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Glycosaminoglycans (GAGs) are important components of the extracellular matrix that have attracted great interest for drug delivery and pharmaceutical applications due to their diverse biological functions. Among GAGs, heparosan (Hep), a biosynthetic precursor of heparin, has recently emerged as a promising building block for the design of nanoparticles with stealth properties. Though this non-sulfated polysaccharide has a chemical structure very close to that of hyaluronic acid (HA), it distinguishes from HA in that it is biologically inert in the extracellular spaces in the body. In this study, we designed Hep- and HA-based nanogels (NGs) that differ only in the chemical nature of the hydrophilic shell. The nanogels were prepared in a very straightforward way from Hep and HA modified with a thermoresponsive copolymer properly designed to induce self-assembly below room temperature. This versatile synthetic approach also enabled further shell-crosslinking allowing to increase colloidal stability. After careful characterization of the un-crosslinked and crosslinked Hep and HA NGs in terms of size (Z-average diameters of un-crosslinked and crosslinked NGs ~ 110 and 150 nm) and morphology, they were injected intravenously into tumor-bearing mice for biodistribution experiments. Interestingly, these show that the liver uptake of Hep nanogels is remarkably reduced and tumor accumulation significantly improved as compared to HA nanogels (intensity ratios of tumor-to-liver of 2.2 and 1.4 for the un-crosslinked and crosslinked Hep NGs versus 0.11 for the un-crosslinked and crosslinked HA ones). These results highlight the key role played by the shell-forming GAGs on the in vivo fate of nanogels, which correlates with the specific biological properties of Hep and HA.

1 Introduction

2 Self-assembled nanogels, nanometer-sized hydrogels obtained
3 by physical self-assembly of interactive hydrophilic polymers, have
4 attracted growing interest for drug delivery as these systems
5 combine the advantages of hydrogels with nanoscale formulations.¹⁻⁶
6⁵ These soft nanoparticles (NPs) can be designed to facilitate the
7 encapsulation of diverse classes of bioactive compounds, and their
8 hydrophilic shell can be exploited to control their biological behavior
9 and targeting ability. In this regard, nanogels made of amphiphilic
10 polysaccharides hold promise as versatile nanocarriers due to the
11 presence of various functional groups on shell-forming
12 polysaccharides in addition to their unique physicochemical
13 properties, including biocompatibility and biodegradability.^{1, 6}
14 Among polysaccharides, glycosaminoglycans such as hyaluronic acid
15 and chondroitin sulfate (CS) are being used increasingly to design
16 self-assembled nanoparticles for tumor-targeted drug delivery.⁷⁻¹⁶
17 These two GAGs can indeed be specifically recognized by cell surface
18 CD44 receptors that are over-expressed by several cancer cells¹⁷⁻²⁰.
19 Yet, these polysaccharides also bind to other receptors in the human
20 body, such HA receptor for endocytosis (HARE) that facilitates

21 efficient receptor-mediated endocytosis^{21, 22}. Moreover, CD44 and
22 other variants are also expressed on a wide variety of normal cell
23 types, including epithelial cells and haematopoietic cells.²³⁻²⁶ In this
24 regard, Bhattacharya et al. recently raised the question of exploiting
25 HA-CD44 interactions to selectively deliver cytotoxic drugs to cancer
26 cells,²⁷ observing the uptake of their HA-derived nanoparticles into
27 CD44-expressing tumors and in other organs, i.e. liver and spleen²⁸.
28²⁹ Based on this, they rationally modified HA through its *N*-
29 deacetylation and selective sulfation to investigate the potential to
30 minimize interaction of HA with CD44, with the aim of achieving
31 maximum tumor accumulation.²⁷

32 In this study, we explored the possibility of using heparosan as a
33 potential alternative to HA for designing nanogels as drug carriers for
34 tumor-targeted drug delivery. This was motivated by the “stealthy”
35 properties of this GAG,³⁰⁻³³ although its chemical structure is very
36 similar to HA. Indeed, Hep has a repeating disaccharide unit of D-
37 glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc) residues
38 such as HA, but the α -1,4 bond between the GlcA and the GlcNAc
39 units replaces the β -1,3 bond found in HA (Scheme 1). Moreover,
40 since Hep is the natural precursor to the heparin/heparan sulfate
41 biosynthesis, it is biocompatible. This non-sulfated GAG appears to
42 be biologically inert in the extracellular spaces; indeed, it is not
43 known to participate in biological binding interactions.³¹ Moreover,
44 it has no known extracellular degradation pathway and thus, it is
45 stable in the bloodstream contrary to HA and heparin/heparan
46 sulfate which are degraded by enzymes in the blood. On the other
47 hand, Hep is degraded by lysosomal enzymes following entry into the

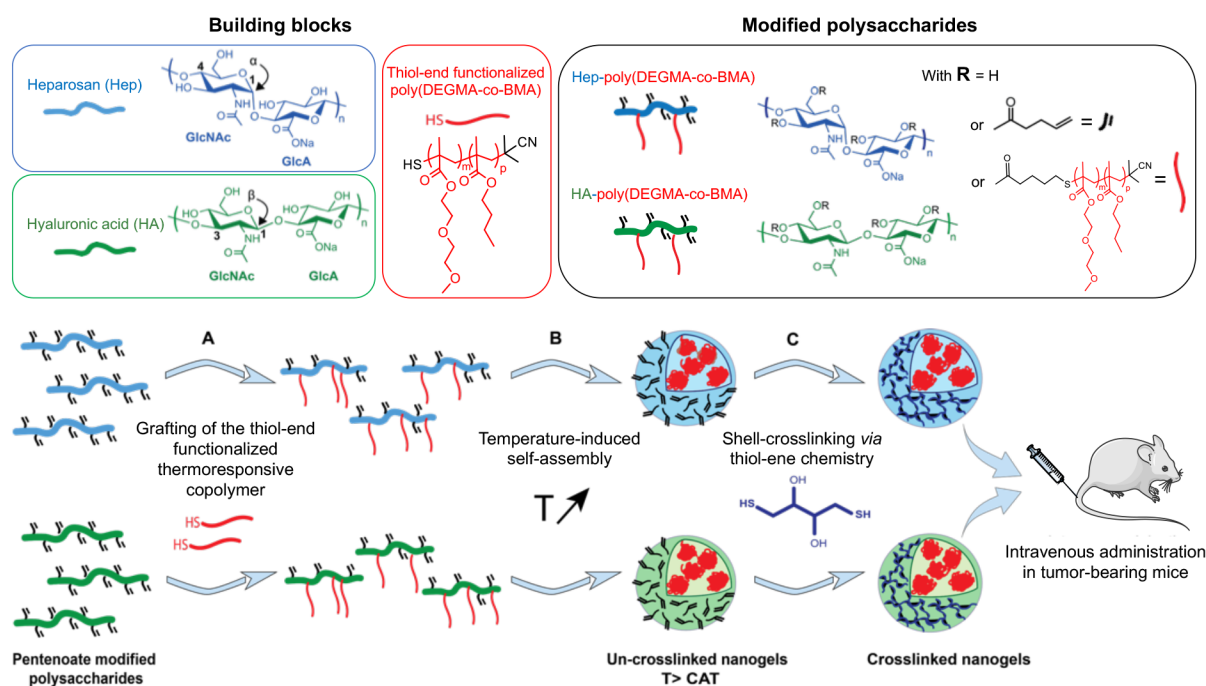
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1 cell, which avoids accumulation in the body.³¹ The peculiar features 23 Hep as a shell-forming polysaccharide of nanocarriers for anti-cancer
 2 of Hep have recently attracted interest for its use as a potential 24 therapy.
 3 alternative to poly(ethylene glycol) (PEG) in the design of 25
 4 nanocarriers for passive tumor targeting in anticancer therapy. 26
 5 Indeed, though the modification of nanoparticles with PEG is a widely 27
 6 adopted approach to extend blood circulation time and improve drug 28
 7 efficacy, several papers from the past decade have suggested that 29
 8 PEG can elicit antibody formation against PEG (anti-PEG) and can also 30
 9 trigger complement activation.³⁴⁻³⁷ Therefore, to propose alternative 31
 10 stealth nanoparticles, some research teams developed NPs by the 32
 11 self-assembly of Hep modified with deoxycholic acid, cholesterol or 33
 12 the anticancer drug doxorubicin (DOX).^{30, 32, 33, 38} These authors 34
 13 showed potential of these systems for intracellular delivery of DOX 35
 14 in vitro. Furthermore, incubation of heparosan-based drug delivery 36
 15 systems with different cancer cells revealed that these nanocarriers 37
 16 have different uptake and subcellular distribution behavior in tumor 38
 17 cells.³² However, the in vivo behaviour of the nanocarriers was not 39
 18 investigated. Besides these self-assembled NPs, Hep-coated 40
 19 liposomes were also prepared by post-insertion of Hep-lipid 41
 20 conjugates.³⁹ Interestingly, these liposomes loaded with DOX were 42
 21 shown to impair tumor growth in a mouse breast cancer model 43
 22 similar to PEG-coated DOX-liposomes, supporting the idea of using 44



Scheme 1. Strategy for the synthesis of un-crosslinked and shell-crosslinked nanogels based on heparosan and hyaluronic acid modified with poly(DEGMA-co-BMA) (Hep-poly(DEGMA-co-BMA) and HA-poly(DEGMA-co-BMA)). A) Grafting of the thermoresponsive copolymer on Hep and HA using radical thiol-ene chemistry; B) formation of nanogels by temperature-triggered self-assembly of modified Hep and HA, and C) shell-crosslinking using radical thiol-ene chemistry.

1 They were prepared in a very straightforward way using thiol-ene 8
 2 chemistry^{40,41} allowing i) the coupling of the thiol-end functionalized 9
 3 copolymer with the polysaccharide modified with alkene groups and, 10
 4 ii) the subsequent crosslinking of the shell-forming polysaccharide by 11
 5 reaction of a bi-functional thiol reagent with the remaining alkene 12
 6 groups. The Hep and HA nanogels were then carefully characterized 13
 7 in terms of size, morphology and cytotoxicity. Finally, their in vivo 14
 8 biodistribution was evaluated by near-infrared fluorescence imaging 15
 9 to shed some light on the role of GAGs on the in vivo fate of the 16
 10 nanogels. To our knowledge, this study provides the first comparison 17
 11 of the in vivo behavior of HA and Hep-based nanoparticles that differ 18
 12 only in the hydrophilic outer shell. 19
 20 **Materials and methods**

1 Materials

2 Hyaluronic acid samples ($M_w = 20$ and 40 kg/mol) were purchased 54 interval. T_{cp} was considered to be the temperature at which the light
 3 from Lifecore (USA). Heparosan ($M_w = 30$ kg/mol) was kindly 55 transmittance was 50 % of that obtained for the same sample at 10
 4 provided by HTL (Javené, France). (Diethylene glycol) methyl ether 56 °C. The critical aggregation temperature (CAT) of HA-poly(DEGMA-
 5 methacrylate, butyl methacrylate, 2-cyano-2-propyl benzodithioate, 57 co-BMA) or Hep-poly(DEGMA-co-BMA) in aqueous solution was
 6 2,2-azobis(2-methylpropionitrile), phosphate buffer saline (PBS, pH 58 assessed using a Zetasizer NanoZS Malvern Instruments apparatus
 7 7.4), sodium chloride, aluminum oxide, tris-(2-carboxyethyl) 59 equipped with a HeNe laser at 173° and a temperature controller. A
 8 phosphine hydrochloride (TCEP), *n*-butylamine, 4-pentenoic 60 solution of HA-poly(DEGMA-co-BMA) or Hep-poly(DEGMA-co-BMA)
 9 anhydride, thiazolyl blue tetrazolium bromide, 1,4-dithiothreitol and 61 in PBS at a concentration of 0.5 mg/mL was filtered through a 0.45
 10 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride 62 µm polycarbonate filter and heated from 10 to 40 °C using a 5 °C
 11 were purchased from Sigma-Aldrich-Fluka (France). 2-Hydroxy-1-[4- 63 interval. The CAT was considered to be the temperature at the
 12 (2-hydroxy-ethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959) 64 intersection between the lower horizontal portion of the plotted
 13 was kindly provided by Ciba Speciality Chemicals (Basel, Switzerland). 65 curve (average scattered intensity versus temperature dependence)
 14 Sulfo-Cyanine7 amine (Cy7-amine) was purchased from Lumiprobe. 66 and the tangent line of the curve. The size and size distribution of
 15 Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine and 67 nanogels were simultaneously measured with the CAT by dynamic
 16 fetal bovine serum (FBS) were provided by Gibco. All chemicals, 68 light scattering (DLS) using a Zetasizer NanoZS Malvern Instruments
 17 except DEGMA and BMA which were purified by running them 69 apparatus operating with a HeNe laser at 173°. The hydrodynamic
 18 through a column packed with aluminum oxide, were used without 70 diameters were calculated from diffusion coefficients using the
 19 any further purification. The positively charged resin, 71 Stokes-Einstein equation. All correlogram analyses were performed
 20 diethylaminoethyl cellulose (DEAE Sepharose CL-6B) was purchased 72 with software supplied by the manufacturer. All the measurements
 21 from GE Healthcare Life Science. Spectra/Por 1 (MWCO 6-8000 73 were performed in PBS (pH 7.4, [NaCl] = 0.15 M).

27 Analytical techniques

22 g/mol) membrane used for dialysis was obtained from Fisher 74 **Synthesis of poly(DEGMA-co-BMA)**
 23 Scientific (Rancho Dominguez, CA). The water used in all experiments 75 DEGMA (10 g, 50 mmol) and BMA (0.398 g, 2.79 mmol), the RAFT
 24 was purified by a Elga Purelab purification system, with a resistivity 76 agent 2-Cyano-2-propyl benzodithioate (0.082 g, 0.372 mmol) and
 25 of 18.2 MΩ cm. Deuterium oxide (D₂O) and deuterated 77 AIBN (3 mg, 0.0186 mmol) in anhydrous toluene (25 mL) were placed
 26 dichloromethane (CDCl₃) were obtained from SDS (Vitry, France). 78 in a round bottom Schlenk flask and oxygen was removed via
 79 bubbling the mixture under nitrogen atmosphere for 30 min. Then,
 80 the flask was sealed and placed in a thermostatic oil bath pre-heated
 81 at 80 °C. The reaction was quenched by cooling and exposure to
 82 oxygen. The resulting copolymer was precipitated in cyclohexane.
 83 The precipitate was dissolved in dichloromethane and the copolymer
 84 was precipitated again in cyclohexane. The resulting precipitate was
 85 finally dried under high vacuum to give 6 g of pure poly(DEGMA-co-
 86 BMA). Samples taken before and during the polymerization were
 87 analyzed by ¹H NMR to determine monomers conversion. The M_n ,
 88 M_w and \mathcal{D} values of the copolymer were determined by size exclusion
 89 chromatography in DMF.

90 Synthesis of HA-poly(DEGMA-co-BMA) and Hep-poly(DEGMA-co-BMA)

90 The pentenoate modified HA20 ($M_w = 20$ kg/mol) and Hep30 ($M_w =$
 91 30 kg/mol) derivatives with a degree of substitution of 0.5 were
 92 synthesized as previously described.⁴¹ The poly(DEGMA-co-BMA)
 93 copolymer was subjected to aminolysis using *n*-butylamine, to
 94 convert the RAFT end-group to a thiol. Briefly, the copolymer (600
 95 mg, 0.375 mmol) was solubilized in dichloromethane (9 mL) and *n*-
 96 butylamine (6.5 mL, 66 mmol) was added. After 5 min under stirring
 97 at room temperature, the reaction mixture was concentrated under
 98 reduced pressure, resulting in a waxy liquid that was solubilized in
 99 dichloromethane. The copolymer was recovered by precipitation in
 100 cyclohexane and dried under vacuum at 45 °C during 4 hours to
 101 remove residual solvent. Next, the thiol-end-functionalized
 102 copolymer (550 mg, 0.0312 mmol) was solubilized in 14 mL of pure
 103 water and TCEP (10 mg, 0.0350 mmol) was added. After 30 min of
 104 cooling/heating cycles from 10 °C to 40 °C using a 1°C/10 min

1 stirring at 4 °C under nitrogen atmosphere, the copolymer solution
2 was added to an aqueous solution of pentenoate modified HA or
3 pentenoate modified Hep (50 mg, 0.116 mmol) in pure water (4 mL),
4 followed by a solution of Irgacure 2959 (2 mL) in water (10 g/L) in
5 order to obtain a final photoinitiator concentration of 0.1 % (w/v) in
6 the reaction media. The reaction mixture cooled in a ice bath, was
7 exposed to UV light ($\lambda = 365$ nm) with an intensity of 20 mW/cm²
8 for 5 min under stirring and nitrogen atmosphere. The resulting HA-
9 poly(DEGMA-co-BMA) and Hep-poly(DEGMA-co-BMA) derivatives
10 were purified via a batch ion exchange process using DEAE Sepharose

11 CL-6B as a weak-anion exchanger. Briefly, DEAE resin (20 mL), stored
12 in a solution of ethanol 30 %, was washed three times with ultrapure
13 water at 4 °C (3 × 20 mL, contact times of 10 min). Excess liquid was

14 removed by centrifugation (10000 rpm, 10 min) at 4° C. Then, the
15 resin was activated by successive washes with a 0.5 M NaCl aqueous
16 solution (20 mL), a 1 M NaCl aqueous solution (20 mL) and finally,
17 four times with ultrapure water (4 × 20 mL). The HA-poly(DEGMA-
18 co-BMA) or Hep-poly(DEGMA-co-BMA) derivative was then added to
19 the resin in a conical tube and allowed to interact with the resin
20 overnight at 4°C under stirring with an orbital shaker. Then, the HA
21 or Hep derivative bound to the resin was subjected to seven washes
22 with ultrapure water (7 × 20 mL) to remove un-grafted copolymer.
23 Finally, the HA or Hep derivative was eluted with a 1 M NaCl aqueous
24 solution (4 × 10 mL). After filtration of the solutions of the HA or Hep
25 derivative through a Buchner funnel with a porous glass filter plate
26 (porosity 4), the solution was dialyzed against deionized water
27 (membrane with cut-off 6-8 Da MW, 72 h). The product was
28 recovered by freeze-drying as a white powder.

29 Synthesis of crosslinked nanogels

30 To a solution of HA-poly(DEGMA-co-BMA) or Hep-poly(DEGMA-co-
31 BMA) (0.015 g, 0.022 mmol) with a DS of 0.03 at a concentration of
32 0.5 g/L in PBS (pH 7.4) at 4°C under nitrogen atmosphere in an ice
33 bath, 564 μ L (1.41 mg, 0.009 mmol) of a solution of DTT in PBS (2.5
34 g/L) was added under stirring. Next, the temperature of the solution
35 was increased to 40°C (above the CAT). After stirring at 40°C for 45
36 min, 3.3 mL of an aqueous solution of Irgacure 2959 (10 mg/mL) was
37 then added to the nanogels suspension to obtain a final photoinitiator
38 concentration of 10 % (w/v). The mixture was exposed to UV light (
39 $\lambda = 365$ nm) with an intensity of 20 mW/cm² for 15 min under
40 stirring and nitrogen atmosphere. The nanogels suspension was
41 transferred into a dialysis bag (MWCO = 6-8000 g/mol) and dialyzed
42 against deionized water for 72 h. The shell cross-linked nanogels
43 were recovered by freeze-drying. The volume of the DTT solution
44 (218 μ L, 652 μ L) was varied to obtain [SH]/[=] ratios of 1 and 2,
45 respectively. Next, the temperature of the solution was increased to
46 40 °C.

47 Determination of the degree of substitution of HA-poly(DEGMA-co- 48 BMA) and Hep-poly(DEGMA-co-BMA) samples by the carbazole 49 reaction

50 The DS was indirectly determined by reaction of D-glucuronic acid
51 units of HA with carbazole²⁶ Briefly, 800 μ L of 25 mM sodium
52 tetraborate solution in sulfuric acid was added to an aqueous

53 solution of HA-poly(DEGMA-co-BMA) or Hep-poly(DEGMA-co-BMA)
54 (200 μ L) at a concentration of 0.1 g/L. After heating at 100 °C for 10
55 min, the solution was cooled at room temperature for 15 min, and
56 then a solution of carbazole (200 μ L) in absolute ethanol 0.125 %
57 (m/v) was added. The sample was heated again at 100 °C for 10 min
58 and its absorbance was determined by spectrophotometry at 530
59 nm. The polysaccharide concentration was calculated from a
60 calibration curve (0.050 to 0.200 g/L), which allowed the indirect
61 determination of copolymer amount in the HA- and Hep-
62 poly(DEGMA-co-BMA) samples.

63 Scanning electron microscopy and transmission electron 64 microscopy

65 For SEM analysis, drops of un-crosslinked and crosslinked nanogels
66 solutions (0.5 mg/mL) in ultrapure water at both 5 and 40 °C were
67 deposited onto mica-coated copper stubs (also precooled/heated at
68 5 or 40 °C, respectively) and allowed to air drying at 5 or 40 °C. The
69 samples were then coated by approximately 2 nm of sputtered Au-
70 Pd and observed in secondary electron imaging mode with a ZEISS
71 Ultra 55 FEG-SEM (Grenoble INP - CMTC). Images were acquired at
72 low voltage of 3 kV using an in-lens detector. For TEM analysis, all
73 samples were dispersed in ultrapure water, stained with 5 % uranyl
74 acetate and observed with a JEOL JEM 1400 (Jeol, USA) transmission
75 electron microscope operating at 120 kV acceleration voltage.

76 Labeling of nanogels based on HA-poly(DEGMA-co-BMA) and Hep- 77 poly(DEGMA-co-BMA) with sulfo-cyanine7

78 Fluorescent nanogels were prepared by grafting the dye Cy7-amine
79 on un-crosslinked and crosslinked nanogels based on HA-
80 poly(DEGMA-co-BMA) and Hep-poly(DEGMA-co-BMA) by an amine-
81 acid coupling reaction using DMTMM as a coupling agent⁴². To this
82 end, crosslinked nanogels (0.008g, 0.0135 mmol) were solubilized in
83 water/DMF (1:1 v/v; 4 mL) and DMTMM (3.7 mg, 0.0135 mmol) was
84 added to the solution, followed by adjusting the pH to 6.5. After 30
85 min of stirring, Cy7-amine (0.5 mg, 0.000676 mmol) solubilized in
86 water/DMF (1:1 v/v) at a concentration of 5 g/L was added to the
87 reaction mixture. After stirring at room temperature for 72 h, the
88 nanogels were purified by dialysis using a membrane MWCO 6-8
89 kg/mol against a mixture of water/ethanol (2/1 v/v) then, against
90 deionized water for 48 h and finally, they were recovered by freeze-
91 drying.

92 Cytotoxicity assay

93 Vero cells (ATCC, Maryland) were maintained in DMEM
94 supplemented with 2 mM L-glutamine and 10% heat-inactivated FBS
95 at 37 °C in a 5% CO₂ atmosphere. In order to investigate a possible
96 toxicity of Hep and HA nanogels towards mammalian cells, Vero cells
97 obtained from confluent cultures were plated (5 × 10⁵ cells/mL) in
98 96-well plates and incubated for 24 h at 37 °C in a 5% CO₂
99 atmosphere. Next, the cells were treated with different
100 concentrations (10 – 1000 μ g/mL) of HA and Hep NGs as well as the
101 native polysaccharides solutions in DMEM. After 72 h of incubation,
102 the cultures were evaluated by MTT assay, as previously described⁴³.
103 After treatment, the medium was removed, and the cellular

1 monolayer was washed with 0.01 M PBS (pH 7.4), and 50 μ L of MTT
 2 solution at 2 mg/mL were added to each well. After incubation for 4
 3 h at 37 $^{\circ}$ C protected from light, MTT solution was removed and 150
 4 μ L of DMSO was added to each well. Absorbance was read in a
 5 microplate reader (BIO-TEK Power Wave XS) at 570 nm. The
 6 percentage of viable cells was calculated compared with controls
 7 (not treated cells). The concentration that decreased 50% of the
 8 absorbance compared with the control cells was considered the toxic
 9 concentration for 50% of cells (CC_{50}).

10 In vivo biodistribution

11 All in vivo procedures were carried out in accordance with the
 12 Brazilian legislation issued by the National Council for Control of
 13 Animal Experimentation (CONCEA) and was approved by the Ethic
 14 Committee on Animal Use of State University of Maringá
 15 (CEUA/UEM), protocol number CEUA 6160200416. Male hairless
 16 mice (3 weeks old, 20-30 g, Brazil) were housed under controlled
 17 conditions of temperature ($22 \pm 1^{\circ}$ C) and humidity, 12:12 h light/dark
 18 cycle and *ad libitum* access to food and water. Solid tumors were
 19 obtained by subcutaneously injecting a suspension of 1×10^7 Ehrlich
 20 ascites carcinoma (EAC) cells/mL in PBS (50 μ L) on the right flanks of
 21 mice. After tumor grown period (≈ 10 days), animals were
 22 anesthetized with isoflurane 2% in an air/ O_2 mixture, and 100 μ L of
 23 suspensions of Cy7-labeled uncrosslinked and crosslinked HA and
 24 Hep NGs (3 g/L in PBS) were administered in the tail vein. For
 25 comparison, Cy7-labeled HA ($M_w = 20$ and 40 kg/mol) and Hep ($M_w =$
 26 30, 3 g/L in PBS) were also injected. Immediately after injection, mice
 27 were evaluated using an In-vivo MS FX PRO (Carestream Molecular

28 Imaging, Carestream Health, United States). Fluorescent images (λ
 29 exc = 750 nm; λ em = 790 nm) were obtained with a CCD camera
 30 (Kodak Image Station) at 0, 1, 3 and 24 h post-injection. Mice were
 31 then sacrificed and the main organs (liver, spleen, lung, kidney, heart,
 32 bladder and tumor) were removed for *ex-vivo* imaging. Images
 33 acquisition and semi-quantification of relative fluorescence intensity
 34 in regions of interest (ROI) were performed using Carestream
 35 Molecular Imaging 5.0 software (Carestream Molecular Imaging,
 36 Carestream Health, United States).

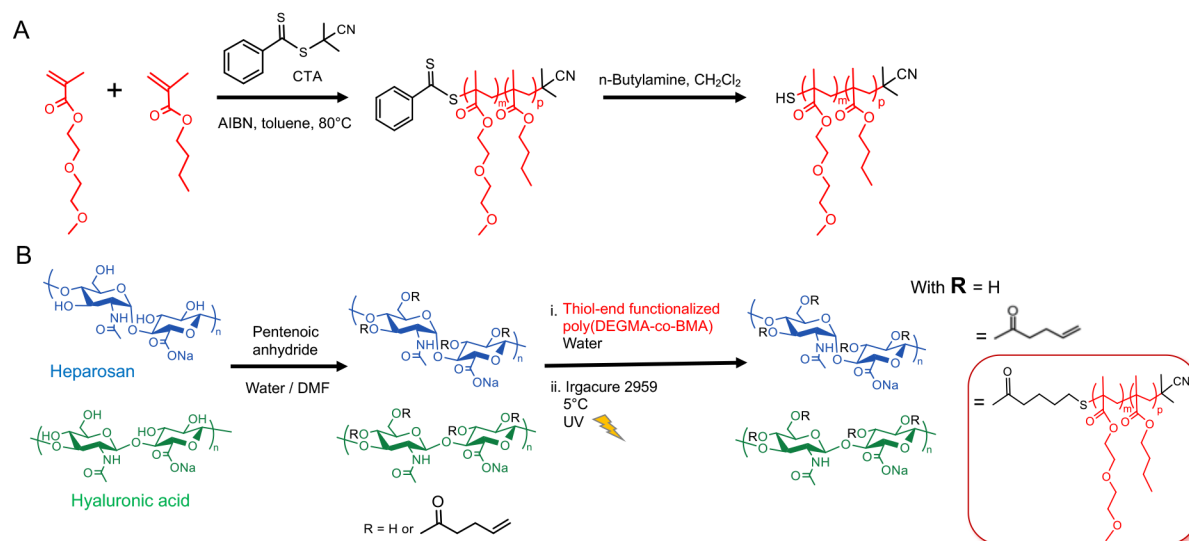
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38 Results and discussion

39 Synthesis of poly(DEGMA-co-BMA)

40 The copolymer poly(DEGMA-co-BMA) was prepared *via* the
 41 reversible addition-fragmentation chain transfer (RAFT) process
 42 from di(ethylene glycol) methacrylate (DEGMA) and
 43 butylmethacrylate (BMA) monomers using 2,2-azobis(2-
 44 methylpropionitrile) (AIBN) as an initiator and 2-cyano-2-propyl
 45 benzodithioate (CPB) as a chain transfer agent (CTA) (Scheme 2A). A
 46 DEGMA/BMA ratio of 95:5 was selected to obtain a copolymer
 47 exhibiting a T_{cp} well below the room temperature and thereby,
 48 allowing formation of nanogels at room temperature (i.e. $\sim 25^{\circ}$ C). 1H
 49 NMR analysis was conducted to monitor the copolymerization
 50 kinetics. The kinetic plots proved that, in these reaction conditions,
 51 CPB allowed a good control over the RAFT copolymerization of
 52 DEGMA and BMA (Figure S1).

53



54

55

56

Scheme 2. Synthetic pathway to Hep- and HA-poly(DEGMA-co-BMA). A) Synthesis of poly(DEGMA-co-BMA) via the RAFT process; B) Grafting of the thermoresponsive copolymer on Hep and HA using radical thiol-ene chemistry

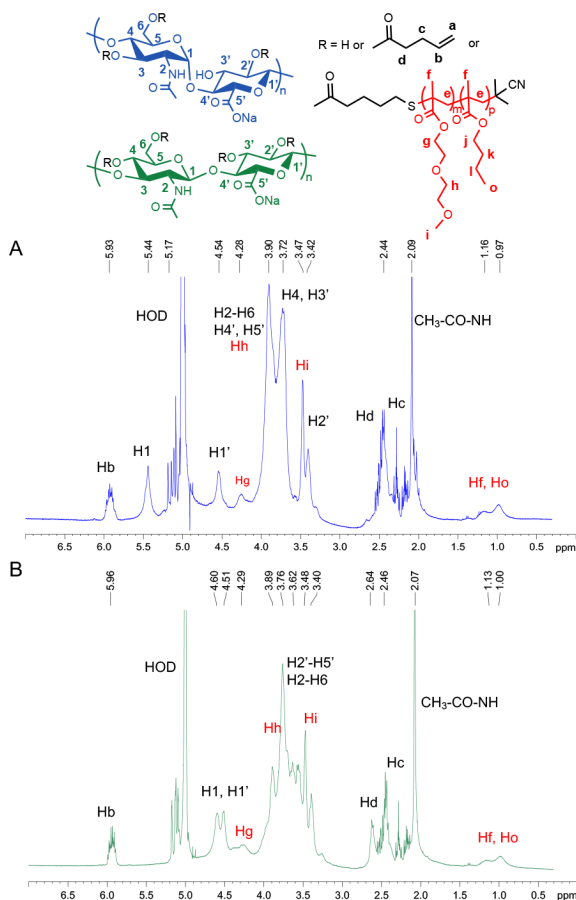
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58 1H NMR and size exclusion chromatography (SEC) analysis of the final
 59 copolymer revealed a final copolymer composition DEGMA/BMA of
 60 95:5, a low dispersity ($\mathcal{D} = 1.12$), and a number average molar mass

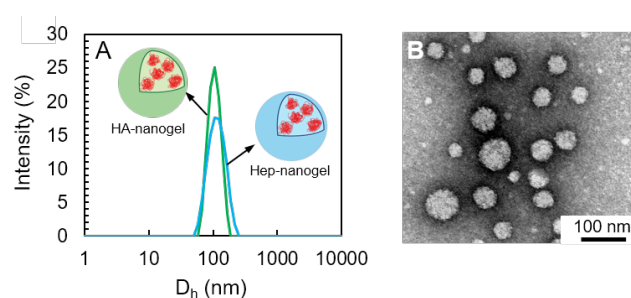
61 M_n of ~ 16 kg/mol ($M_{n,NMR} = 15500$ g/mol, $M_{n,SEC} = 15740$ g/mol,

64 Synthesis of HA and Hep-based nanogels

1 In order to couple poly(DEGMA-co-BMA) to HA and Hep, the
 2 polysaccharides were first esterified with pentenoic anhydride to
 3 produce ene-functional derivatives that were previously shown to
 4 react efficiently with various thiol-containing molecules via thiol-ene
 5 photochemistry.^{40,41} The pentenoate modified HA and Hep (HA-p and
 6 Hep-p, respectively), possessing a degree of substitution (DS,
 7 average number of substituents per repeating unit) of 0.5, were
 8 subsequently reacted with poly(DEGMA-co-BMA) on which the RAFT
 9 end-group was converted to a thiol by aminolysis using *n*-butylamine
 10 (Scheme 2). The thiol-ene coupling reaction was conducted under
 11 UV-light irradiation ($\lambda = 365$ nm) in water, in the presence of Irgacure
 12 2959 as a photoinitiator. The HA- and Hep-poly(DEGMA-co-BMA)
 13 conjugates were then purified *via* a batch ion exchange process
 14 performed at low temperature (< 10 °C) to ensure effective removal
 15 of the non-grafted copolymer during washes with water, followed by
 16 a rapid dialysis to remove salt. This purification process, completed
 17 in less than four days, afforded the final products in 50 % yield.
 18 Successful grafting of the copolymer was confirmed by ¹H-NMR
 19 analysis. In the ¹H-NMR spectrum, the proton signals at 4.25 ppm,
 20 3.76 ppm, 3.39 ppm and in the region of 0.99-1.2 ppm arising from
 21 poly(DEGMA-co-BMA) can easily be observed (Figure 1). The DS of
 22 the conjugates, determined by the carbazole assay,⁴⁴ were found to
 23 be 0.02 ± 0.01 for the HA- and Hep-poly(DEGMA-co-BMA)
 24 conjugates.



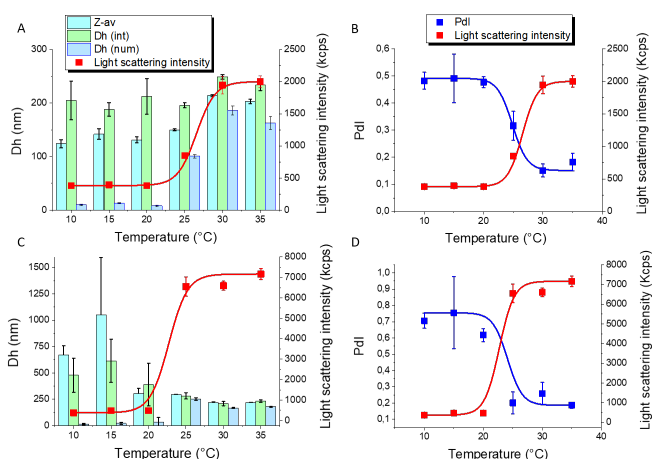
25
 26 **Figure 1.** ¹H NMR spectra (400 MHz, 6 mg/mL in D₂O) of Hep-poly(DEGMA-co-
 27 BMA) (A) and HA-poly(DEGMA-co-BMA) (B) at 5°C.



40

41 **Figure 2.** Temperature responsiveness of Hep- and HA-poly(DEGMA-co-BMA). A)
 42 Dynamic light scattering analysis of Hep and HA-poly(DEGMA-co-BMA) solutions
 43 in PBS ($C_p = 0.5$ g/L) at 37 °C. B) TEM image of Hep NGs in water at 40 °C ($C_p = 0.5$
 44 g/L).

45 The critical aggregation temperature (CAT) of the HA- and Hep-
 46 poly(DEGMA-co-BMA) conjugates was determined by measuring the
 47 light scattering intensity (LSI) of aqueous solutions of the derivatives
 48 as a function of temperature (Figure 3). The CAT, defined as the
 49 temperature at the intersection between the lower horizontal
 50 portion of the plotted curve and the tangent line, was found to be 20
 51 °C and 22 °C for HA- and Hep-poly(DEGMA-co-BMA), respectively.
 52 Both HA and Hep derivatives are thus able to self-assemble into
 53 nanogels at room temperature.



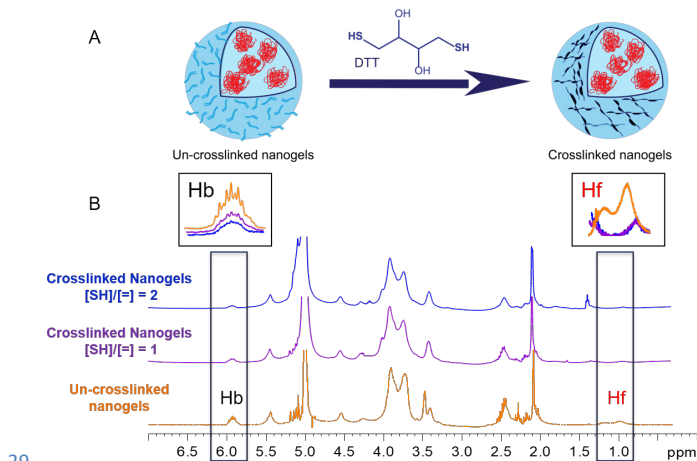
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55 **Figure 3.** Analysis by DLS of the temperature sensitivity of Hep-poly(DEGMA-co-
 56 BMA) (A, B) and HA-poly(DEGMA-co-BMA) (C, D) in PBS (pH 7.4, $C_p = 0.5$ g/L). A
 57 and C) Variation of the D_h , Z-Ave and LSI values upon cooling from 35 °C to 10 °C
 58 (0.5 °C/min). B and D) Variation of the LSI and PDI upon cooling from 35 °C to 10
 59 °C.

1 Self-assembly into well-defined nanostructures is also reflected in
2 the sharp decrease of the polydispersity index (PDI) above the CAT as
3 well as in the values of mean diameter in different distributions
4 (intensity and number distribution) and of Z-average size, which are
5 similar. In contrast, strong discrepancies between the mean size
6 values are observed below the CAT.

7 Synthesis and characterization of shell-crosslinked nanogels

8 In order to avoid a fast destabilization of the nanogels structure after
9 intravenous administration due to dilution of the polymers solution
10 in the blood or by the interaction with blood components, the
11 polysaccharide shell of Hep and HA NGs was crosslinked using thiol-
12 ene chemistry by reaction of the remaining pentenoate groups with
13 dithiothreitol (DTT) as a bis-thiol crosslinker (Figure 4A). This
14 crosslinking step was performed under relatively dilute conditions
15 ($C_p = 0.5$ g/L) to avoid inter-nanogel coupling, and by varying the
16 [SH]/[=] ratio from 1 to 2 to ensure that all nanogels were sufficiently
17 crosslinked to be stable at low temperature. Comparison of ^1H NMR
18 spectra before and after the thiol-ene reaction with DTT provided
19 evidence of successful shell-crosslinking (Figure 4B). Indeed, the
20 addition of thiols to alkenes during the crosslinking step leads to the
21 formation of thioether bonds and concomitantly, to the
22 disappearance of the double bonds of the pentenoate groups.
23 Consequently, the intensity of the alkene protons at 5.9 and 5.1-5.2
24 ppm is decreased and the signals of the methylene protons of the
25 pentenoate groups at 2.1-2.3 ppm undergo a chemical shift.
26 Furthermore, the proton signals of the polysaccharide backbone and
27 the copolymer are significantly broadened since the crosslinking
28 reaction decreases the mobility of the polymer chains.

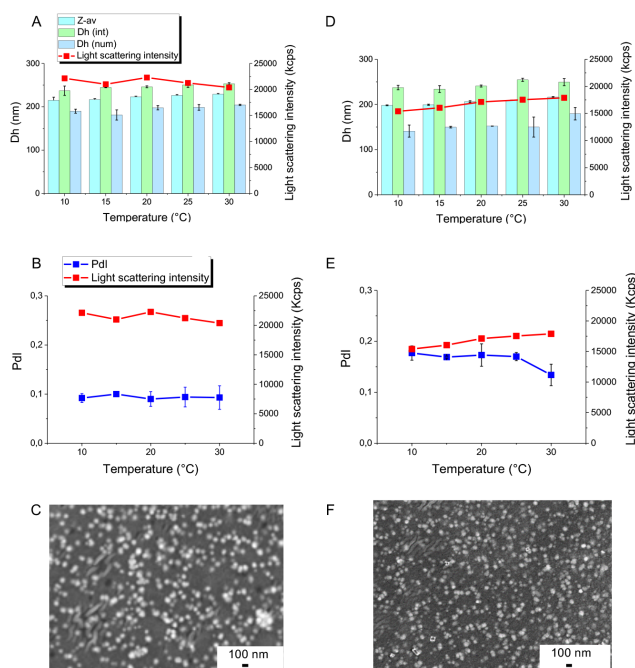


29
30 **Figure 4.** Shell crosslinking of NGs by reaction between DTT and alkene groups of
31 the shell-forming polysaccharide. A) Reaction conditions: Irgacure 2959, UV-light
32 ($\lambda = 365$ nm) exposure for 15 min, $C_p = 0.5$ g/L in PBS. B) Comparison of ^1H NMR
33 spectra of un-crosslinked Hep NGs and crosslinked Hep NGs by varying the [SH]/[=]
34 ratio from 1 to 2. The signal intensities were normalized relative to the intensity
35 of the CH_3CO signal of HA

36 Successful crosslinking was further confirmed by FT-IR spectroscopy
37 (Figure S4). In the IR spectra of the crosslinked nanogels, additional
38 bands at 839 cm^{-1} and 911 cm^{-1} are observed after reaction with DTT,
39 which can be attributed to C-S bend, C-S stretching and H-C-S bend,
40 respectively. The new band observed at 1253 cm^{-1} (CH_2 vibrations)
41 also suggests incorporation of DTT.^[19-20]

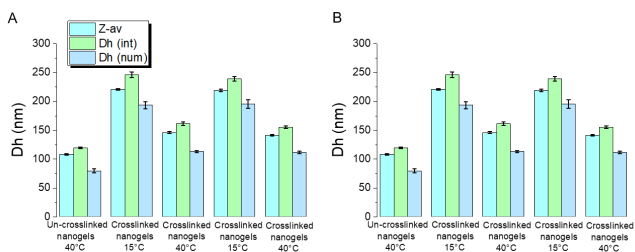
42 Temperature responsiveness of crosslinked nanogels

43 While the un-crosslinked nanogels of Hep-p-poly(DEGMA-co-BMA)
44 and HA-p-poly(DEGMA-co-BMA) disassemble below the CAT (~ 22
45 $^{\circ}\text{C}$), their counterparts crosslinked with excess DTT ([SH]/[=] of 2)
46 were found to be stable below the CAT. This was clearly
47 demonstrated by DLS measurements at temperatures varying from
48 30 to $10\text{ }^{\circ}\text{C}$ with a cooling rate of $0.3\text{ }^{\circ}\text{C}/\text{min}$ (Figure 5). The LSI and
49 PDI of the nanogels were constant upon cooling, demonstrating
50 efficient nanogel crosslinking. Furthermore, scanning electron
51 microscopy (SEM) images revealed the presence of nanogels at $5\text{ }^{\circ}\text{C}$
52 (Figure 5C and 5F). It should be noted that a ratio of [SH]/[=] of 2 is
53 required to ensure complete stability of nanogels. Indeed, the LSI of
54 the nanogels crosslinked with a [SH]/[=] ratio of 1 slightly decreased
55 upon cooling, suggesting dissociation of some nanogels (Figure S5).



56
57 **Figure 5.** Analysis by DLS and SEM of crosslinked Hep NGs (A, B, C) and HA NGs (D,
58 E, F) in PBS (pH 7.4, $C_p = 0.5$ g/L). A and D) Variation of the Dh, Z-Ave and LSI values
59 upon cooling from $35\text{ }^{\circ}\text{C}$ to $10\text{ }^{\circ}\text{C}$ ($0.3\text{ }^{\circ}\text{C}/\text{min}$). B and E) Variation of the LSI and PDI
60 upon cooling from $35\text{ }^{\circ}\text{C}$ to $10\text{ }^{\circ}\text{C}$. C and F) SEM observation at $5\text{ }^{\circ}\text{C}$.

61 Interestingly, the SCL nanogels showed temperature-dependent
62 swelling/deswelling transitions between 15 and $40\text{ }^{\circ}\text{C}$. The swelling-
63 deswelling transition was fully reversible over multiple
64 heating/cooling cycles.



66 **Figure 6.** Analysis by DLS of the swelling-deswelling transition of crosslinked Hep-
67 NGs (A) and HA-NGs (B) over multiple heating/cooling cycles ($C_p = 0.5$ g/L in PBS).

1 In vitro cytotoxicity, in vivo biodistribution and tumor targeting of 2 crosslinked and uncrosslinked Hep-NGs and HA-NGs

3 The in vitro cytotoxicity of un-crosslinked and shell-crosslinked Hep 4 NGs and HA NGs, as well as of native HA and Hep, was evaluated in 5 Vero cells after 72 h of incubation by MTT assay. For all the samples, 6 the toxic concentration for 50 % of the cells was higher than 1000 7 $\mu\text{g}/\text{mL}$, the maximum assessed concentration, demonstrating the 8 very low cytotoxicity and biocompatibility of our HA and Hep 9 derivatives (Figure S6).

10 The in vivo biodistribution and tumor targeting of Hep- and HA-based 11 nanogels were then evaluated in Ehrlich solid tumor (EST)-bearing 12 mice⁴⁵ by a non-invasive fluorescence imaging system. The main 13 characteristics of the nanogels in terms of mean size and Pdl near 14 body temperature are summarized in Table 1. Importantly, the 15 similar size of both HA- and Hep-based un-crosslinked nanogels as 16 well as of both crosslinked ones together with their similar chemical 17 composition, except the hydrophilic shell structure, allowed a proper 18 comparison of the biodistribution of HA- and Hep NGs. The nanogels 19 and the native polysaccharides, HA ($M_w = 20$ and 40 kg/mol) and Hep 20 ($M_w = 30$ kg/mol), were labeled with the NIR dye Sulfo-Cyanine7 21 (Cy7) to visualize their biodistribution. Briefly, the samples were 22 chemically modified with Cy7-amine using 4-(4,6-dimethoxy-1,3,5- 23 triazin-2-yl)-4-methylmorpholinium chloride as an amine-acid 24 coupling agent.⁴⁶ The content of Cy7 molecules in the polysaccharide 25 derivatives (DS) was 0.002, as determined by UV/Vis spectroscopy at 26 680 nm. After Cy7 labeling, the nanogels maintained their size as 27 shown by nanoparticle tracking analysis (NTA),⁴⁷ which enables the 28 visualization and recording of nanoparticles in solution (Figure S7). 29 Particle size distribution obtained by NTA ranged from 60 to 250 with 30 an average of 150 ± 40 nm and from 60 to 300 with an average of 31 190 ± 43 nm for the SCL NGs based on HA and Hep, respectively 32 (Figure S7).

33 **Table 1.** Size and polydispersity of nanogels based on Hep-poly(DEGMA-co-BMA) and 34 HA-poly(DEGMA-co-BMA) determined by dynamic light scattering at 40 °C (0.5 g/L in 35 PBS).

	Nanogels	Z-Average size (nm)	Pdl
HA	Un-crosslinked nanogels	108 ± 1	0.174 ± 0.03
	Crosslinked nanogels (1 eq DTT)	151 ± 2	0.176 ± 0.01
Hep	Un-crosslinked nanogels	119 ± 1	0.095 ± 0.006
	Crosslinked nanogels (1 eq DTT)	146 ± 2	0.095 ± 0.013

36
37 Time-dependent biodistribution of Cy7-labeled native Hep, HA 38 and nanogels on EST-bearing mice was observed after intravenous 39 administration. Ex vivo fluorescence images of excised organs and 40 tumors showed a higher accumulation of Hep NGs in the tumor than 41 HA NGs. As shown in Figure 7A, the intensity ratio of tumor-to-liver 42 between 1 h and 24 h was in the order un-crosslinked Hep NGs > 43 crosslinked Hep NGs > un-crosslinked HA NGs \approx crosslinked HA NG, 44 suggesting lower liver uptake and higher tumor accumulation for the 45 nanogels based on Hep. These results were consistent with those 46 obtained from native polysaccharides (Figure 7B). Indeed, as can be 47 seen from Figure 7B, the intensity ratio of tumor-to-liver is much

48 higher for Hep ($M_w = 30$ kg/mol) than HA, whatever the molar mass 49 of HA (20 or 40 kg/mol). Interestingly, the intensity ratio of tumor- 50 to-liver for the Hep-NGs and native Hep progressively increased 51 during the whole period of time studied (24 h).

52 The higher intensity ratio of tumor-to-liver at 24 h post-injection for 53 the Hep NGs and native Hep in comparison to the HA NGs and native 54 HA correlates with their very low liver uptake and significant tumor 55 accumulation (Figure 7C-F). Notably, the ex vivo fluorescence images 56 of the main organs displayed in Figure 7E clearly show that the liver 57 uptake of nanogels based on Hep is remarkably reduced and tumor 58 accumulation significantly improved. These results highlight the key 59 role played by the shell-forming GAG on the in vivo fate of nanogels, 60 taking into account the fact that the HA and Hep NGs are similar in 61 terms of size and morphology. Regarding the in vivo behavior of the 62 HA NGs, several research teams previously observed accumulation 63 of HA-based nanoparticles and drug conjugates in the liver in 64 addition to the uptake in the tumor.^{28, 29, 48-52} This was found to be 65 also the case with HA NPs administered in EST-bearing mice model, 66 which is a well-established animal model for CD44 receptor 67 overexpressing tumors.^{45, 53-55} Two possible reasons for such 68 accumulation in the liver were raised: cellular uptake by phagocytic 69 cells of the reticuloendothelial system (RES) and interaction of HA 70 with the HARE receptor which is expressed by liver sinusoidal 71 endothelial cells and mediates systemic clearance of HA^{21, 56}. Here, 72 the fact that Hep NGs and native Hep are not sequestered by the liver 73 rules out the idea that the hepatic accumulation of HA NGs and 74 native HA is mainly due to non-specific uptake by phagocytic cells of 75 the RES. Moreover, it is worth noting that a similar liver uptake was 76 also observed for native unconjugated HA after intravenous 77 administration in healthy mice, while native Hep did not accumulate 78 in major organs (Figure S8). The preferential accumulation of native 79 HA in the liver of healthy mice was previously reported by other 80 research groups.⁵⁷⁻⁵⁹ These data support the notion that the liver is 81 the major site of circulating HA uptake and degradation in contrast 82 to Hep which does not bind to HARE²¹. Actually, Hep stands out 83 among other GAGs (HA, CS, heparin, dermatan sulfate) that 84 selectively interact with HARE, resulting in their clearance from 85 circulating lymph and blood.²¹ This unique feature of Hep may be 86 related to the fact this GAG is only temporarily present in the 87 organism as it is the biosynthetic precursor of heparin and heparan 88 sulfate. Therefore, as Hep is biologically inert in the extracellular 89 environment, it can circulate in the bloodstream for a prolonged 90 period of time. In this regard, it was previously reported that its half- 91 life can vary from 15 h to 2 days depending on its molar mass (60 or 92 100 kg/mol).⁶⁰ As a result, the prolonged circulation of Hep in the 93 bloodstream may increase its probability of reaching the tumor 94 tissue after systemic administration in vivo as well as that of Hep 95 NGs.

96 Finally, comparison of un-crosslinked and crosslinked NGs did not 97 show significant differences in their in vivo fate, despite the slightly 98 higher size and presumably decreased deformability of the 99 crosslinked nanogels (Figure 7C, 7F). All together, these results 100 demonstrate that the HA and Hep NGs exhibit the same in vivo

1 behaviour as the native GAGs despite their modification with alkene
2 groups for thiol-ene coupling reactions. Notably, the selective
3 accumulation of Hep NGs in the tumor site make them very
4 promising as nanocarriers for cancer diagnosis and treatment.

5 backbone with a thermoresponsive copolymer, poly(DEGMA-co-
6 BMA), properly designed to obtain stable nanogels at room
7 temperature. The versatile synthetic route to nanogels also allowed
8 their further shell-crosslinking to further store the nanogels at low
9 temperature. After intravenous administration in tumor-bearing
10 mice, both un-crosslinked and crosslinked Hep NGs were able to
11 accumulate in the tumor at a much higher level than their
12 counterparts based on HA. Importantly, the well-defined properties
13 of both Hep and HA NGs families in terms of chemical structure
14 (except the hydrophilic outer-shell), size and morphology allowed to
15 reliably assess the effect of the shell-forming glycosaminoglycan on
16 their in vivo biodistribution. These results thus showed that Hep NGs
17 provide an exciting new class of drug delivery platform for anticancer
18 therapy. To the best of our knowledge, our study provides the first
19 analysis of the in vivo behavior of self-assembled nanoparticles
20 based on Hep, demonstrating significant differences compared to
21 HA-based self-assembled nanoparticles. Regarding the effect of
22 shell-crosslinking on in vivo biodistribution, higher accumulation
23 levels in most of organs, especially in liver and tumor, were observed
24 for the crosslinked Hep NGs compared to the un-crosslinked ones, 24
25 h after administration. At this stage, it is difficult to explain these
26 results as the un-crosslinked and crosslinked NGs are different not
27 only in size but also in shell stiffness/deformability. So far, the impact
28 of nanoparticles flexibility/stiffness on their function has been very
29 little explored, and the potential benefits of tuning nanoparticle
30 elasticity are not clear.²³⁻²⁵ Based on these considerations, these Hep
31 NGs represent an attractive platform to investigate the impact of
32 design parameters such as shell crosslinking, incorporation of
33 combination regimens as well as inorganic nanoparticles (magnetic
34 nanoparticles, gold nanoparticles) in order to more optimally exploit
35 the biocompatibility and the beneficial distribution of these novel
36 nanocarriers.

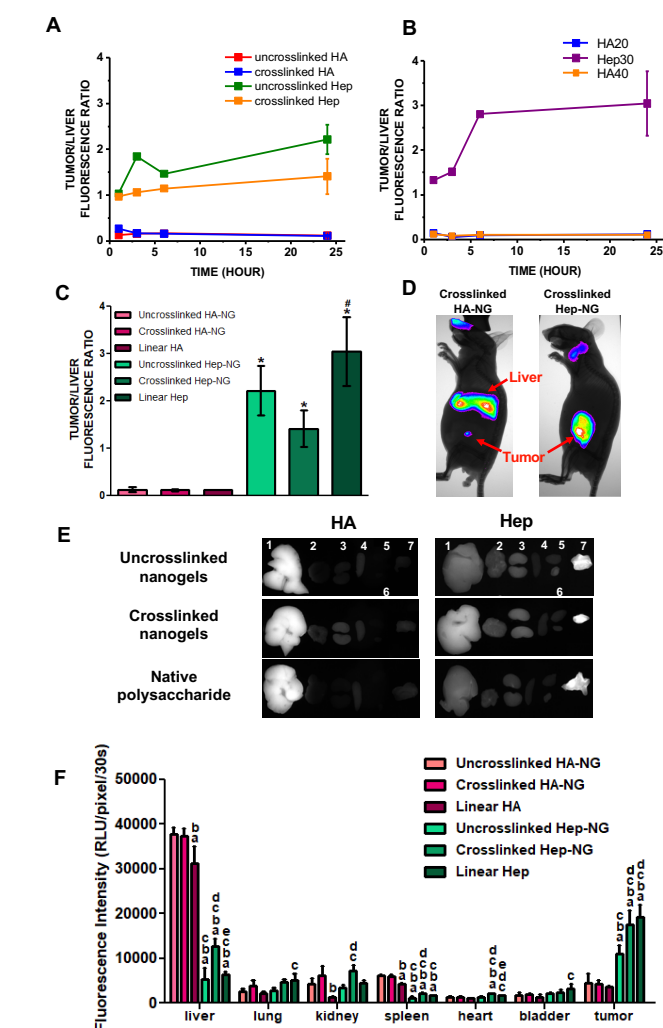
58

59 Conflicts of interest

60 There are no conflicts to declare.

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5

6 **Figure 7.** *In vivo* biodistribution of Hep- and HA-NGs in Ehrlich solid tumor-bearing mice.
7 Fluorescence intensity ratio of the excised tumor to liver at 1 h, 3 h, 6 h ($n = 1$) and 24 h
8 ($n = 3$) post-injection of Hep- and HA-NGs (A), and of native Hep and HA (B). C)
9 Fluorescence intensity ratio of the excised tumor-to-liver at 24 h post-injection of Hep-
10 and HA-NGs as well as of native Hep and HA ($n = 3$); error bars represent standard
11 deviations (SD). One-way ANOVA followed by Tukey post hoc test. *# $P < 0.05$.
12 *Compared to HA (native polysaccharide and nanogels). #Compared to crosslinked Hep-
13 NG. D) *In vivo* fluorescence images of the tumor-bearing mice after intravenous injection
14 crosslinked HA-NGs and Hep-NGs. E) *Ex vivo* fluorescence images of main organs and
15 tumors retrieved from the tumor-bearing mice at 24 h post-injection of NGs and of the
16 native polysaccharides: (1) liver; (2) lung; (3;3') kidney; (4) spleen; (5) heart; (6) bladder;
17 (7) tumor. F) *Ex vivo* fluorescence intensity of main organs at 24 h post-administration of
18 uncrosslinked and crosslinked HA- as well as Hep-NGs and native polysaccharides (HA
19 and Hep) ($n = 3$). The results were expressed as mean \pm SD. One-way ANOVA followed
20 by Tukey post hoc test: ^aCompared to uncrosslinked HA-NG; ^bcompared to crosslinked
21 HA-NG; ^ccompared to linear HA; ^dcompared to uncrosslinked Hep-NG; ^ecompared to
22 crosslinked Hep-NG.

23 Conclusion

24 In this study, novel biocompatible and biodegradable nanogels based
25 on heparosan were developed by modification of the polysaccharide

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3 Notes and references

- 4
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