Multifunctional carrier based on Halloysite/Laponite hybrid hydrogel for kartogenin delivery

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Experimental

Halloysite nanotubes used in this study are from Sigma Aldrich. Laponite RD ($Na_{0.7}^{+}(Si_8Mg_{5.5}Li_{0.3})O_{20}(OH)_4]^{0.7-}$) is a Rockwood product. All the materials were used without further purification.

Thermogravimetric (TG) analyses were performed by using a Q5000 IR apparatus (TA Instruments) under nitrogen flow of 25 cm^3 min^{-1} for the sample and 10 cm^3 min^{-1} for the balance. The explored temperature interval ranged between 25 and 600 °C at a heating rate of 20 °C min^{-1}. The mass of each sample was ca. 5 mg. The calibration was carried out by means of Curie temperature of standards (nickel, cobalt, and their alloys). FT-IR spectra (KBr) were recorded with an Agilent Technologies Cary 630 FT-IR spectrometer. Specimens for these measurements were prepared by mixing 5 mg of the sample powder with 100 mg of KBr.

Dynamic light scattering (DLS) measurements were carried out by means of a Zetasizer NANO-ZS (Malvern Instruments). The field-time autocorrelation functions were fitted by Laplace transformation, which provided an intensity-weighted apparent hydrodynamic radius (R_h). In detail, the fitting of the field-time autocorrelation functions provided the decay rate (Γ) of the diffusive mode. For the translational motion, the collective diffusion coefficient at a given concentration is $D_t = \Gamma/q^2$ where q is the scattering vector given by $4\pi n\lambda^{-1} \sin(\theta/2)$, with n being the water refractive index, λ the wavelength (632.8 nm), and θ the scattering angle (173°). The apparent hydrodynamic radii were calculated by using the Stokes−Einstein equation as $R_h = k_bT/(6\pi\eta)$, k_b being the Boltzmann constant, T the absolute temperature, and η the water viscosity.

For TEM observation the samples were prepared using a drop of suspension solution on formvar-coated copper grid (400 mesh) and allowing the drop to dry completely in a vacuum desiccator. The TEM images of the samples were obtained using a Philips TEM CM 100 transmission electron microscope at accelerating voltage = 80 kV.

ζ-potential measurements were carried out by means of a Zetasizer NANO-ZS (Malvern Instruments) at 25.0 ± 0.1 °C using folded capillary cells (DTS1070). The concentration of the dispersions was 10^{-3} wt%.

Synthesis of kartogenin

Compound **1**: HNT/Pd catalyst (3.84 mg, 0.1 mol%), phenylboronic acid (0.547 mmol, 0.99 equiv.), K_2CO_3 (0.615 mmol, 1.12 equiv.), 4-bromoaniline (0.550 mmol, 1 equiv.), ethanol (0.6 mL) and water (0.6 mL) were placed in a microwave test tube provided with a cap. The mixture was inserted into microwave apparatus at a temperature of 120 °C under constant stirring for 10 min. The reaction

mixture was cooled at room temperature and the crude was poured into water and extracted by centrifuge with diethyl ether. The organic layers were dried with $MgSO_4$ and the solvent removed under vacuum and the residue was purified by short flash column chromatography (SiO_2, petroleum ether/ethyl acetate). Conversion was determined by ^{1}H-NMR. Kartogenin: Isobenzofuran-1,3-dione (0.550 g, 3.720 mmol) was dissolved in 35 mL of acetic acid at 60 °C, and then a 1,1′–biphenyl-4-amine (**1**) (0.700 g, 4.140 mmol in 15 mL of acetic acid) solution was added dropwise. The reaction mixture was stirred at 60 °C for 24 h, followed by cooling down at room temperature, whereupon a pale white suspension was formed. The resulting crude product was filtered, and the residue was recrystallized with EtOH to afford pure kartogenin.

Mp: 278-279 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.02-7.99 (m, 2H, C**H**): 7.85-7.81 (m, 2H, C**H**), 7.77-7.73 (m, 2H, C**H**); 7.76-7.56 (m, 2H, C**H**); 7.54-7.47 (m, 4H, C**H**); 7.43-7.39 (m, 1H, C**H**); FT-IR (KBr): 3056 cm^{-1} stretching **H–C**=C; 1787 cm^{-1} stretching HO–**C=O**; 1702 cm^{-1} stretching H_2N–**C=O**); 1494 and 1531 cm^{-1} stretching and bending C=C, respectively; UV-*vis*: maximum absorption band at 261 nm.

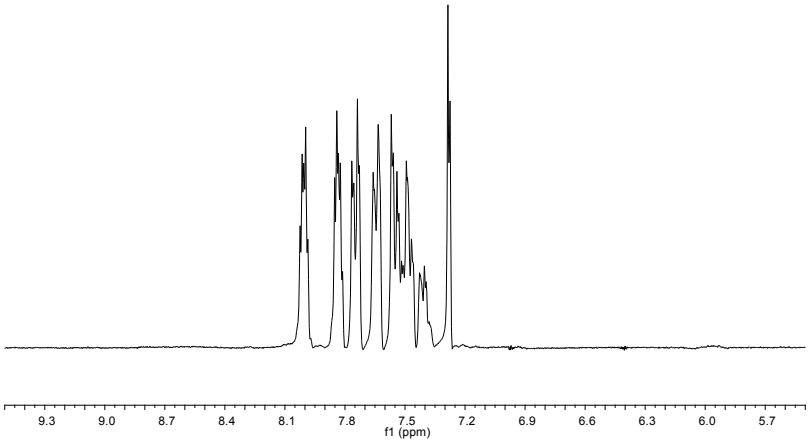


Figure S.1. ^{1}H-NMR spectrum of kartogenin.

Loading of kartogenin on HNT nanomaterials.

To a dispersion of p-HNT in deionized water (5 mL), 1 mL of kartogenin solution 10^{-2} M in $CHCl_3$ was added. The suspension was sonicated for 5 min, at an ultrasound power of 200 W and at 25 °C and then was evacuated for 3 cycles. The obtained dispersion was left under stirring for 24 h at room temperature. After this time, the powder was washed with water and then dried at 60 °C under vacuum. The loading efficiency was calculated according to the original concentration of kartogenin and the

concentration of unloaded molecule quantified by means of UV-*vis* spectrophotometry at the monitoring wavelength of 261 nm. The loading percentage of KGN into HNT lumen was also calculated by dividing the mass of the encapsulated molecules by the mass of the molecules-loaded into HNT nanomaterials**.**

Preparation of gels.

Pure gels were prepared by weighing into a screw-capped sample vial (diameter 1.5 cm) the amount of laponite and solvent (~ 1 g). The mixture was first dispersed for 5 minutes with ultrasound irradiation and left at room temperature until a gel was obtained. Hybrid gels were prepared by weighing into a screw-capped sample vial (diameter 1.5 cm) the amount of laponite, halloysite and solvent (~ 1 g). The mixture was first dispersed for 5 minutes with ultrasound irradiation and subsequently left at room temperature until a gel was obtained.

Rheological measurements.

Rheology measurements were recorded at room temperature on ARES G2 (TA Instruments) strain-controlled rheometer using a plate-plate (PP 25-2) tool, the sample was placed between the shearing plates of the rheometer. Rheological properties, such as strain sweep and frequency sweep, were recorded three times on three different aliquots of gels. Strain sweeps were carried at angular frequency of 10 rad/s and frequency sweeps at strain of 0.25%. These values were chosen to be within the linear viscoelastic region (LVR) of gels.

Thixotropic and Sonotropic Behaviour.

The gel phases obtained were subjected to two different external stimuli. The mechanical stimulus was involved by stirring the gel phase with a stirring bar of 8 mm of length and 3 mm of height at 1000 rpm for 5 min. The sonotropic behaviour of the gel phases was tested by irradiating in an ultrasound water bath for 5 min with a power of 200 W and a frequency of 45 kHz. Thereafter, the materials were stored at room temperature overnight.

When the samples were stable to the tube-inversion test, the gels were defined as thixotropic or sonotropic.

Kinetic Release.

The release of KGN from the HNT/KGN hybrids was performed as follows: 20 mg of the sample were dispersed in 1 mL of phosphate buffer pH 7.4 and transferred into a dialysis membrane (Medicell International Ltd MWCO 12-14000 with diameter of 21.5 mm). The membrane was then put in a round bottom flask containing 9 mL of the release medium at 37 °C and stirred.

At fixed times, 1 mL of the release medium was withdrawn and analyzed. To keep constant the volume of the release medium 1 mL of fresh solution was added each time to replace the withdrawn one.

KGN release from gel matrix.

Hybrid gels obtained in PBS at 2 wt % of Lap and 5 wt% of HNT/KGN ([KGN] = 1.13×10^{-3} M) were prepared, as discussed above, in a total volume of 5 mL. 5 mL of the gelation solvent were casted on gel matrix. The release kinetic was carried out at 37 °C.

At fixed intervals of time, 250 μL of supernatant solution were taken out to be spectrophotometrically analysed controlling KGN peak at 261 nm, and simultaneously refilled with other 250 μL of the same solvent pre-warmed at 37 °C. In this way, alterations of the final concentration of kartogenin on supernatant solution were minimized.

Synovial fluid extraction

Signed informed consent from all participating subjects was obtained. Synovial fluid samples were extracted aseptically with ultrasound guidance from the knee joint of four patients with OA.

KGN release in synovial fluid

The release of KGN from the HNT/KGN hybrids in synovial fluid was performed as follows: 5.8 mg of the sample were dispersed in 0.5 mL of synovial fluid and transferred into a dialysis membrane (Medicell International Ltd MWCO 12-14000 with diameter of 21.5 mm). The membrane was then put in a round bottom flask containing 1.5 mL of the release medium at 37 °C and stirred. At fixed times, the release medium was withdrawn and analyzed and replaced with fresh synovial fluid (1.5 mL).

Calculation of concentration of KGN released

The KGN concentration in the solution was determined by UV-vis spectrophotometry (λ = 271 nm) using the Lambert-Beer law.

Total amount of drug released (F_t) were calculated as follows:

$$F_t = V_m C_t + \sum_{i=0}^{t-1} V_a C_i \qquad (2)$$

where V_m and C_t are volume and concentration of the drug at time t. V_a is the volume of the sample withdrawn and C_i is drug concentration at time i ($i<t$).

Cell culture

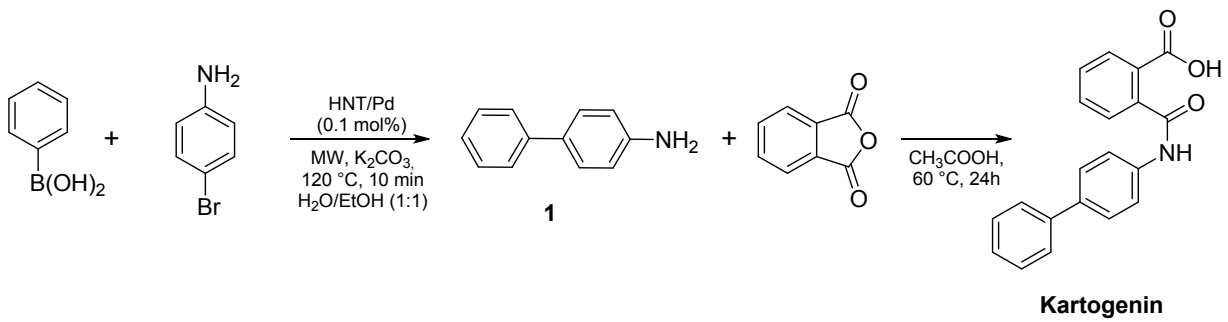
Human hepatocellular liver carcinoma cell line HepG2 was cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin, as well as 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

MTS assay and evaluation of cell viability

HepG2 cells were plated at a density of 1×10^4 cells per well on 96-well plates, and treated with serial concentrations ranging from 100 nM to 10 μM final concentration and incubated overnight at 37 °C in 5% CO_2 atmosphere for 72 h. After treatment, cell viability was assayed using CellTiter 96®AQ_{ueous} One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI), allowed to develop for 1 h 37 °C. Colorimetric measurements were performed at 490 nm using an ELISA plate reader.

Statistics

All experiments were performed in triplicate and repeated at least twice. The results were expressed as mean ± SEM. The responses were calculated as a percentage compared to the untreated group results.



Scheme S.1. Synthetic procedure for the synthesis of kartogenin molecule.

Figure S.1 shows the FT-IR spectra of pure kartogenin and of the HNT/KGN composite. In the pure KGN spectrum (Figure S.1a), the specific bands of the C=O groups of carboxylic and amide groups can be found at ca. 1770 and 1700 cm^{-1}, respectively. The stretching vibrations of aromatic C=C and C-N appear at ca. 1480 and 1380 cm^{-1}, respectively.[1] The assignments for the bands of the pristine halloysite can be done on the basis of literature data.[2] In particular, HNTs show two bands at 3622 and 3693 cm^{-1} corresponding to the O–H stretching of inner hydroxyl groups and outer surface hydroxyl groups, respectively. Additionally, a broad signal at 1640 cm^{-1} is attributed to the H–O–H bending of H-bonded water on the halloysite structure, which corresponds to the broad O–H stretching signal at 3550 cm^{-1}. Apical Si–O and Si–O–Si stretching vibrations provided the bands at 1115 and 1031 cm^{-1}, respectively. The bands at 753 and 690 cm^{-1} derive from the perpendicular Si–O stretching vibration. The O–H deformation vibration of inner Al–O–H groups generates the band at 912 cm^{-1}.

The FT-IR spectrum of the HNT/KGN system (Figure S.1b) resembled the superimposition of the spectra corresponding to the drug and halloysite confirming the successful loading of KGN into the halloysite nanomaterials.

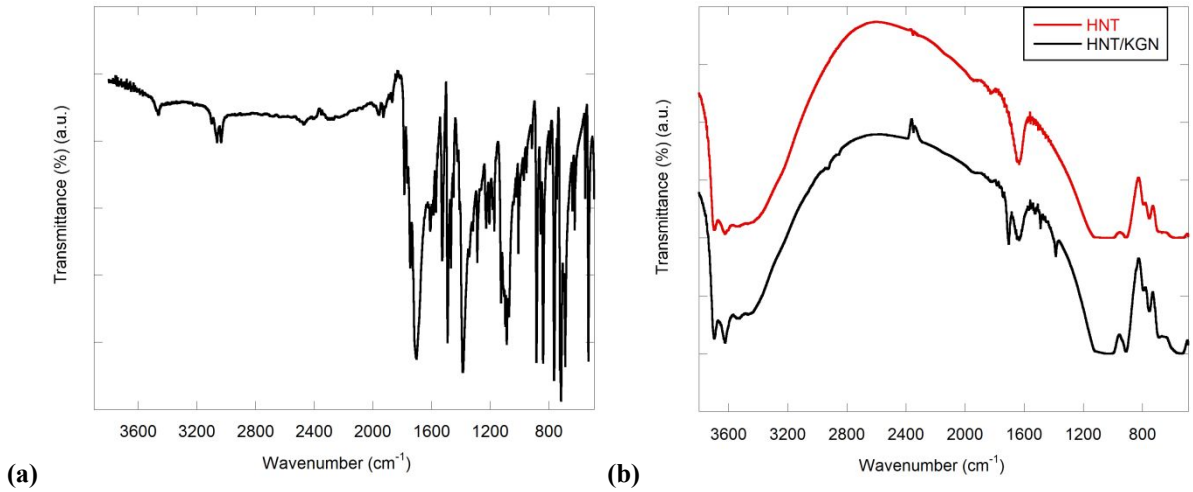


Figure S.2. FT-IR spectra of (a) KGN; (b) HNT and HNT/KGN composite.

Table S.1. Mass losses and residual masses at 600 °C for HNT/KGN composite and their pure components.

Material	$ML_{25–150}$ (wt%)	$ML_{200–300}$ (wt%)	$ML_{400–600}$ (wt%)	MR_{600} (wt%)
HNT	1.66	0	11.7	83.8
KGN	0	96.9	0	0.8
HNT/KGN composite	1.96	8.11	10.9	77.8

Table S.2. Degradation temperatures of kartogenin from TG and DTG curves.

Material	T_{ons} (°C)	T_{peak} (°C)
KGN	268	299
HNT/KGN composite	248	272

Table S.3. Gelation tests of Lap in the presence of different amounts of HNT.

	H_2O		***PBS (1x)***		***Phosphate buffer pH 7.4***	
	Laponite (%)[a]	**Appareance[c]**	**Laponite (%)[a]**	**Appareance[c]**	**Laponite (%)[a]**	**Appareance[c]**
None	3	TG	3	OG	3	OG
	2.5	TG	2.5	OG	2.5	OG
	2	TG	2	OG	2	I
HNT[b]	3	OG	3	OG	3	OG
	2.5	OG	2.5	OG	2.5	OG
	2	OG	2	OG	2	OG

[a]Concentration of laponite in the gel, (%, w/w). [b]5 wt% of HNTs with respect to laponite. [c]Appearances: TG = transparent gel; OG = opaque gel; I = insoluble.

Table S.4. G′ and G″ at γ=0.25%, tanδ=G″/G′ and values of γ at G′=G″ for gels investigated at 2 wt% gelator concentrations and T = 25 °C. Error limits are based on average of three different measurements with different aliquots of gels.

Gel	G′[a]	G″[a]	tan δ [a]	γ at G′= G″
pristine laponite(PBS)	73 ±3	4.1±0.2	0.056±0.006	55±7
HNT/Lap (PBS)	92±8	5.6±0.1	0.062±0.006	62.8±0.1

[a] values at γ = 0.25% and ω = 10 rad/s

Table S.5. Self-healing abilities at 2 wt% laponite filled with 5 wt% of HNT.

Gel	Thixotropy	Sonotropy
Laponite in H_2O	yes	
Laponite in PBS	yes	Stable
Laponite in PB	yes	
HNT/Lap in H_2O	yes	
HNT/Lap in PBS	yes	Stable
HNT/Lap in PB	yes	

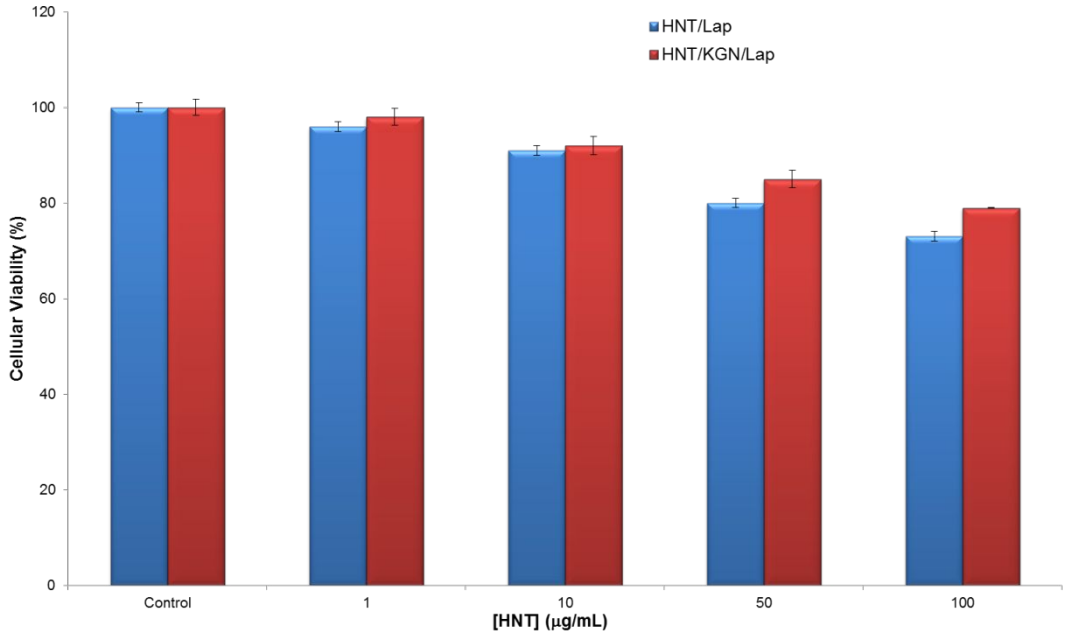


Figure S.3. MTS test for cell viability of (a) HepG2 cells cultured for 72 h in presence of HNT/Lap and HNT/KGN/Lap hybrid systems. The data are mean values obtained from at least three estimations.

References

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2. Massaro, M.; Riela, S.; Guernelli, S.; Parisi, F.; Lazzara, G.; Baschieri, A.; Valgimigli, L.; Amorati, R. A Synergic Nanoantioxidant Based on Covalently Modified Halloysite-Trolox Nanotubes with Intra-Lumen Loaded Quercetin. *J. Mater. Chem. B* **2016,** *4* (13), 2229-2241.