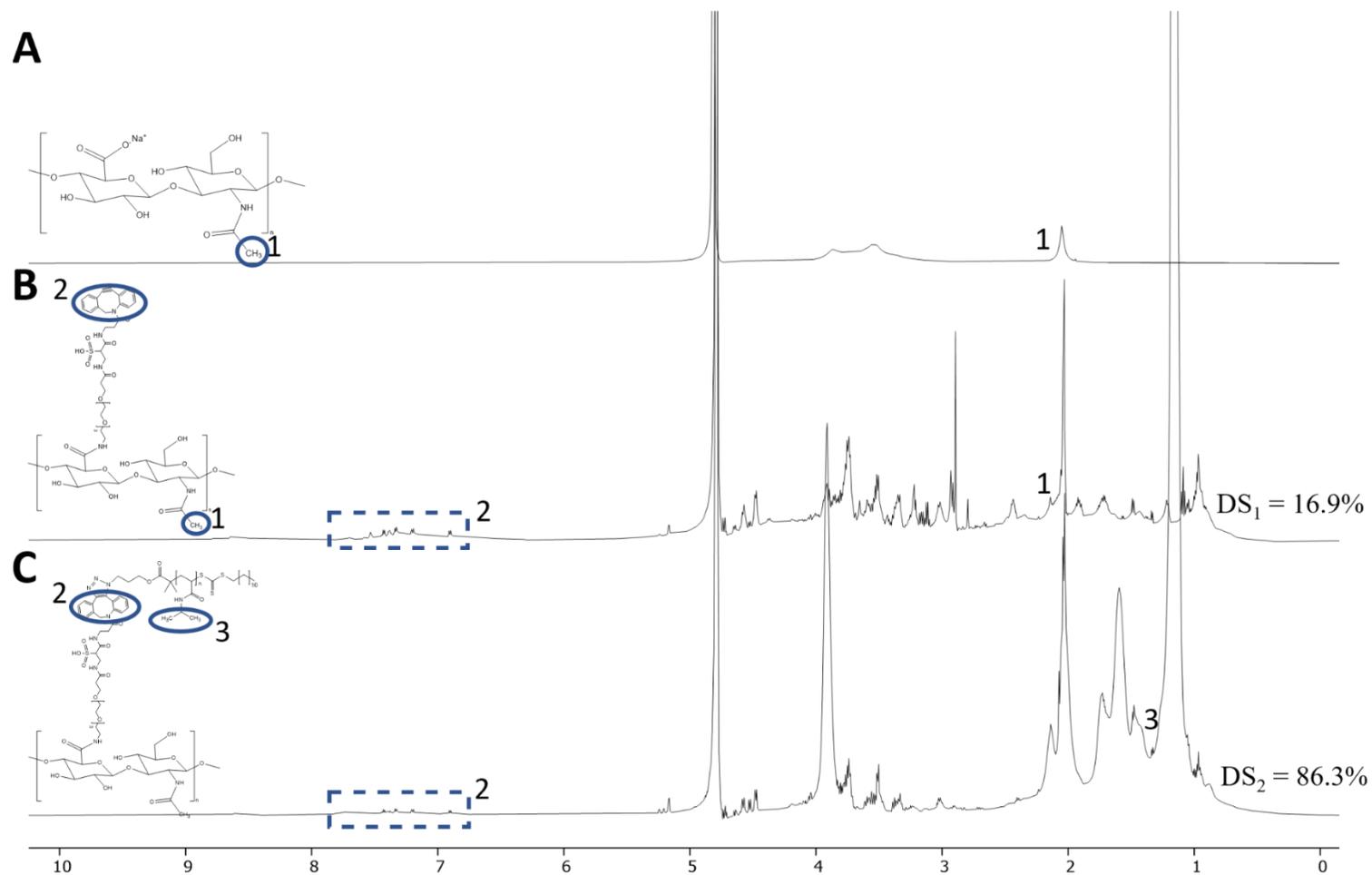


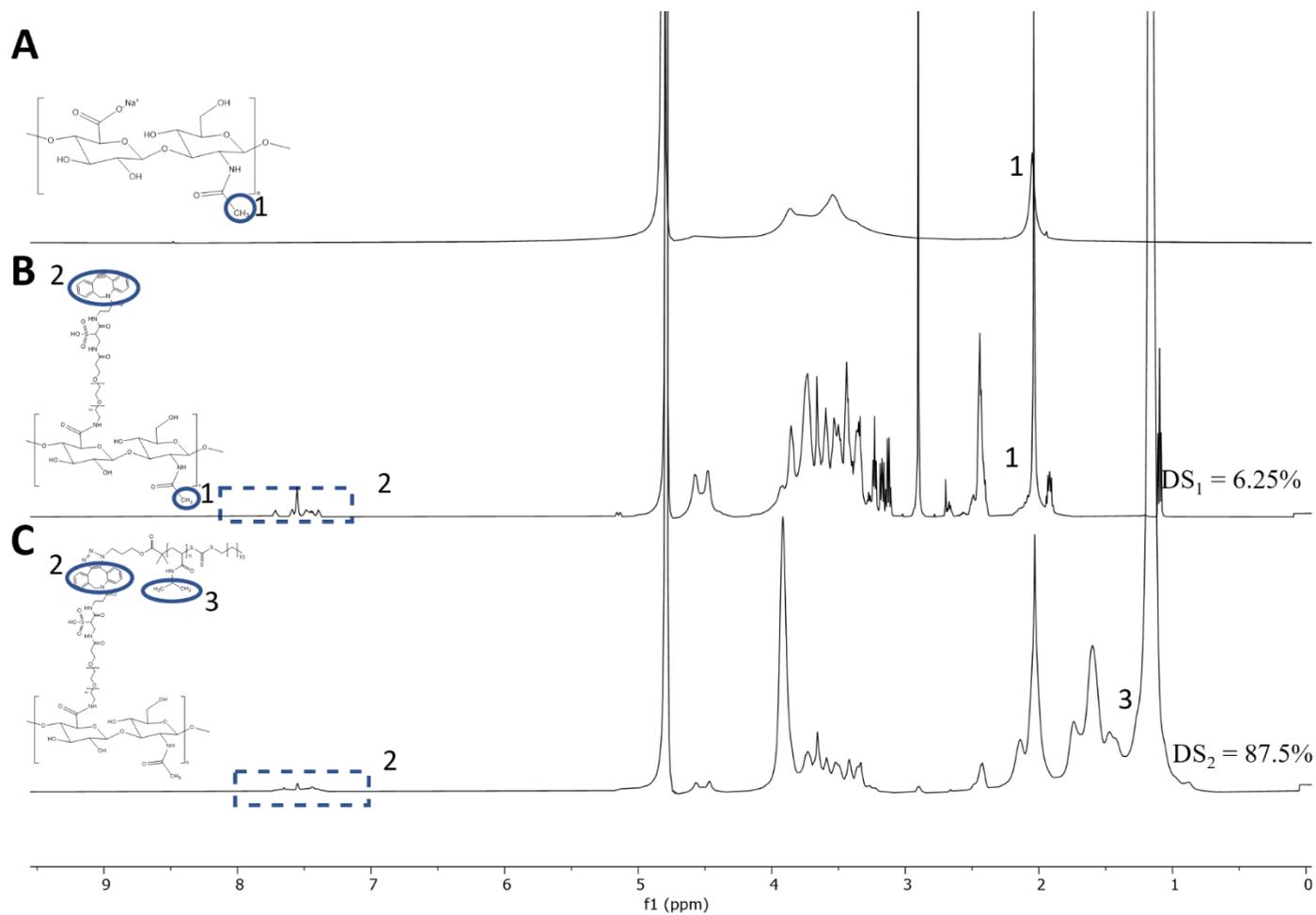
---

# **Supplementary Materials: Nanoforming Hyaluronan-Based Thermoresponsive Hydrogels: Optimized and Tunable Functionality in Osteoarthritis Management**

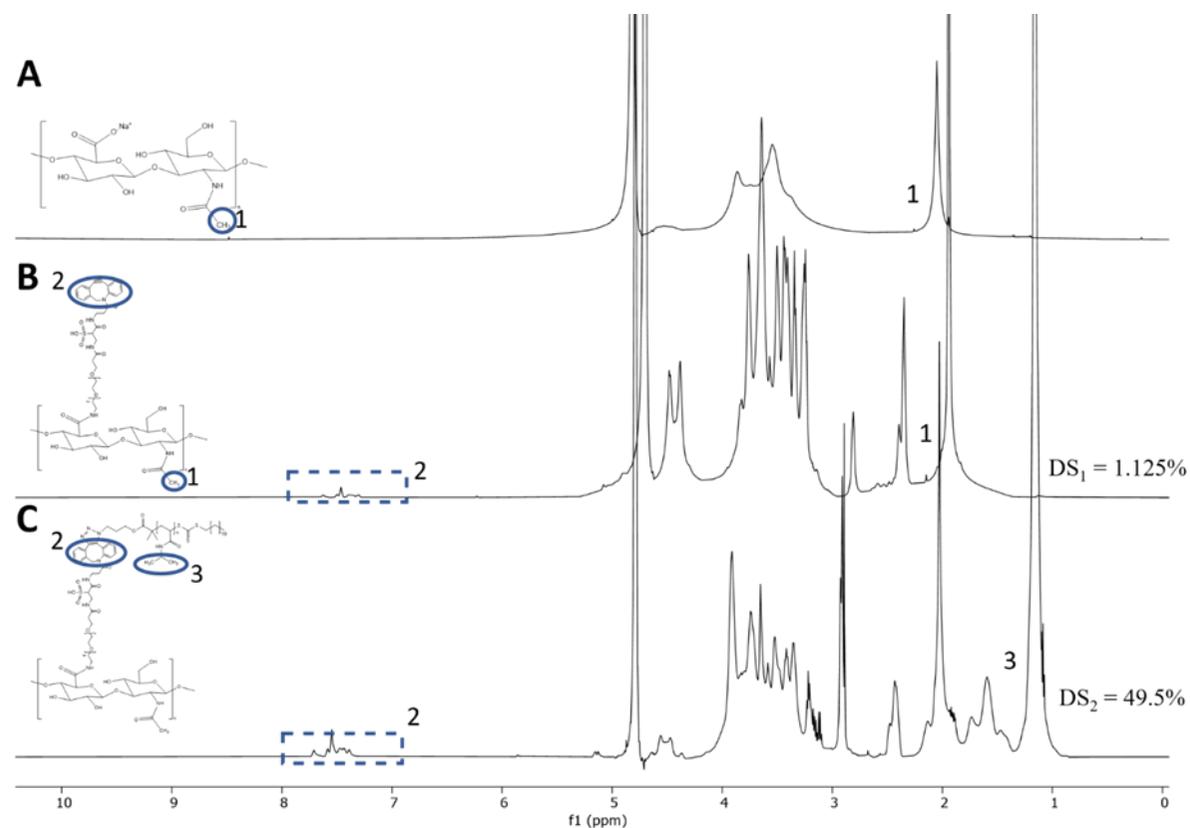
Alexandre Porcello, Paula Gonzalez-Fernandez, Olivier Jordan and Eric Allémann



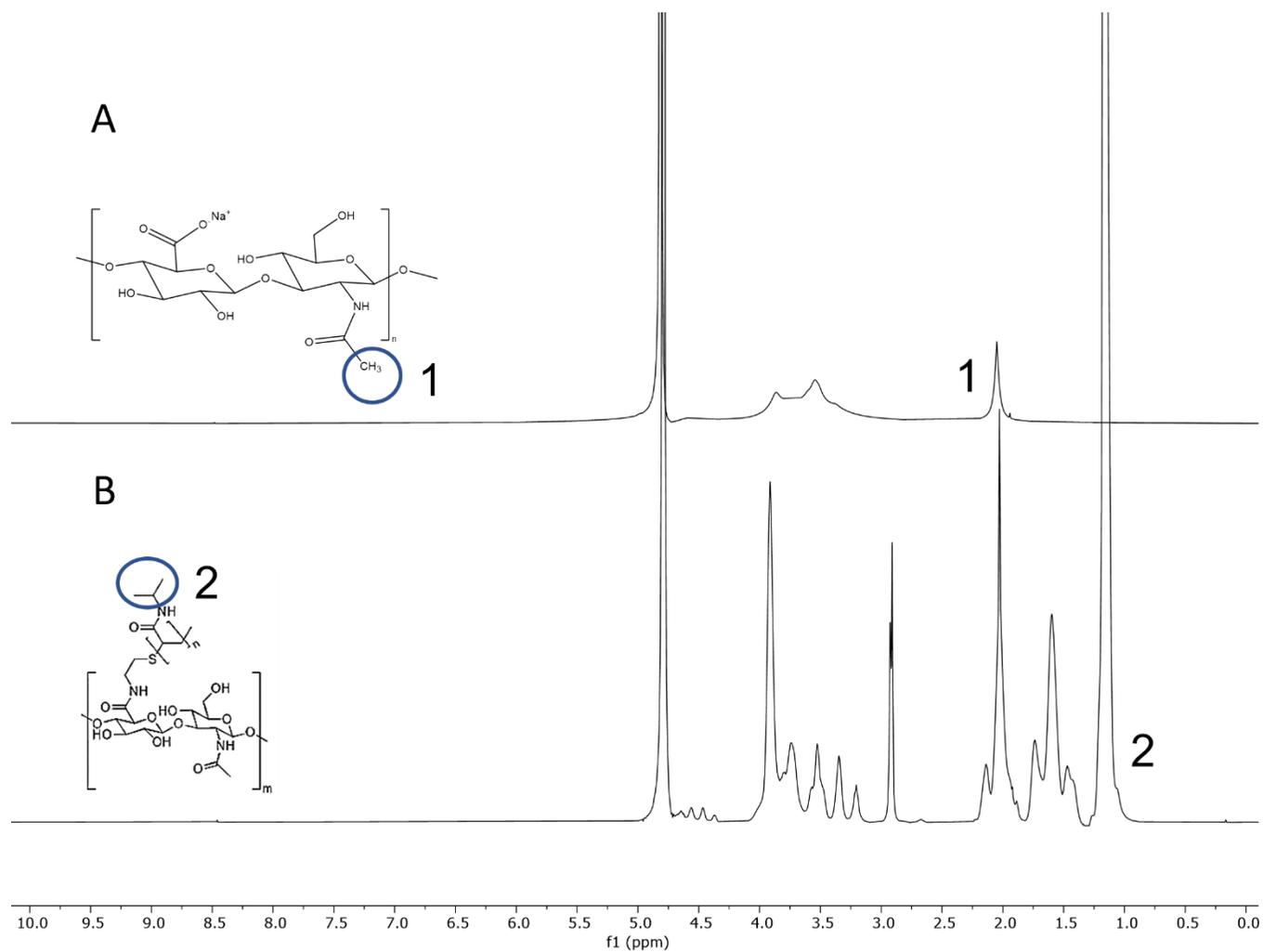
**Figure S1.** <sup>1</sup>H-NMR spectra in D<sub>2</sub>O of HA (A), HA - Sulfo DBCO-PEG4 with the DS1 (B), and HA-L-PNIPAM 0.5 with the DS2 (C). For B and C, the polymers were submitted to hyaluronidase before NMR (see methods). The specific assignments of the proton NMR resonance peaks are  $\delta = 2.0$  ppm (1), 4.7–2.7 ppm (HA-backbone/PEG-DBCO), 6.8–7.7 ppm (2), 1.1 ppm (3). DS<sub>1</sub> (HA - Sulfo DBCO-PEG4) = 16.9%. DS<sub>2</sub> (HA-L-PNIPAM 0.5) = 86.3%.



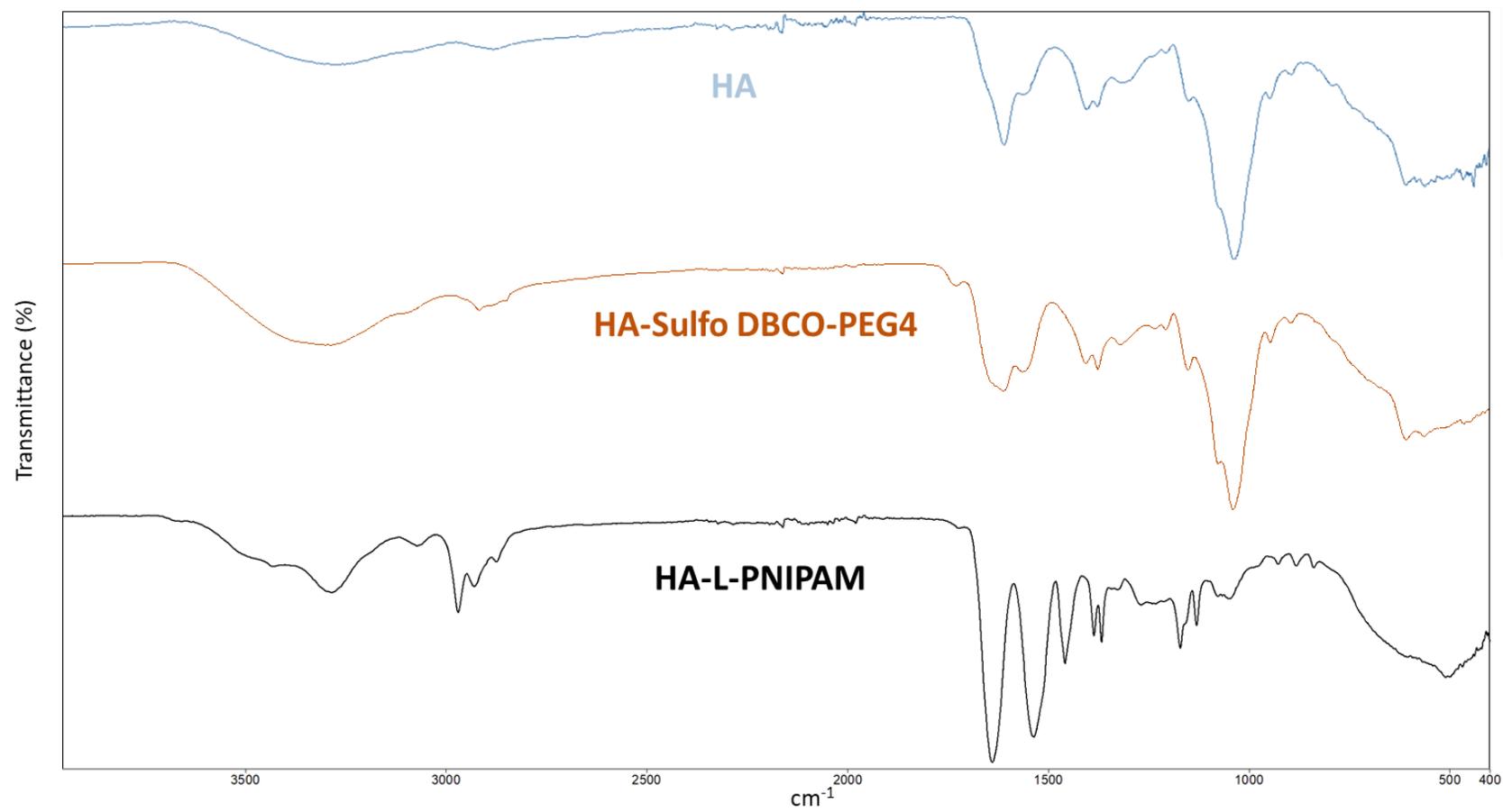
**Figure S2.** <sup>1</sup>H-NMR spectra in D<sub>2</sub>O of HA (A), HA - Sulfo DBCO-PEG4 with the DS<sub>1</sub> (B), and HA-L-PNIPAM 0.5 with the DS<sub>2</sub> (C). For B and C, the polymers were submitted to hyaluronidase before NMR (see methods). The specific assignments of the proton NMR resonance peaks are  $\delta = 2.0$  ppm (1), 4.7–2.7 ppm (HA-backbone/PEG-DBCO), 6.8–7.7 ppm (2), 1.1 ppm (3). DS<sub>1</sub> (HA - Sulfo DBCO-PEG4) = 6.25%. DS<sub>2</sub> (HA-L-PNIPAM 0.5) = 87.5%.



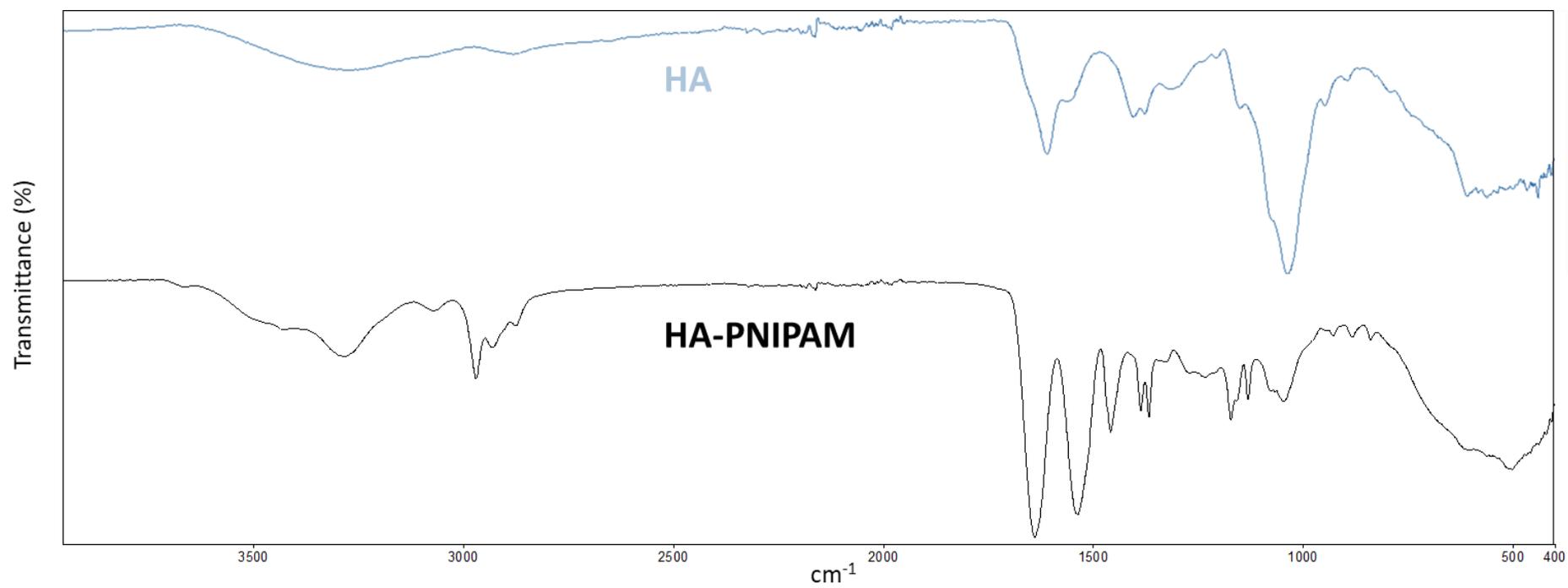
**Figure S3.**  $^1\text{H-NMR}$  spectra in  $\text{D}_2\text{O}$  of HA (A) HA - Sulfo DBCO-PEG4 0.1 (B) and HA-L-PNIPAM 0.1 (C). For B and C, the polymers were submitted to hyaluronidase before NMR (see methods). The specific assignments of the proton NMR resonance peaks are  $\delta = 2.0$  ppm (1), 4.7–2.7 ppm (HA-backbone/PEG-DBCO), 6.8–7.7 ppm (2), 1.1 ppm (3). DS (HA - Sulfo DBCO-PEG4) = 1.125%. DS (HA-L-PNIPAM 0.1) = 49.5%.



**Figure S4.** <sup>1</sup>H-NMR spectra in D<sub>2</sub>O of HA (A) and HA - PNIPAM (B). The specific assignments of the proton NMR resonance peaks are  $\delta = 2.0$  ppm (1) (i.e., acetamido moiety of the N-acetyl-D-glucosamine residue of HA), 1.1 ppm (2) (i.e., methyl protons of PNIPAM).



**Figure S5.** ATR/FT-IR spectra recorded on HA (blue curve), HA-Sulfo DBCO-PEG4 (orange curve) and HA-L-PNIPAM 0.5 (black curve).



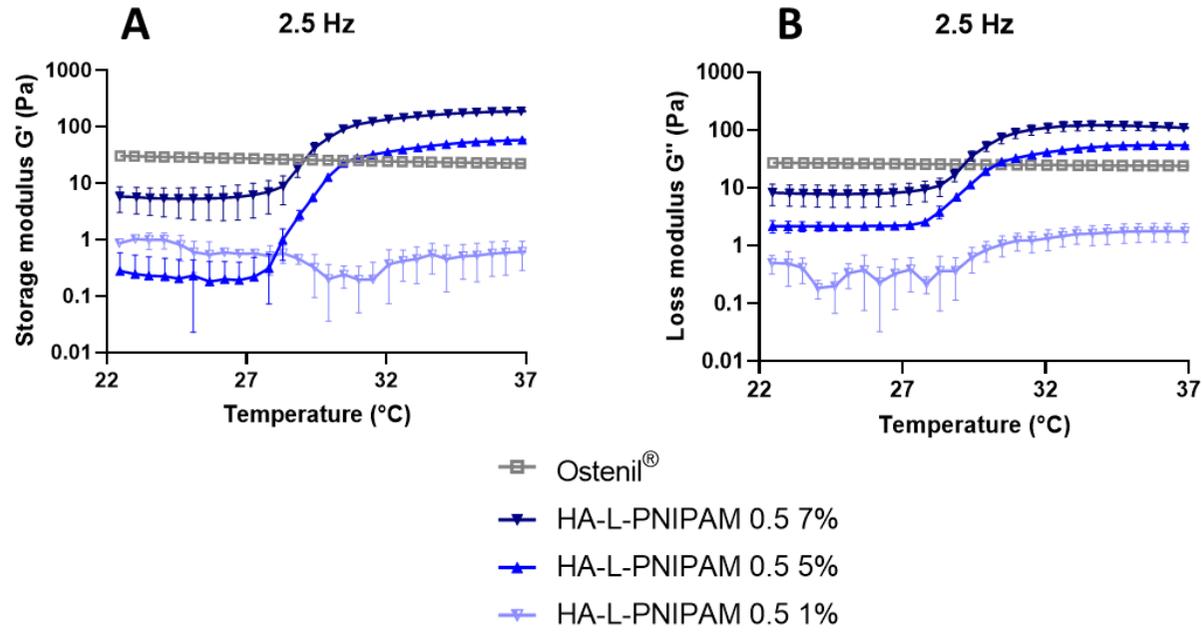
**Figure S6.** ATR/FT-IR spectra recorded on HA (blue curve) and HA-PNIPAM (black curve).

**Table S1.** Degrees of substitution (DS) of the intermediate HA - Sulfo DBCO-PEG4 (DS1) for the formulations of HA-L-PNIPAM determined by UV-vis spectroscopy at 307 nm.

<b>Formulation</b>	<b>DS<sub>1</sub> (307 nm)</b>
HA-L-PNIPAM 0.5	13.8
HA-L-PNIPAM 0.25	6.2
HA-L-PNIPAM 0.1	0.9

**Table S2.** Degrees of substitution (DS) of HA-PNIPAM determined by UV-vis spectroscopy at 286 nm.

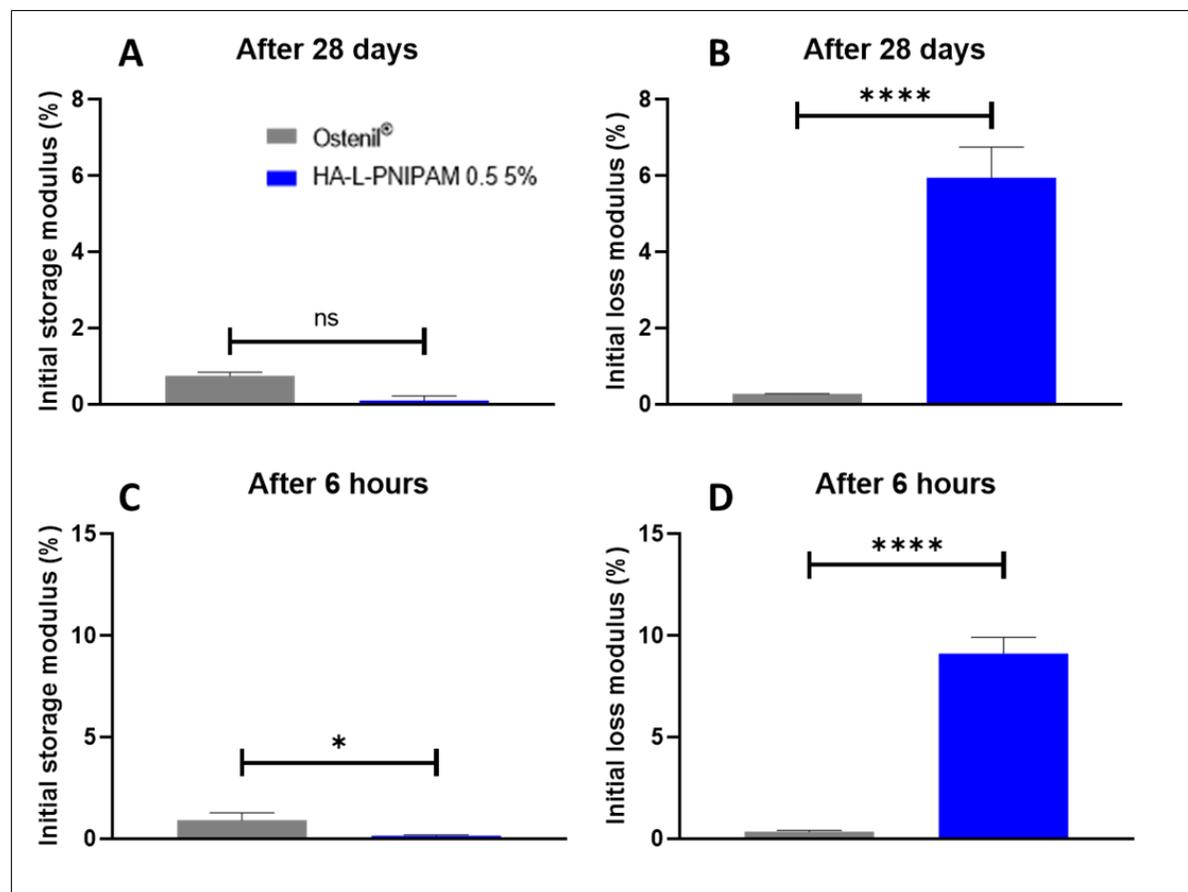
<b>Formulation</b>	<b>DS (286 nm)</b>
HA-PNIPAM	8.0



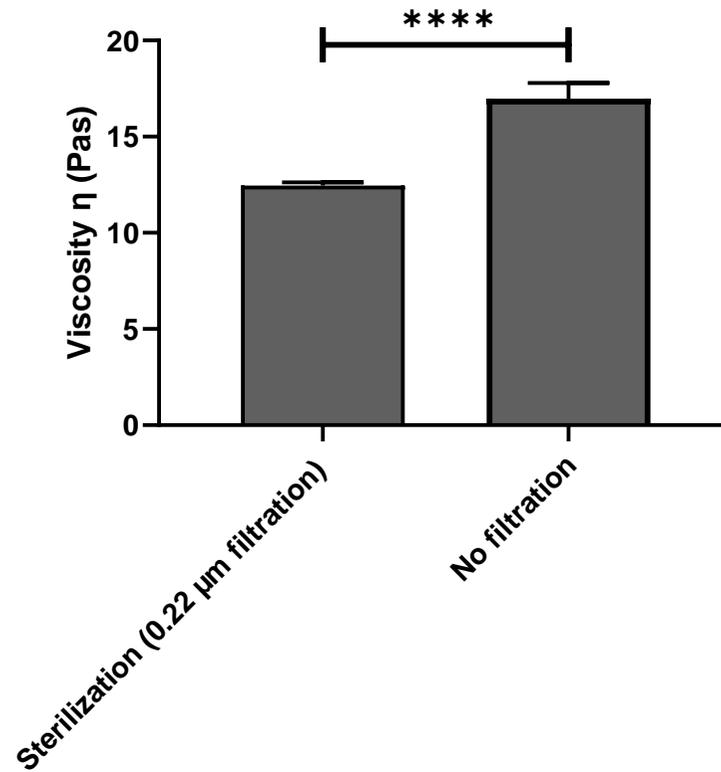
**Figure S7.** Temperature dependence of the storage modulus ( $G'$ ) and loss modulus ( $G''$ ) at a frequency of 2.5 Hz of Ostenil<sup>®</sup>, HA-L-PNIPAM 0.5 1 %, 5 %, and 7 % ( $n = 3; \pm \text{SD}$ ) (A and B).

**Table S3.** Summary of viscoelastic properties obtained in rheology for Ostenil® and different HA-L-PNIPAM 0.5 concentrations at 25 °C and 37°C. HA contents for Ostenil® were calculated according to values obtained in <sup>1</sup>H NMR spectroscopy.

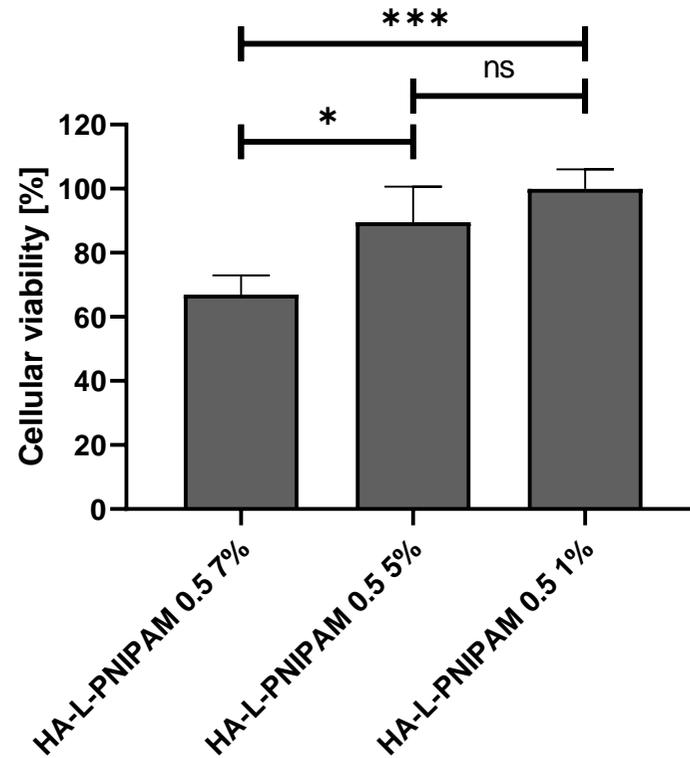
Viscosupplement	HA content (mg/mL)	Temperature (°C)	Frequency 2.5 Hz	
			G' (Pa)	G' (Pa)
Ostenil®	10.0	25	28.52 ± 1.1	28.52 ± 1.1
		37	22.1 ± 0.67	22.1 ± 0.67
HA-L-PNIPAM 0.5 7%	9.9	25	5.28 ± 3.03	5.28 ± 3.03
		37	189.51 ± 21.31	189.51 ± 21.31
HA-L-PNIPAM 0.5 5%	7.1	25	0.23 ± 0.21	0.23 ± 0.21
		37	59.23 ± 5.97	59.23 ± 5.97
HA-L-PNIPAM 0.5 1%	1.4	25	0.68 ± 0.27	0.68 ± 0.27
		37	0.63 ± 0.33	0.63 ± 0.33



**Figure S8.** Ostenil® and HA-L-PNIPAM 0.5 5 % initial storage modulus ( $G'$ ) and initial loss modulus ( $G''$ ), after 28 days of storage, at 0.5 Hz and 37°C and after 4 additions of 10  $\mu$ l of hyaluronidase (50 U/mL) (A and B). Ostenil® and HA-L-PNIPAM 0.5 5% initial storage modulus ( $G'$ ) and initial loss modulus ( $G''$ ), as function of time at 0.5 Hz and 37°C, after an addition of 40  $\mu$ l  $H_2O_2$  (30% w/w) (E and F) and after 6 hours (C and D). Significant difference (i.e.,  $p < 0.05$ ) was evidenced by one asterisk (“\*”). Highly statistically significant differences (i.e.,  $p < 0.0001$ ) was evidenced by four asterisks (“\*\*\*\*”).  $n = 3 \pm SD$



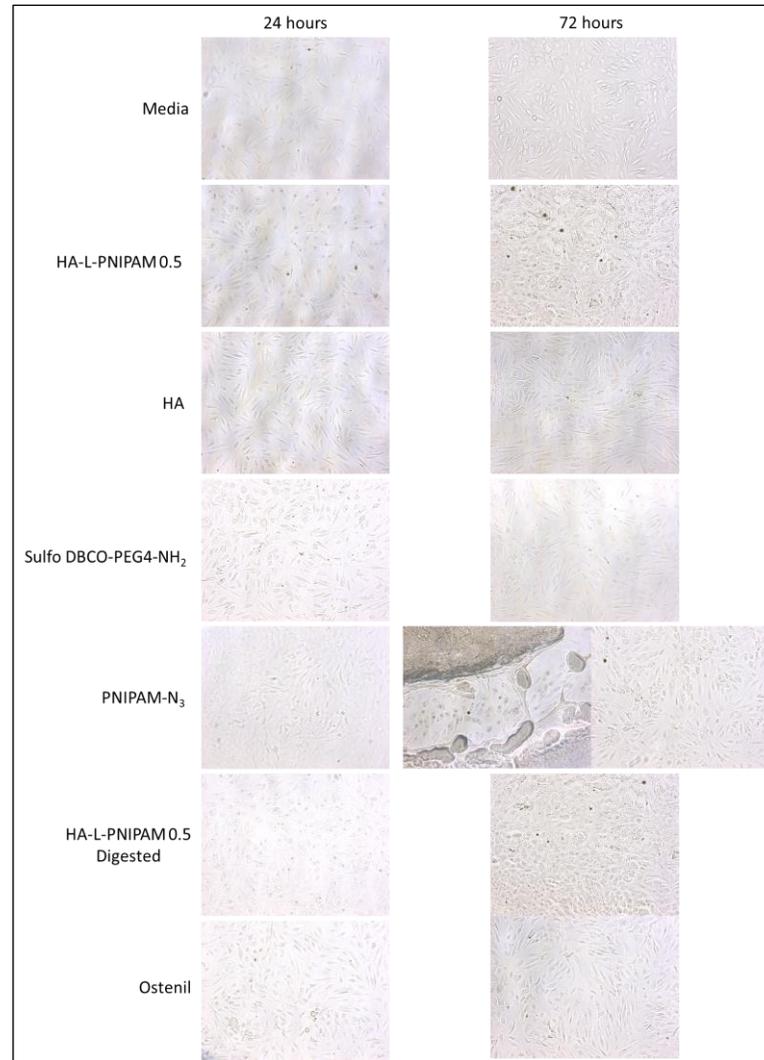
**Figure S9.** Viscosity of HA-L-PNIPAM 0.5 5% under a constant shear stress of  $0.1 \text{ s}^{-1}$  at  $37 \text{ }^\circ\text{C}$  before and after  $0.22 \text{ }\mu\text{m}$  filtration. Highly statistically significant differences (i.e.,  $p < 0.0001$ ) was evidenced by four asterisks (“\*\*\*\*”).  $n = 3 \pm \text{SD}$



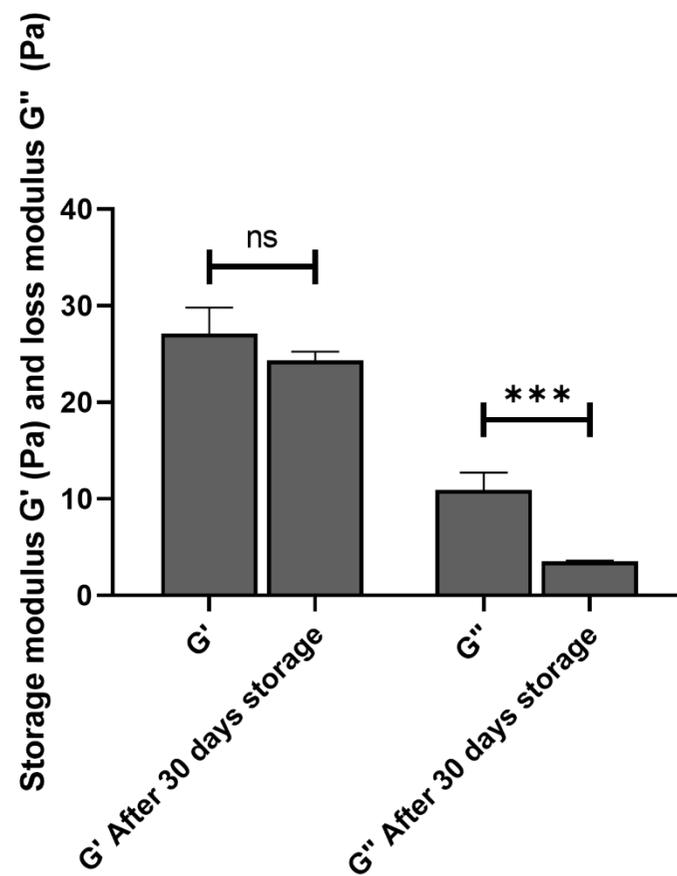
**Figure S10.** The cellular viability of human fibroblast like synoviocytes (HFLS) incubated with HA-L-PNIPAM 0.5 formulations at 1 %, 5 % and 7 % (w/v) after 24 h. Significant difference (i.e.,  $p < 0.05$ ) was evidenced by one asterisk (“\*”). Highly statistically significant differences (i.e.,  $p < 0.001$ ) was evidenced by three asterisks (“\*\*\*”).  $n = 4 \pm SD$

**Table S4.** Results of viability obtained by Countess® after 0.4% trypan blue staining of human fibroblast-like synoviocytes (HFLS) incubated with HA-L-PNIPAM 0.5 5%, HA-L-PNIPAM 0.5 5% digested by hyaluronidase, Hyaluronic acid 0,71 % (2200–2400 kDa), Sulfo DBCO-PEG4-NH<sub>2</sub> 0.21 %, PNIPAM-N<sub>3</sub> 4.08 % (15'000 Da) and Ostenil® after 24 h and 72 h. (n = 3; ± SD).

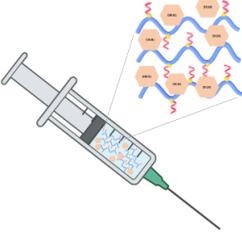
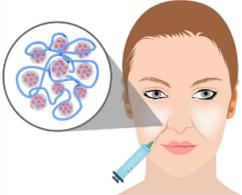
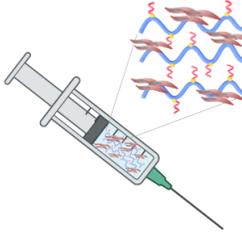
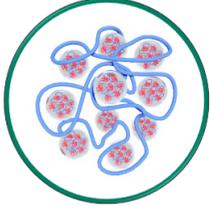
Compounds	Viability	
	24 hours	72 hours
Media	100 ± 0	99.3 ± 0.5
HA-L-PNIPAM 0.5	98.7 ± 1.2	93.6 ± 9.3
HA	99.3 ± 1.2	98.7 ± 1.2
Sulfo DBCO-PEG4-NH <sub>2</sub>	98.7 ± 1.2	90 ± 13
PNIPAM-N <sub>3</sub>	96.0 ± 5.3	78 ± 16.0
HA-L-PNIPAM 0.5 Digested	96.0 ± 2.6	81 ± 18.2
Ostenil®	96.6.23 ± 4.1	85 ± 6.1



**Figure S11.** Aspects of the cells after 24 hours and 72 hours of incubation with the different products.



**Figure S12.** HA-L-PNIPAM 0.5 5% storage and loss modulus after 30 days of storage at 4 °C. Highly statistically significant differences (i.e.,  $p < 0.001$ ) was evidenced by four asterisks ("\*\*\*\*").  $n = 3 \pm \text{SD}$ .

	<b>Musculoskeletal indications</b>	<b>Drug delivery system</b>	<b>Aesthetic indications</b>	<b>Therapeutic cell delivery</b>	<b>Other applications</b>
					
<b>Identified specific applications</b>	<ul style="list-style-type: none"> <li>• Viscosupplementation</li> <li>• Volumetric supplementation</li> <li>• Tendinous tissue disorders</li> </ul>	<ul style="list-style-type: none"> <li>• Small drug</li> <li>• Protein</li> <li>• Peptide</li> </ul>	<ul style="list-style-type: none"> <li>• Facial dermal filler</li> <li>• Body filler</li> <li>• Cosmetics</li> </ul>	<ul style="list-style-type: none"> <li>• Living cells</li> <li>• Cellular derivatives</li> </ul>	<ul style="list-style-type: none"> <li>• Stress urinary incontinence</li> </ul>
<b>Main advantages</b>	<ul style="list-style-type: none"> <li>✓ Resistance against hyaluronidases</li> <li>✓ Resistance against ROS</li> <li>✓ High <i>in situ</i> viscosity</li> </ul>	<ul style="list-style-type: none"> <li>✓ Injectability</li> <li>✓ <i>In situ</i> forming implant</li> </ul>	<ul style="list-style-type: none"> <li>✓ Ease of injection</li> <li>✓ Thinner needle</li> <li>✓ Resistance against hyaluronidases</li> <li>✓ Resistance against ROS</li> <li>✓ High <i>in situ</i> viscosity</li> </ul>	<ul style="list-style-type: none"> <li>✓ Ease of injection</li> <li>✓ Viability after extrusion</li> </ul>	<ul style="list-style-type: none"> <li>✓ Ease of injection</li> <li>✓ <i>In situ</i> forming implant</li> </ul>

**Figure S13.** Summary of possible future application for HA-L-PNIPAM.