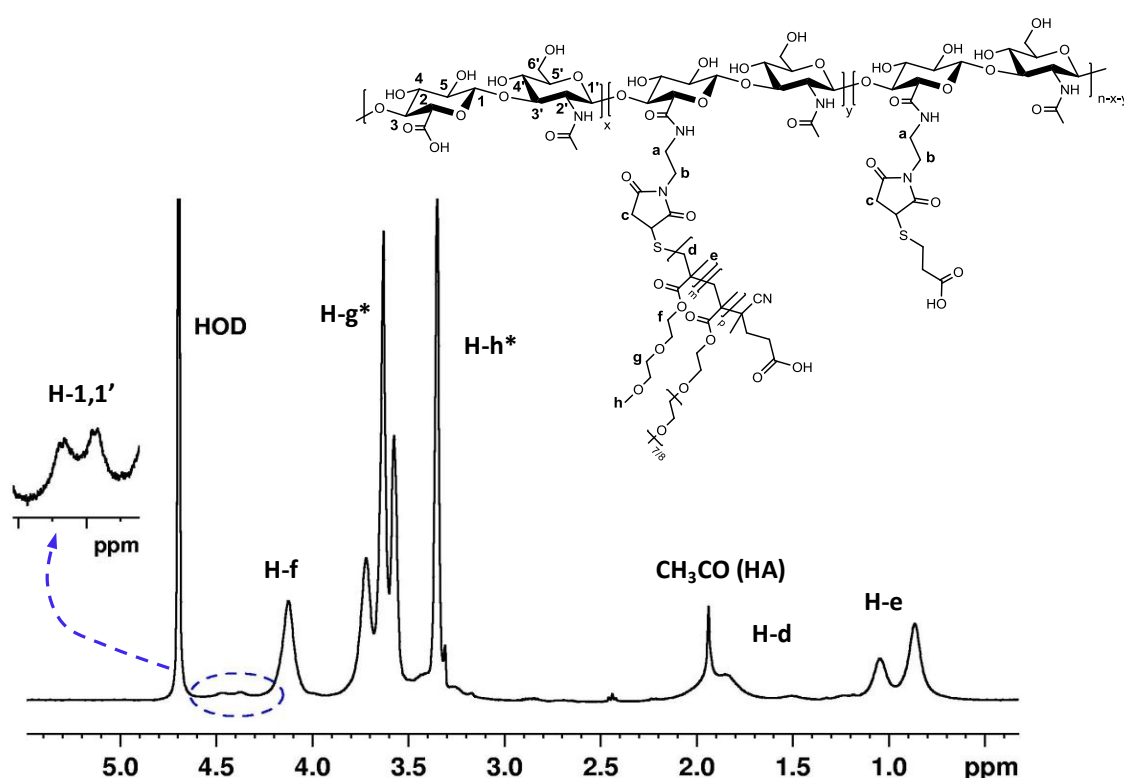
$$12 \quad R_h = \frac{k_B T}{6\pi\eta\Gamma} q^2 = \frac{k_B T}{6\pi\eta D} \quad (3)$$

13 where k_B is the Boltzmann constant, T is the temperature, and η is the viscosity of the medium. Confocal Laser
14 Scanning Fluorescence Microscopy (CLSM) was performed on a Leica SP5 confocal microscope equipped with
15 a 63x oil immersion objective.

16 **Synthesis of poly(DEGMA-co-OEGMA).** A mixture of 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid
17 (0.114 g, 0.4066 mmol), DEGMA (8 g, 42.50 mmol), OEGMA (1.06 g, 2.23 mmol) and AIBN (0.004 g, 0.024
18 mmol) was dissolved in toluene (20 mL) and N_2 gas was purged for 30 min to the solution. The reaction vessel
19 was sealed and then put into a pre-heated oil bath at 80 $^\circ\text{C}$ for 21 h 30. The resulting mixture was precipitated in
20 cold diethyl ether (200 mL) to give the random copolymer as a waxy liquid in 82 % yield (6.56 g). SEC (THF)
21 M_n : 16900 g/mol. PDI: 1.1. ^1H NMR (400 MHz, CDCl_3) δ (ppm) : 7.47, 7.38 (H of aromatic ring), 4.11
22 (CH_2OCO), 3.69-3.57 (CH_2O), 3.40 (CH_3O), 1.92-1.63 (CH_2), 1.26-0.88 (CH_3).

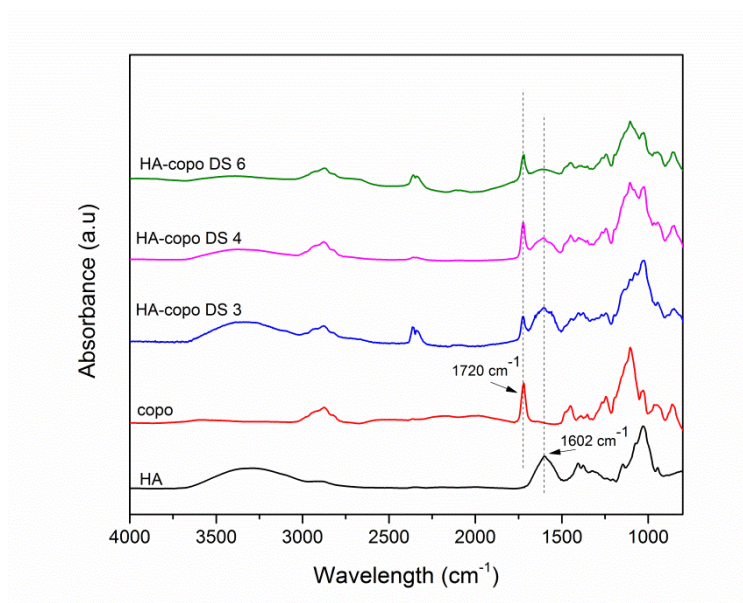
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24 **Synthesis of HA-poly(DEGMA-co-OEGMA).** The first step consisted in the aminolysis reaction of
25 poly(DEGMA-co-OEGMA) with *n*-butylamine, which leads to thiol-capped copolymer chains. Thus, the
26 copolymer (1 g) was introduced in a flask placed under a nitrogen atmosphere. CH_2Cl_2 (10 mL) was added, and
27 after complete dissolution of the polymer, *n*-butylamine (10 mL, 0.1 mol) was added to the solution. The
28 reaction mixture was stirred for 10 min at room temperature. During this period, the originally pink solution
29 became pale yellow. A waxy liquid (0.80 g) was recovered by precipitation in diethyl ether. The thiol-capped
30 copolymer was then reacted with HA-AEM (DS = 10). This derivative was prepared by dissolving HA (0.60 g,
31 1.49 mmol) in pure water to a concentration of 6 g/L. *N*-(2-Aminoethyl)maleimide trifluoroacetate salt (0.285 g,
32 1.12 mmol) and *N*-hydroxysulfosuccinimide sodium salt (0.162 g, 0.75 mmol) were added to this solution. The
33 pH of the reaction medium was then adjusted to 4.75. Next, an aqueous solution of *N*-(3-dimethylaminopropyl)-
34 *N'*-ethylcarbodiimide hydrochloride (0.054 g, 0.28 mmol) was added slowly to the mixture. The pH of the
35 reaction medium was maintained at 4.75 by addition of 0.5 M HCl. The reaction was allowed to proceed at room
36 temperature until no further change in pH was observed (i.e. 4 hours). The modified HA was purified by

1 diafiltration through a membrane Amicon YM 30. The diafiltration was stopped when the filtrate conductivity
2 was lower than 10 μ S. Next, the solution of the HA-AEM derivative was diluted with pure water to obtain a final
3 volume of 360 mL, and divided into portions of 60 mL (i.e. into portion of 0.10 g of HA-AEM). After addition
4 of a 10-fold concentrated solution of PBS (6.6 mL) to one of the portions, poly(DEGMA-co-OEGMA) (0.412 g,
5 0.024 mmol) was added and the mixture was stirred at 4 °C (pH 7.4) for 12 h. After addition of MPA (21 μ L,
6 0.24 mmol) and stirring again at 4 °C for 6 h, the HA-poly(DEGMA-co-OEGMA) was purified by diafiltration
7 through an membrane Amicon YM 30. The product was then recovered by freeze-drying as a white powder in
8 (0.340 g) with a DS in copolymer chains of 6 from 1 H NMR integration.
9 The synthesis of the other poly(DEGMA-co-OEGMA) samples with DS=3 and 4 was performed using a similar
10 procedure, using different amounts of the copolymer, i.e. 0.206 and 0.288 g, respectively, per HA-AEM portion.
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14 **Figure S1.** 1 H NMR spectrum (400 MHz, 6 mg/mL in D_2O , 25 °C) of HA-poly(DEGMA-co-OEGMA), DS = 6.
15 *These signals mask the H-2, H-2', H-3, H-3', H-4, H-4', H-5, H-5' and H-6 proton signals of HA.

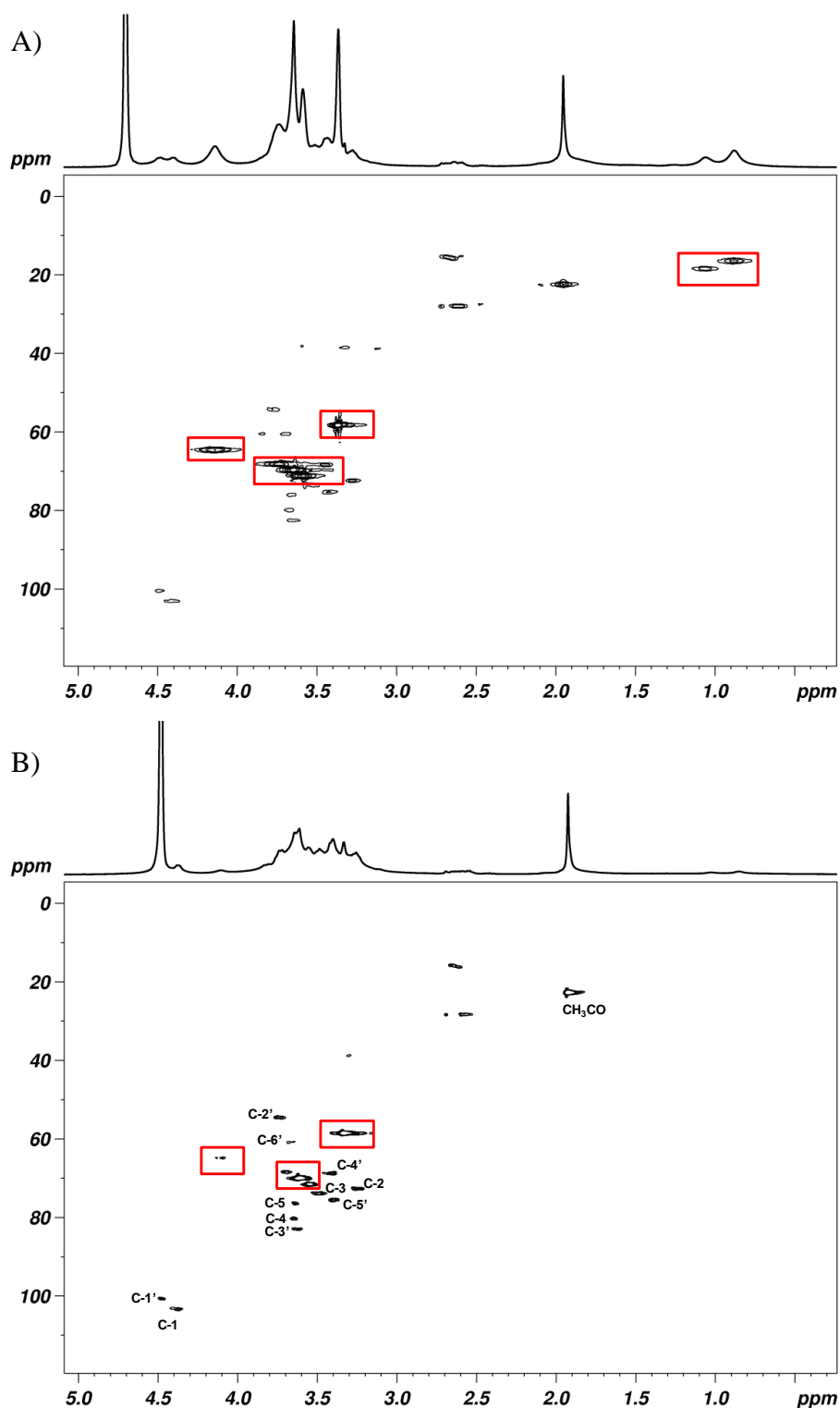
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Figure S2. FT-IR spectra of initial HA, poly(DEGMA-co-OEGMA) and of the HA-copolymer with DS = 3, 4 and 6.

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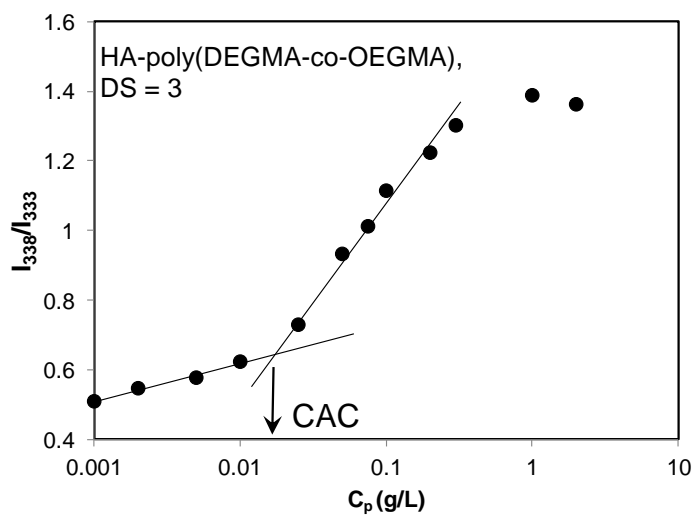


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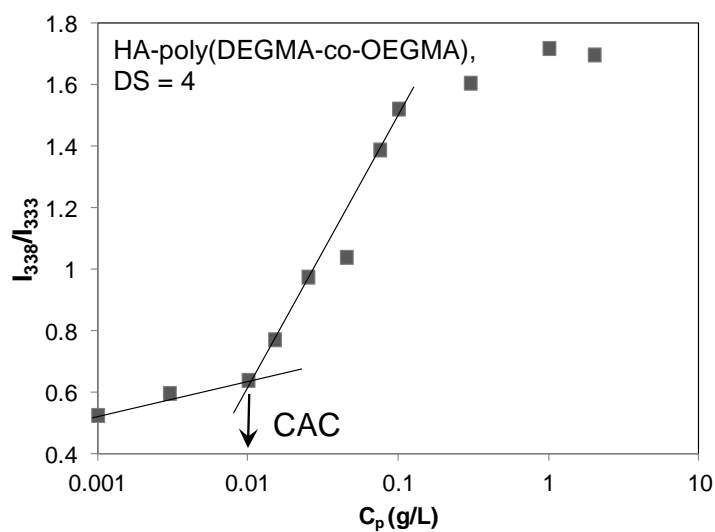
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4 **Figure S3.** 1D ^1H NMR and 2D ^1H - ^{13}C HSQC NMR spectra (400/100 MHz, 20 mg/mL in D_2O) of HA-
5 poly(DEGMA-co-OEGMA) with DS = 3 at 25 °C (A) and 40 °C (B). The HA protons located in the δ 3.1-3.6
6 ppm region, which were masked by the copolymer proton signals in the 1D spectrum at 25 °C, became clearly
7 visible at 40°C due to the decrease of the proton signals of the copolymer. The assignment of the carbon signals
8 of HA is given in spectrum B and the cross-peaks corresponding to the copolymers are marked in red.

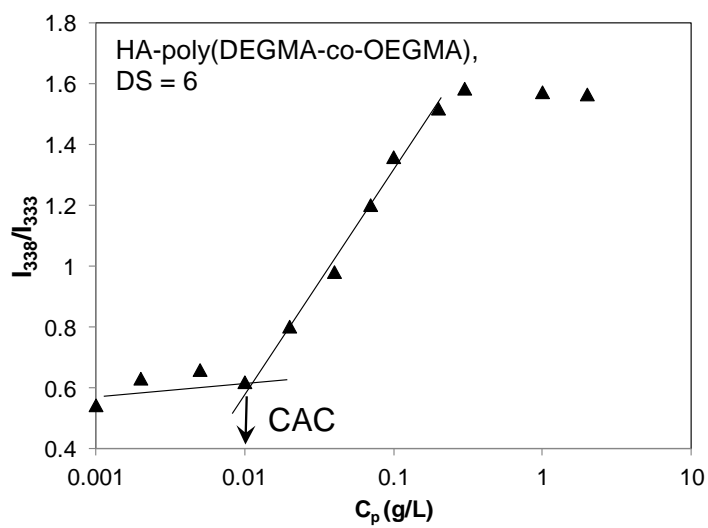
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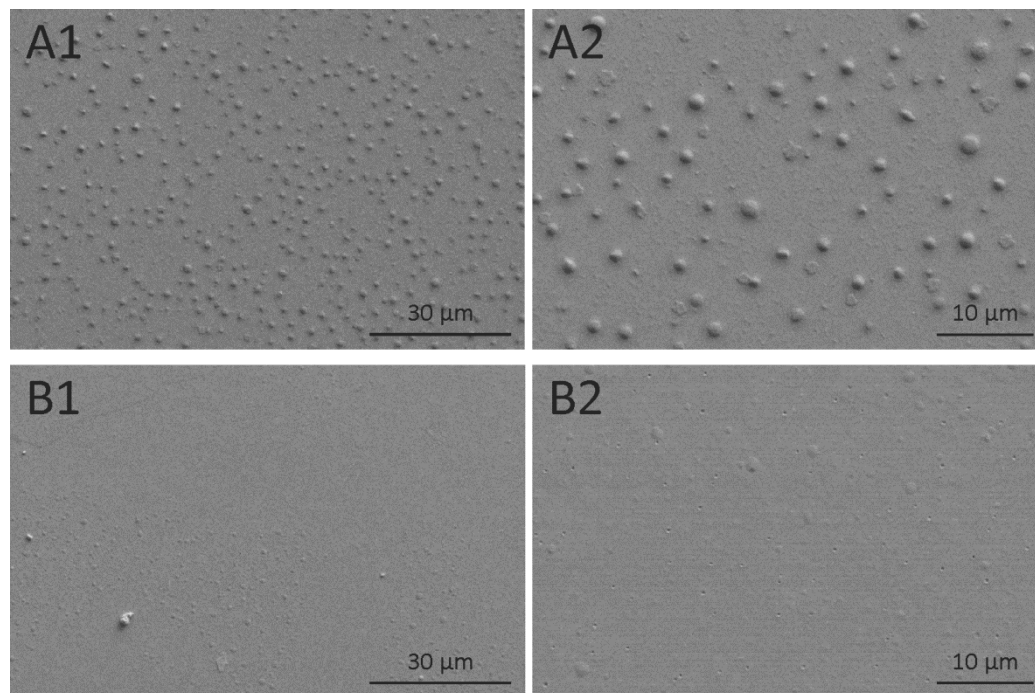
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Figure S4. Plots of the intensity ratios of I_{338}/I_{333} for three different HA-poly(DEGMA-co-OEGMA) from pyrene excitation spectra as a function of concentration.

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2 **Scanning electron microscopy observation of nanogels.**
3 Drops of solutions in pure water of respectively poly(DEGMA-co-OEGMA) modified HA and non-modified HA,
4 pre-heated at 45 °C were deposited onto mica-coated copper stubs (also pre-heated at 45 °C) and allowed to air
5 drying at 45 °C. The samples were coated by 2 nm of electron beam evaporation carbon and observed in
6 secondary electron imaging mode with a Zeiss ultra 55 FEG-SEM (CMTC-INPG, Grenoble) at an accelerating
7 voltage of 3 kV, using an in-lens detector.



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9 **Figure S5.** Scanning electron microscopy images of (A) poly(DEGMA-co-OEGMA) modified HA (DS 4) and
10 (B) non-modified HA.
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13 **Encapsulation of hydrophobic molecules in thermosensitive HA Nanogels.**

14 To a solution (0.5 mL) of HA-poly(DEGMA-co-OEGMA) (DS = 3) at 2 g/L at room temperature, a solution of
15 Nile Red in ethanol (1 mg/mL or 3.14 mM) was added to obtain a NR concentration of 10 μM. The mixture was
16 stirred in a water bath at 40 °C and ethanol was allowed to evaporate slowly under a stream of nitrogen.

17 To solutions (0.5 mL) of HA-poly(DEGMA-co-OEGMA) (DS = 3) at 2 g/L at room temperature, a solution of
18 paclitaxel in methanol (1 mg/mL or 1.17 mM) was added to obtain PTX concentrations of 80, 60, 30 and 20 μM.
19 The mixtures were stirred in a water bath at 40 °C and methanol was allowed to evaporate slowly under a stream
20 of nitrogen. The solutions containing PTX at 30 and 20 μM remained clear whereas that containing PTX at 60
21 and 80 μM contained small particles in dispersion. *In vitro* cell experiments were thus conducted using the 30
22 μM sample for which the entrapment efficiency (EE) was found to be 80 %. It was determined by centrifugation
23 (10000 rpm for 10 min) to remove non-encapsulated PTX and quantification by high-performance liquid
24 chromatography (HPLC) of PTX loaded in the nanogels. To this end, PTX was extracted from the nanogels with
25 acetonitrile. Isocratic reverse-phase HPLC was performed using a Nucleodur C18 column at 25 °C. The mobile
26 phase consisted of acetonitrile/water (45:55, v/v) with a flow rate of 0.9 mL/min. The signals were recorded by

1 UV detector at 227 nm. A calibration line was conducted to determine the PTX concentration in the range of
2 0.1–40 μM , and the r^2 -value of peak area against PTX concentration was at least 0.999.

3 The encapsulation efficiency was calculated based on the following equation :

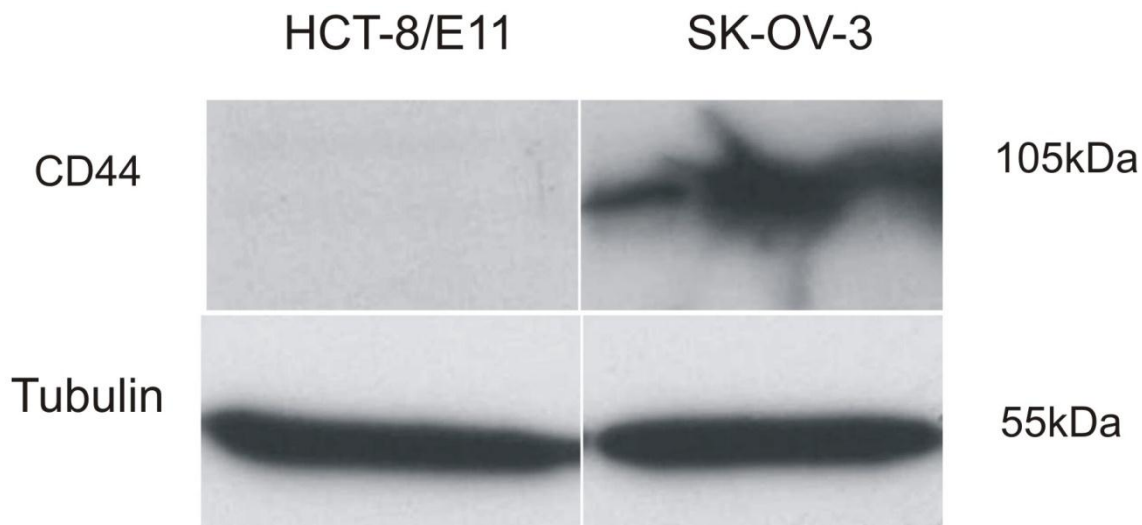
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$$\text{EE (\%)} = \text{weight of PTX in nanogels} / \text{weight of the feeding PTX} \times 100\%$$

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7 ***In vitro* cell culture assays.**

8 **Cell lines.** Human colorectal cancer cell line HCT-8/E11,(Vermeulen, S.J.; Bruyneel, E.A.; Bracke, M.E.;
9 Debruyne, G.K.; Vennekens, K.M.; Vleminckx, K.L.; Berx, G.J.; Vanroy, F.M.; Mareel, M.M. *Cancer Research*
10 1995, **55**, 4722-4728) and human ovarium cancer cell line SKOV-3 (ATCC, Manassas, VA) were maintained in
11 DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and antibiotics.

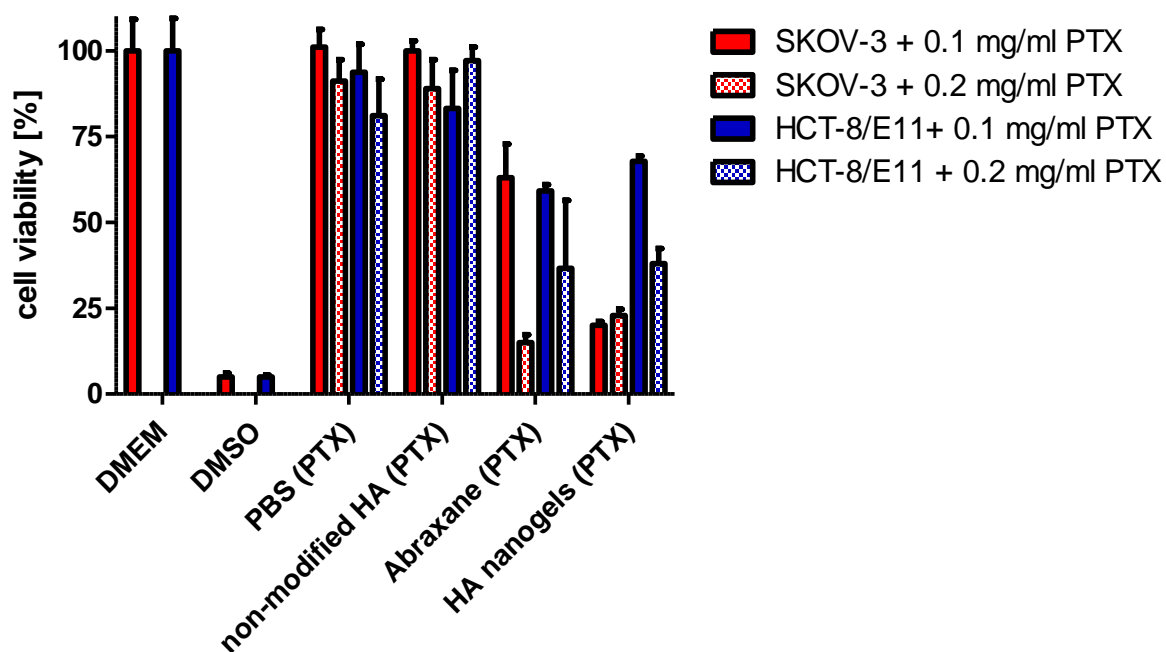
12 **Protein analysis.** For Western blot analysis cell lines were harvested in Laemmli lysis buffer. Cell lysates (25
13 μg) were suspended in reducing sample buffer and boiled for 5 minutes at 95 °C. Samples were run on 8% SDS-
14 PAGE, transferred to PVDF membranes, blocked in 5% non-fat milk in PBS with 0.5% Tween-20, and
15 immunostained with the following primary antibodies: mouse monoclonal anti-CD44s (clone BBA10) (R&D
16 systems, Minneapolis, MN), and mouse monoclonal anti-tubulin (Sigma-Aldrich, St.-Louis, MO). **Figure S4**
17 shows a photograph of the obtained blots.



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19 **Figure S6.** Western blot analysis of cell lysates.

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21 **Cell viability assay.** Cells were seeded at a density of 10000 cells / well into 24-well plates. After 24 h, cells
22 were treated for 2.5 h with PTX dispersed in PBS, PTX mixed with non-modified HA, PTX formulated as
23 Abraxane (purchased from Celgene) and PTX formulated in thermosensitive HA nanogels. The latter
24 formulation was kept at 40 °C to assure the presence of nanogels. The cells were thermostated at 37 °C during
25 the addition of the respective samples, again to assure the HA to be preset as nanogels. All experiments were run
26 in five fold. Cultures were washed and conditioned with fresh medium for 6 days. The effect on cell viability
27 was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) metabolic
28 viability assay. This is a sensitive, quantitative and reliable assay that measures the conversion of the yellow
29 MTT substrate into a dark blue formazan salt by cellular dehydrogenases. Optical densities were obtained by
30 measuring the dissolved formazan crystals with a spectramax paradigm multi-mode microplate detection

1 platform (Molecular devices, Sunnyvale, CA) at 570 nm. **Figure S5** shows a resume of the cell viability data
2 obtained from adding different concentrations of PTX.



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4 **Figure S7.** Cell viability of SKOV-3 and HCT/8E-11 cells treated with controls (no PTX added) and the
5 respective PTX formulations.
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