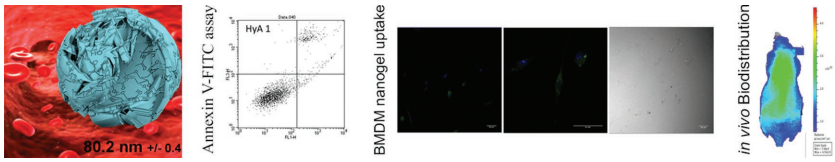
Biocompatibility of a Self-Assembled Crosslinkable Hyaluronic Acid Nanogel

Sílvia Santos Pedrosa,* Paula Pereira, Alexandra Correia, Susana Moreira, Hugo Rocha, Francisco Miguel Gama*

Hyaluronic acid nanogel (HyA-AT) is a redox sensitive crosslinkable nanogel, obtained through the conjugation of a thiolated hydrophobic molecule to the hyaluronic acid chain. Engineered nanogel was studied for its biocompatibility, including immunocompatibility and hemocompatability. The nanogel did not compromise the metabolic activity or cellular membrane integrity of 3T3, microvascular endothelial cells, and RAW 264.7 cell lines, as determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide and lactate dehydrogenase release assays. Also, we didn't observe any apoptotic effect on these cell lines through the Annexin V-FITC test. Furthermore, the nanogel cell internalization was analyzed using murine bone marrow derived macrophages, and the in vivo and ex vivo biodistribution of the Cy5.5 labeled nanogel was monitored using a non-invasive near-infrared fluorescence imaging system. The HyA-AT nanogel exhibits fairly a long half-live in the blood stream, thus showing potential for drug delivery applications.



1. Introduction

Nanotechnology is a fast growing field with particular interest for biomedicine research. Nanoparticles and other nanomaterials in general, offer numerous advantages due to their small size, drug loading ability and special pharmacodynamics. Due to their potential and peculiar characteristics are often employed to: i) target-specific delivery of drugs, or other molecules; ii) improve drug stability or solubility, in vitro or in vivo; iii) reduce side effects of biologically active compound. Therefore, it is not strange to realise that so many nano-systems are being investigated; having several products reached the market, and many more undergoing clinical trials.[1,2]

However, nanoparticles attractive properties can also be the source of problems, mainly by the interaction with immune system. Engineered nanoparticles can specifically be designed either to target or avoid the immune system.[1] Interactions with immune system are considered beneficial when advantageous medical reactions are obtained, e.g. vaccination or the treatment of autoimmune disorders. However, nanoparticles and especially polymeric nanoparticles may escape the immune system recognition and perform its duty e.g., as drug delivery system.[1,2] Hyaluronic acid is a naturally occurring polysaccharide, ubiquitous in the human body, and with appealing biological properties.[3]

In 2010, Kohane and Langer defined biocompatibility as "an expression of the benignity of the relation between

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a material and its biological environment". Generally biocompatibility is achieved when materials interact with the body and do not induce unacceptable toxic, immunogenic, thrombogenic and/or carcinogenic effects.[4]

An HyA-AT crosslinked through redox sensitive bond was prepared in our research group in previous work, demonstrating potential for drug delivery applications.[5] Here, we assess the biocompatibility, immunocompatibility and hemocompatibility of the engineered nanogel. Also, nanogels biosdistribution in healthy mice model was assessed by non-invasive near-infrared (NIR) fluorescence imaging system. Nanogel was labeled with a NIR probe – Cy5.5 hydrazide – and its biodistribution was studied for the course of 48 h.

2. Experimental Section

2.1. Materials

All reagents used were of analytical grade. Sodium hyaluronate (M_W = 7.46 KDa) was purchased from Lifecore Biomedical (USA). AG 50W – X8 resin was purchased from Bio-Rad (USA). Dimethyl sulfoxide (DMSO), Tetrabutylammonium fluoride hydrate (TBA-F), 11-Amino-1-undecanethiol hydrochloride (AT), N-hydroxysulfosuccinimide (NHS), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), hydrogen nicotinamide adenine dinucleotide (NADH), pyruvate, haemoglobin from bovine blood and Drabkin's reagent were acquired from Sigma-Aldrich (Italy). Fluorescein-5-thiosemicarbazide and Cy5.5 hydrazide were purchased from Life Technologies Ltd (UK). Cell Culture reagents and culture medium were purchased from Biochrom (Germany). The water used was distilled and ultrapurified (Milli-Q system).

2.2. Nanogel Assembling

HyA-AT nanogel was produced as described previously,[5] by chemical conjugation. Briefly, a thiolated hydrophobic molecule (AT) was grafted in the hyaluronic acid backbone (DS = 11%) by carbodiimide chemistry. The resulting amphiphilic hyaluronic acid conjugate was dispersed in water and stirred for a few minutes at room temperature. Dispersion was further filtrated by cellulose acetate serynge filter (pore size 0.22 μm). Engineered nanogel was characterized thoroughly[5] and the nanogel colloidal dispersion in water displayed an average size distribution of 80.2 +/– 0.4 nm. The nanogel dispersions used for in vitro and in vivo studies were filtered through cellulose acetate syringe filter (pore size 0.22 μm) in aseptic conditions.

HyA-AT nanogel morphology (1.0 mg mL^{-1}) was analyzed by Cryo-Field-Emission Scanning Electron Microscopy (Cryo-FESEM) in an electron microscope (SEM/EDS: FESEM JEOL JSM6301F/Oxford Inca Energy 350). Sample was frozen in liquid nitrogen and then fractured and sublimated for 10 min at –95 °C to expose the nanogel particles. Finally, samples were sputter-coated with gold and palladium at –140 °C using an accelerating voltage of 10 kV. The observation was performed at –140 °C and 15 kV.

With the intent to evaluate the interaction of the nanogel with protein components of the culture medium, its size distribution profile by intensity was evaluated through dynamic light scattering (DLS). Briefly, HyA-AT nanogel was dispersed in water at 1.0 mg mL^{-1} and incubated at room temperature with FBS 1%(v/v). Dispersions were evaluated as to their size distribution profile at: 0, 24, 48, and 72 h. Also, control samples of nanogel 1 mg mL^{-1} and FBS 1% were also analyzed, following the same protocol.

2.3. Synthesis Of Hya-AT-Fluorescein Labeled Nanogel

For in vitro murine bone marrow derived macrophages (BMDM) cellular uptake evaluation, HyA-AT nanogel and native HyA were labeled with Fluorescein-5-thiosemicarbazide. Briefly, thiosemicarbazide group reacted with carboxylic group of hyaluronic acid nanogel in the presence of EDC as a coupling agent.[6–10] The molar ratio of Fluorescein-5-thiosemicarbazide to free carboxylic acid groups of HyA-AT nanogel was 0.25. The coupling agent (EDC) was added to the reaction mix at an equimolar ratio to the free carboxylic acid groups of HyA-AT nanogel and native HyA. The reaction was allowed to occur overnight at room temperature, in the dark. The reaction mixture was thoroughly dialysed (MW cut-off 1000 Da) against distilled water to remove non-desired reaction products. Nanogel and polymer labelling was confirmed by UV/vis spectroscopy at 492 nm.

2.4. Synthesis Of Hya-At-Cy5.5 Labeled Nanogel

For in vivo biodistribution study using near infrared fluorescence (NIRF) technology, HyA-AT nanogel and native HyA were labeled with Cy 5.5 – hydrazide. Hydrazide reactive moiety was conjugated with carboxylic groups of HyA-AT nanogel[8–11] in presence of EDC, as coupling agent. The molar ratio of Cy 5.5 – hydrazide to free carboxylic acid groups of HyA-AT nanogel and native HyA was 0.25. EDC was added in an equimolar ratio to the free carboxylic acid groups. The reaction was allowed to occur overnight at room temperature, in the dark. The reaction mixture was thoroughly dialysed (MW cut-off 2 000 Da) against distilled water to remove non-desired reaction products. Nanogel and polysaccharide labelling was confirmed by UV/vis spectroscopy at 649 nm.

2.5. Cell lines, Cell Culture, and Maintenance

Mus musculus, mouse embryonic fibroblasts (NIH/3T3) cell line was maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% (v/v) newborn calf serum, 100 IU mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin. Mouse leukemic monocyte macrophage (RAW 264.7) cell line was grown in DMEM supplemented with 10% (v/v) of heat-inactivated (FBS), 100 IU mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin. Human microvascular endothelial cells (HMEC) were grown in RPMI-1640 supplemented with 10% FBS, Epidermal Growth Factor (10 ng mL^{-1}), Hydrocortisone (1 μg mL^{-1}), 100 IU mL^{-1} penicillin, and 0.1 mg mL^{-1} streptomycin. All cells were maintained at a 37 °C and 95% humidified air with 5% CO2, environment. RAW 264.7 cells were a courtesy of Dr Hugo Rocha (Federal University of Rio Grande do Norte, Brazil). NIH/3T3 cells and HMEC were already available in the laboratory.

During subculture, cells were detached by trypsinization with 0.05% (w/v) trypsin-EDTA after reaching 80% confluency. RAW 264.7 cells were dislodge with a cell scraper.

Murine Bone Marrow-Derived Macrophages were collected from femoral and tibial mouse bone marrow of female Balb/c mice. Mouse femurs and tibias were collected under aseptic conditions and flushed with RPMI -1640. The resulting cell suspension was centrifuged at 500 g for 10 min. Pellet was resuspended in RPMI-1640 supplemented with 10×10^{-3} M HEPES, 10% heat-inactivated FBS, 60 μg mL^{-1} penicillin/streptavidin, 0.005×10^{-3} M β-mercaptoethanol (Complete RPMI) and 10% L929 cell conditioned medium (LCCM). To remove adherent bone marrow cells, cell suspension was incubated on cell culture dishes, overnight at 37 °C at 95% humidified air containing 5% CO2 atmosphere. The non-adherent cells were centrifuged at 500 g (10 min) and seeded in 24 well plates at 5×10^5 cells per well, in RPMI complete medium containing 10% of LCCM, and incubated at 37 °C in a 5% CO_2 atmosphere. 4 d after seeding 10% of LCCM was re-added and the medium was renewed on the seventh day, once again with complete RPMI and 10% LCCM. After 10 d, cells were completely differentiated into macrophages.[12,13]

2.6. In Vitro Cell Toxicity

2.6.1. Cell Proliferation Assay

Assessment of cell proliferation impairment on 3T3, HMEC, and RAW 264.7 cells was performed using the MTT reduction assay, adapted from Mosmann.[14] Cells were seeded in 24-well cell culture plates at a density of 1×10^4 cells per well for 3T3 and RAW 264.7 cells and of 2×10^4 for HMEC cells, and left adhering in 0.5 mL of adequate culture medium overnight. HyA-AT nanogel 0.1, 0.5, and 1 mg mL^{-1} dispersions were suspended in adequate culture medium, resulting in a 1:5 fresh medium dilution. For RAW 264.7 cell line the maximum concentration tested was 0.5 mg mL^{-1}. Untreated cells were used as control of 100% cell viability. Another control with 20% distilled water was used to access the effect of water dilution of samples containing the nanogel. A positive control with 20% of DMSO was used in every analysis. The samples were incubated for 24, 48, and 72 h and the cells metabolic activity was calculated due to the reduction of tetrazolium salt of MTT by mitochondrial succinate dehydrogenase enzymes of metabolically active cells. To which well, 10% (v/v) of a MTT solution (5 mg mL^{-1} in PBS) was added and it was incubated at 37 °C and 5% CO_2 for a period of 4 h. In this period of time, the tetrazolium salt was bioreduced to a formazan product that consists of dark blue crystals that were insoluble in the culture medium. The supernatant was discarded slowly and the crystals were solubilized in dimethyl sulfoxide and quantified spectrophotometrically at 570 nm. The experiments were performed in triplicates as the results were presented as percentage in which 100% viability corresponds to the non-treated cells.

2.6.2. Ldh Release Assay

The lactate dehydrogenase (LDH) release assay measures the membrane integrity as function of the amount of cytoplasmic LDH leaked through membrane-impaired cells. The lactate was converted to pyruvate in the presence of LDH with parallel reduction of nicotinamide adenine dinucleotide (NAD), detected as a change in absorbance at 340 nm.[15] Cells were seeded in 12-well plate at a density of the 2×10^5 cells per well for 3T3 and HMEC cell lines and 1×10^5 for RAW and allowed to settle overnight in 0.5 mL of adequate culture medium. The cells were treated with nanogel dispersions with a concentration of 0.1 and 1.0 mg mL^{-1} in suitable culture medium and supplementation. The exception was RAW 264.7 cells that were incubated with 0.1 and 0.5 mg mL^{-1} nanogel dosages. Untreated cells were used as control of 100% cell viability. Another control with 20% distilled water was used to access the effect of the water dilution of the samples containing the nanogel. A control with 20% of DMSO was used in every analysis as a positive control. The samples where incubated for 24 h and after that period each culture medium from every well was collected and centrifuged at 13 000 rpm for 1 min and the cell free supernatant was collected and stored on ice for further analysis – Extracellular LDH. Cells were scraped with a Tris solution (15×10^{-3} M) extracellular and further lysed by sonication. The resulting supernatants were used to quantify the LDH present – Intracellular LDH. An aliquot of extracellular (40 μL) or intracellular (10 μL) LDH were assigned into a microplate and 250 μL of the NADH solution 0.31×10^{-3} M in phosphate buffer 0.05 M, pH7.4 added to each well. Lastly, 10 μL of an 8.96×10^{-3} M piruvate solution in phosphate buffer (substrate solution) was added and immediately afterward the variation of the absorbance at 340 nm was read in a microplate spectrophotometer system, as to determine the rate of NADH consumption (slope of the line). LDH leakage was expressed as the ratio between extracellular and total LDH, corresponding the inverse value to the cell membrane integrity. Each experiment was performed in triplicate.

2.6.3. Apoptosis Assay

The FITC Annexin V Apoptosis Detection Kit was used to detect apoptotic and necrotic cells in 3T3, HMEC, and RAW cell lines. Cells (2×10^5/well) were seeded in a 12-well plate and incubated overnight. Nanogel samples were added to the respective wells in a concentration range of 0.1 to 1.0 mg mL^{-1} dispersed in suitable culture medium and incubated for 24 h. A negative control without any nanogel sample but with 20% distilled water was used – since preliminary studies revealed that untreated cells and cells incubated with 1:5 distilled water, culture medium ratio had similar results. The positive apoptotic control was prepared by culturing the control cells in medium containing H_2O_2 with different incubation times and concentration accordingly to the cells line (0.5×10^{-3} M and 6 h incubation in RAW; 0.2×10^{-3} M and 24 h incubation for HMEC; and 5×10^{-3} M and 3 h incubation for 3T3).[16,17] Cells were then collected by trypsinization 250 μL trypsin/EDTA 0.25%/0.02% in PBS. Cell suspension was transferred to flow cytometry sample tubes (Beckman Coulter) and washed twice with cold PBS. Each sample was incubated with 40 μL of the work solution (1.8 μL of the Annexin V and PI diluted in 36.4 μL of the Annexin V binding buffer) for 15 min at room temperature, in the dark. Finally, 200 μL of Annexin V binding buffer was added to the samples that were then analyzed by flow cytometry using a Coulter Epics XL Flow Cytometer (Beckman Coulter Inc., Miami, FL, USA). Cells were set as positive depending on the fluorescence intensity of Annexin V-FITC or PI. The positive of Annexin V-FITC

indicates the out-releasing of phospholipid phosphatidylserine (PS), which happens in the early stage of apoptosis. The positive of PI indicates the damage of cell membrane, which occurs either in the end stage of apoptosis, in necrosis or in dead cells. Therefore, the apoptotic cells were identified as Annexin positive, and PI negatives – early apoptosis or Annexin and PI positive PI – late apoptosis. Nonviable cells were identified as PI positive and viable cells as Annexin and PI negative.[17]

2.7. Complement Activation Assay

Complement cascade was studied as reported previously[1] and based on the NCL (Nanotechnology Characterization Laboratory) protocol for qualitative determination of total complement activation by Western blot analysis. Briefly, a pool of human plasma from healthy donors was incubated with 1 mg mL^{-1} of GC nanogel in the presence of veronal buffer. Equal volumes (50 μL) of plasma, buffer and sample were mixed and incubated at 37 °C for 60 min. Cobra venom factor from Quidel Corporation (San Diego, CA, USA), and PBS were used as positive and negative controls, respectively. Proteins were resolved by 10% SDS-PAGE, and then transferred to a membrane (Immun-Blot PVDF Membrane, Biorad, Hercules, USA) using the transblot semidry BioRad transfer equipment (Trans blot SD, BioRad, Hercules, USA). The membranes were incubated for 90 min with a mouse monoclonal antibody against human C3 diluted 1:1000 (Abcam, Cambridge, UK), washed and incubation with secondary polyclonal goat anti-mouse IgG antibodies conjugated with alkaline phosphatase diluted 1:2000 (Dako, Glostrup, Denmark). The membrane was finally revealed with 5-Bromo- 4-Chloro-3-Indolyl Phosphate (BCIP) (Sigma). For further analysis, membranes were scanned with ChemiDoc XRS+ System (Bio-Rad; Hercules, CA). The percentage of the lower band was then quantified with Image Lab Software 3.0.

2.8. Murine Bone Marrow Derived Macrophages Nanogel Uptake

The hyaluronic acid nanogel cytocompatibility was further analyzed by the phagocytic activity of murine BMDM. Macrophages were seeded in 24 well plates (5×10^5 cells/well) on top of coverslip discs and were left adhering overnight. Further, cells were incubated for 6 h with 0.2 mg mL^{-1} fluorescein labeled nanogel dispersion in culture medium. Dextrin-FITC labeled nanoparticles (0.2 mg mL^{-1}) were used as a positive control for phagocytic uptake, as described by Gonçalves et al.[18] Fluorescein labeled native HyA (0.2 mg mL^{-1}) was used to compare its phagocytic internalization with the HyA-AT nanogel. Full medium was removed from all the wells and coverslips were washed twice with PBS at room temperature and fixed with 2% paraformaldehyde solution for 25 min. After, 4′,6-diamidino-2-phenylindole (DAPI, 120 ng mL^{-1}) was used to stain the nucleus for 3 min at room temperature. Cells were observed in a confocal laser scanning microscope Leica SP2 AOBS SE (Leica Mycrosystems, Germany).

2.9. Haemolysis Index

The haemolysis assay was performed in agreement to the procedure described by the American Society for Testing Materials[19] and used in previous works.[20] Whole blood was collected from three independent healthy donors using citrated blood collection tubes. Briefly, 0.5 mL of diluted blood at 10 mg mL^{-1} was added to 3.5 mL of the nanogel solution in PBS at 0.1, 0.5, and 0.1 mg mL^{-1} and incubated at 37 °C for 3 h. The tubes were gently mixed at 30 min frames to homogenize the mixture. Ultrapure water and Phosphate-buffered saline (PBS) were used as positive and negative haemolytic control, respectively. The suspension was centrifuged at 750 g for 15 min and 0.5 mL of the supernatant was collected. Then, 0.5 mL of Drabkin's reagent was added and the solution was left incubating for 15 min at room temperature. Finally, the absorbance at 540 nm was measured by UV–vis spectroscopy (JASCO V560). Haemoglobin standard solutions were prepared from bovine blood haemoglobin to elaborate a calibration curve to infer the haemoglobin content of the samples. Experience was made in triplicates.

2.10. In Vivo And Ex Vivo Near-Infrared Fluorescence (NIRF) Imaging

All experiments with live animals were performed in compliance with the Portuguese General alimentary and Veterinarian Board (authorization number 006315/27/03/2014, from DGAV-Portugal) and animals were kept and used strictly in accordance with National rules and the European Communities Council Directive (86/609/EEC), for the care and handling of laboratory animals. Athymic nude mice CD1-Foxn1nu mice (6-weeks old) were purchased from Charles River Laboratories International, Inc. HyA-AT-Cy5.5 labeled nanogel dispersion and native HyA-Cy5.5 solution were injected intravenously into the mice via tail vein ($n = 5$) at a 5 mg kg^{-1} animal body weight. At established time points (2, 7, 24, and 48 h) mice were anesthetised with Ketamine 75 mg kg^{-1} BW and Medetomidine 1 mg kg^{-1} BW solution prior to its analysis and blood sample collection.

The time-dependent biodistribution and accumulation profiles of samples were observed by using a Xenogen's IVIS Lumina Series and Living Image Software. To evaluate the blood clearance at all time points, 50 μL venous blood was collected from the retro-orbital vein and transferred to a 96-well plate and analyzed at each time point. The minimum amount of blood was collected, respecting the animal size and time schedule (50 μL per analysis). To observe the organ distribution of the samples, each group of mice was sacrificed with a lethal dose of anaesthesia 48 h post samples injection. Then, major organs were excised and transferred to a 6-well plate and observed using the Xenogen's IVIS Lumina Series and Living Image Software. NIR fluorescence images obtained with a 12-bit CCD camera equipped with a special C-mount lens and Cy5.5 bandpass emission filter (680–720 nm).

2.11. Statistical Analysis

The results were expressed as mean ± SD of 3 independent experiments ($n = 3$). Statistical analysis was performed with t-test or two-way ANOVA followed by Tukey's comparison test using using GraphPad Prism version 6.00 for Mac OS X, GraphPad Software, La Jolla California USA. Significance of the results was indicated according to P values with one, two, three or four of the used symbols (*, # or +) corresponding to $P = 0.01$ to 0.05; $P = 0.001$ to 0.01; $P = 0.0001$ to 0.001 and $P < 0.0001$, respectively).

3. Results And Discussion

3.1. Hya-At Nanogel Characterization

The chemical conjugation of a hydrophobic chain to HyA was already fully characterized in our previous study.[5] The resulting amphiphilic molecule (HyA-AT) self assembles in aqueous environment onto nanosized structures. The morphology and size of the nanogel was evaluated by Cryo-FESEM and DLS analysis regarding its size distribution by intensity, as shown in Figure 1a. The particles were apparently spherical and well dispersed without any aggregation, and the nanogel reveals a bimodal size distribution with a mean size diameter of 80.2 +/– 0.4 nm ($n = 5$).[5]

It is well known that protein adsorption to nanomaterials has high impact on the interaction of nanomaterials with cells, both in vivo and in vitro. Therefore, we wanted to assess if the serum supplementation used in culture medium affected the size distribution profile

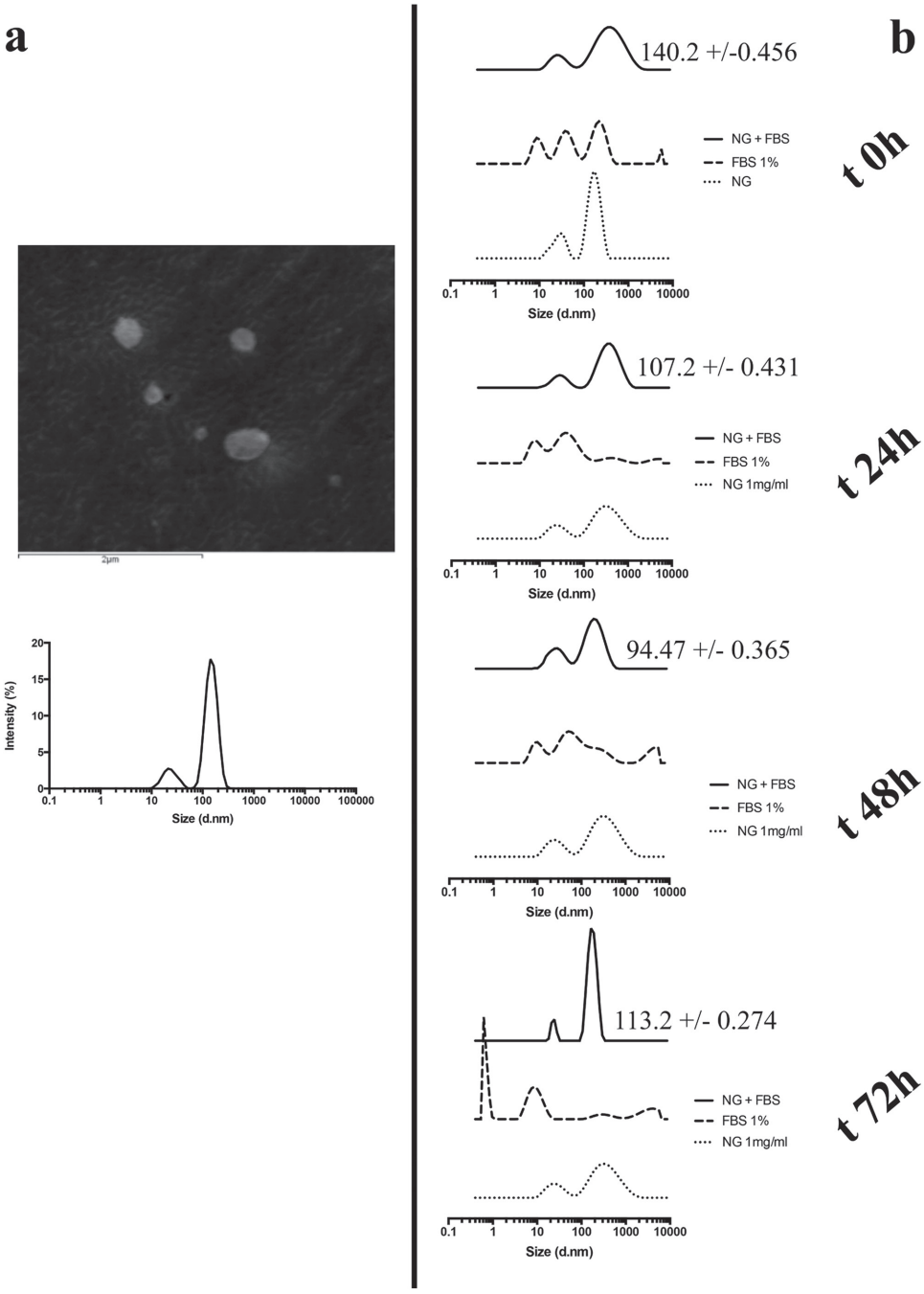

Figure 1. Nanogels size and morphology characterization and serum stability.

of the nanogel. As can be seen in Figure 1b, we observed the interaction of the nanogel with FBS 1% (v/v) by DLS analysis, in the course of 72 h. Indeed, nanogel showed colloidal stability and its average size diameter, around 80 nm, maintained constant throughout time. The serum proteins size distribution profile fluctuated through time, probably due to the formation of unstable aggregates.

Section a: Nanogels size distribution profile by intensity through DLS analysis and Cryo-Field-Emission Scanning Electron Microscopy (Cryo-FESEM).

Section b: Nanogel size stability by intensity through DLS analysis, in the presence of serum proteins (FBS), over time.

3.2. Cytotoxicity Studies

Cytotoxicity studies were performed in three cell lines: 3T3, HMEC, and RAW 264.7. 3T3 fibroblasts were chosen as a model for stromal cells, which can be found in matrix and connective tissue throughout the body. Human microvascular endothelial cell line (HMEC-1) was used to investigate the possible cytotoxic effects in vasculature. RAW 264.7 are murine macrophages cell line, which are commonly included in nanomaterial toxicity investigations as an inflammatory cell type.

In the case of RAW macrophages we only tested a maximum 0.5 mg mL^{-1}, since macrophages can readily phagocytose nanomaterials at a very high rate and lead to overload and cell death. Thus, we didn't feel the need to test higher concentrations in this case.[21,22] In addition to different nanogel concentrations, DMSO was used as a positive control and two negative controls were performed – one consisting of 100% culture medium and the other of culture medium diluted with 20% water. This last control is actually the most relevant one, since it mimics the water dilution effect with the nanogel samples (which slightly affects the cell growth).

3.2.1. Cell Proliferation

MTT is a colorimetric, easy, fast and safe assay that measures the mitochondrial metabolic activity of viable cells (Figure 2). We observed that the water-diluted control had a slightly lower cell growth or activity may be due to the dilution of nutrients of the culture medium. This effect was most noticed in the longer incubation time (Figure 2). The metabolic activity was not, overall, evidently affected be the nanogels presence, however at longer incubation time (72 h) and highest dose, a reduced cell proliferation or activity was observed in 3T3 and RAW cells – in comparison to the diluted medium control. RAW cells seem to be more susceptible to nanogel treatment, but even in this case a slightly lower proliferation is observed only with highest incubation time.

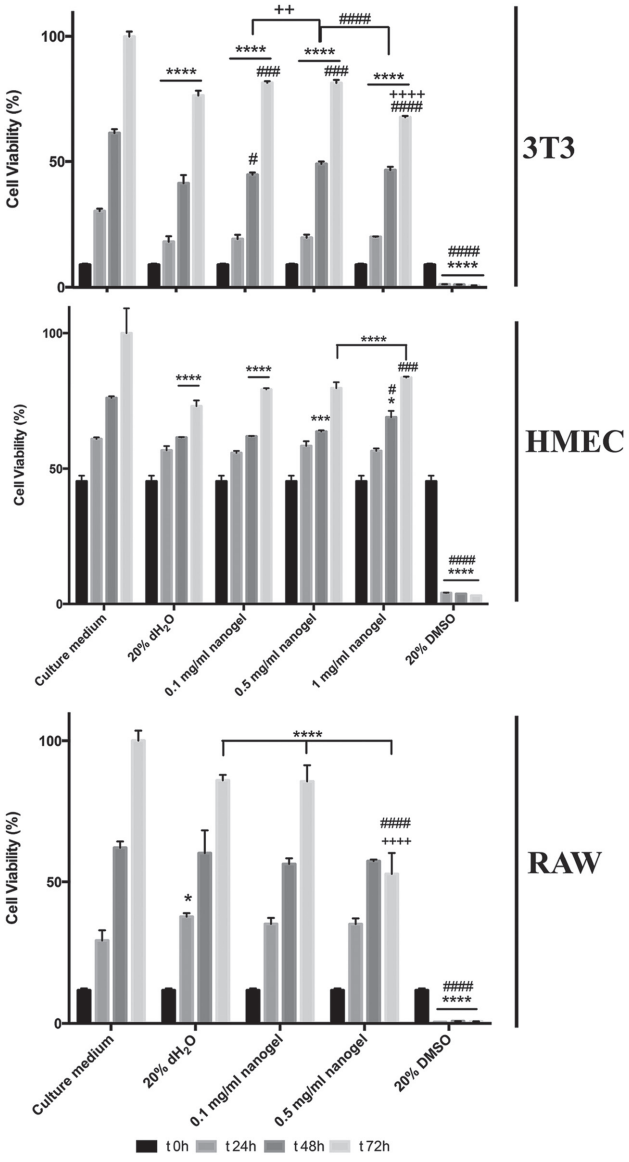

Figure 2. Cell viability of 3T3, HMEC, and RAW cells determined by MTT assay as to exposure to HyA-AT nanogel at 0.1 to 1 mg mL^{-1} concentration. Non-treated cells referred to as culture medium are considered 100% cell viability at 72 h. Statistical analysis was performed using a two-way ANOVA and a Tukey's comparison test. Differences between samples and culture medium at any given time point are represented by (*); whereas differences between samples and 20% dH_2O diluted control at any given time point are represented by (#); differences between nanogel concentration are represented by (+).

Similar results were obtained by other researchers[20,23] when studying nanoparticles effect on macrophage cell lines. For instance, poly(ethylene glycol) (PEG)-conjugated hyaluronic acid nanoparticles, showed dose-dependent cytotoxicity to cancer cells (MDA-MB-231, SCC7, and HCT 116) and significantly lower cytotoxicity against normal fibroblasts (NIH-3T3).[24] Fairly high nanogel concentrations were tested (up to 1.0 mg mL^{-1})

to effectively detect toxic effects and objectively assess the safety of the material.

3.2.2. Evaluation Of Membrane Cell Integrity

The evaluation of cell membrane integrity was performed by LDH release assay. As shown in Figure 3, membrane integrity was preserved at all nanogel concentrations for all cell lines. This indicates that the nanogel did not affect membrane stability in any of the cells tested – 3T3, HMEC, and RAW. According to Fotakis and Timbrell,[15] LDH release is not as sensitive as the MTT assay and requires higher concentration of sample or longer incubation time for the detection of cytotoxic effects. However, even at high nanogel dosage such as 1 mg mL^{-1}, any effect was observed.

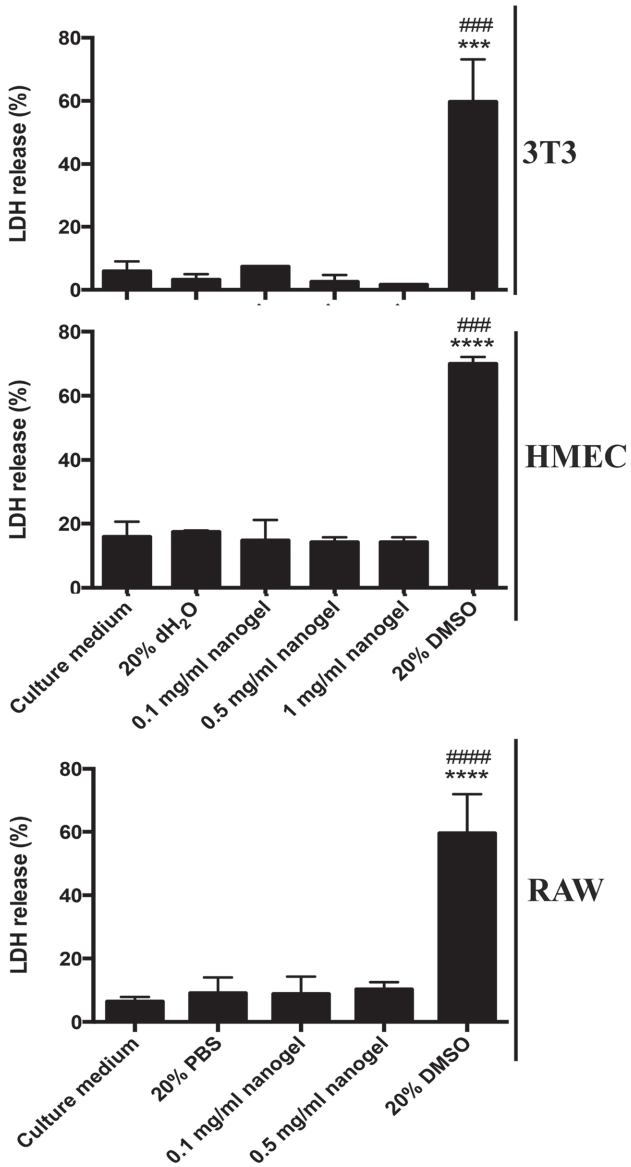

Figure 3. Cell membrane integrity of 3T3, HMEC, and RAW cells determined by LDH release assay as to exposure to HyA-AT nanogel at 0.1 to 1 mg mL^{-1} concentration. Results are present as LDH release percentage after 24 h sample incubation. Statistical analysis was performed using a t-test. Differences between samples and culture medium are represented by (*); whereas differences among samples and 20% dH_2O control are represented by (#).

3.2.3. Apoptosis Assay

Nanogel induced apoptosis was determined by annexin V-FITC and PI double staining resorting to flow cytometry, as shown in Figure 4. Early apoptosis is characterized by plasma membrane reorganization (translocation of phosphatidylserine to the external surface), detected by positive staining for Annexin V-FITC. In later stage of apoptosis cells present membrane damage, therefore PI can bind to DNA in cytoplasm resulting in positive staining for both Annexin V and PI.[25] In all cell lines tested no significant effect by nanogels presence was noticed in comparison with the negative control (20% water diluted culture medium). Nanogel interaction with HMEC cells caused a slight increase in only late apoptic population (Annexin V positive) regarding control cells. The observed effect was indeed dose dependent.

3.3. Complement Activation

The nanogel effect on the complement cascade activation was evaluated by the cleavage of C3, which is a marker for both activation pathways. Western blot analysis for the presence of the C3 fragment was performed after incubation of the HyA-AT nanogel (at 1 mg mL^{-1} concentration) with human plasma. The results are shown in Figure 5. The upper band of 115 kDa corresponds to the intact C3 factor and the one with 43 kDa to the main degradation product. The protein degradation was quantified considering the intensity of the band at 43 kDa normalized to the value obtained with the positive control (cobra venom factor). As could be observed in Figure 5, the percentage of C3 cleavage product(s) was similar to those found in the negative control, so it may be concluded that the nanogel does not activate the complement system.

3.4. Murine Bone Marrow Derived Macrophages Nanogel Uptake

The phagocytic recognition and nanogel uptake by BMDM was investigated by confocal microscopy. Murine BMDM are extensively used as a phagocytic model due to its peculiar capacity of internalizing extracellular materials by a wide range of mechanisms and entry routes.[26] Macrophages were incubated with Dextrin-FITC labeled nanogel (Figure 6a), native HyA-Fluorescein labeled (Figure 6b), and HyA-AT-Fluorescein labeled nanogel (Figure 6c), the different formulations (used at the same

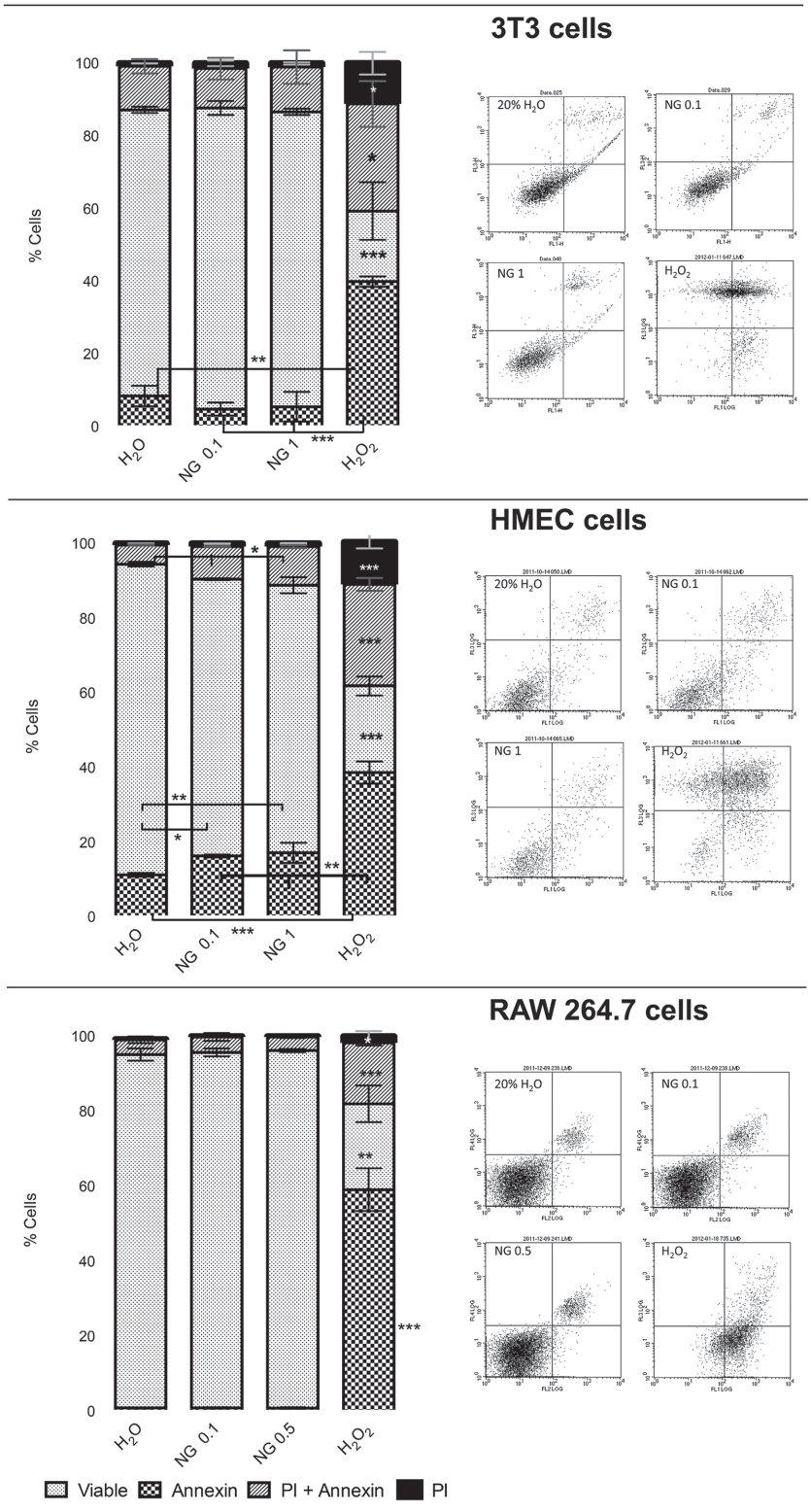

Figure 4. Flow cytometry analysis of 3T3, HMEC, and RAW cell line for the presence or absence of the Annexin v-FITC and/or PI markers. Cells were previously incubated with two different nanogel concentrations for 24 h. A negative control with 1:5 distilled water-diluted culture medium and hydrogen peroxide was used as apoptosis positive control. Statistical analysis was performed using a t-test and a Tukey's comparison test. Differences between samples and culture medium are represented by (*). Dot Plots of the correspondent cell lines are presented at the right side of the image. Top left quadrants matches annexin V negative and PI positive cells (legend: PI); top right quadrants corresponds to late apoptotic cells that express annexin V and PI positive (legend: Annexin + PI); bottom right quadrants pairs with apoptotic cells that express annexin V positive and PI negative (legend: annexin); and for last, bottom left quadrants, viable cells that does not express neither annexin V or PI.

concentration) presenting similar levels of fluorescence. Also, untreated cells were observed, as a control (figure 6d). Gonçalves, et al.[18] have demonstrated that dextrin nanogels were extensively recognised and internalized by BMDM. Therefore, dextrin-FITC labeled nanogel was used as a positive control (Figure 6A). As it was expected, the dextrin nanogel incubated BMDM cells presented an intense green staining, demonstrating much higher internalization than the HyA-AT nanogels. Interestingly, the native HyA was slightly more internalized by BMDM than HyA-AT nanogel (Figure 6). This result suggests a promising behaviour in vivo, i.e., the ability of the nanogel to escape blood clearance and exhibit a large circulation time in the vascular system.

3.5. Hemocompatibility Study

Experiment was performed in agreement with the Standard Practice for Assessment of Haemolytic Properties of Materials from the American Society for Testing Materials (ASTM F756-00, 2000). Nanogel proved to be non-haemolytic at the concentrations tested, since the corrected haemolytic index is inferior to 5% (Figure 7). Still, as compared to the negative control, no visible haemolytic effect was observed in the presence of the nanogel.

3.6. In Vivo Nanogel Biodistribution Profile

In vivo biodistribution analysis is an important tool to assess the potential of nanocarriers as delivery systems.[27]

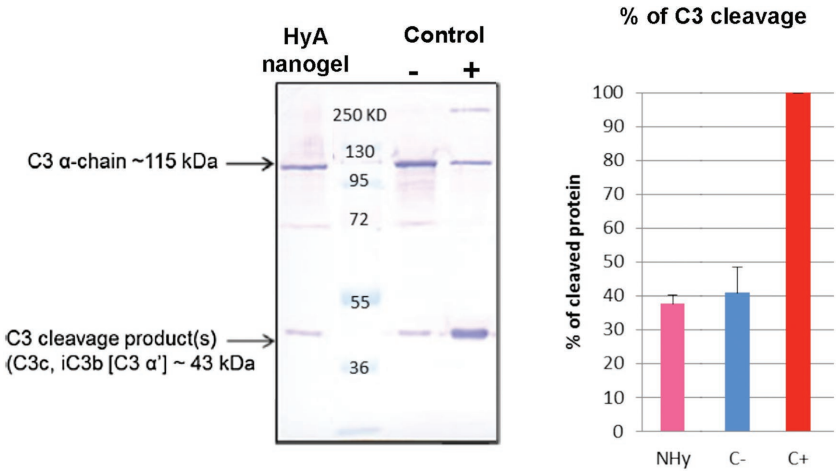

Figure 5. Analysis of HyA-AT nanogel complement activation through C3 protein cleavage by western blot. A) Western blot membrane is presented on the left and B) graphical representation of the % of C3 protein as comparison to PBS and Cobra venon, negative and positive controls, respectively.

Nude mice were intravenously injected in the tail vein (5 mg kg^{-1} animal weight). Native HyA-Cy 5.5 was used as a control and administered following the same protocol.

In vivo biodistribution was monitored non-invasively as a function of time, over a period of 48 h (Figure 8a). At each time point, NIRF images of the whole animal were obtained and blood samples were also collected, from every animal, by retro-orbital punction. By the analysis of the whole body images we can say that the animals treated with the nanogel seem to exhibit higher fluorescence intensity at all time points, in comparison to native HyA treated animals, as further confirmed by observing the blood-collected samples. In fact, 24 h post injection, the native HyA fluorescence was almost absent from mice whole body (Figure 8a) and undetectable in the blood (Figure 8c), proving its fast clearance.

It is interesting to note that some fluorescence signal is detectable in nanogel treated animals even after 24 h, indicating a fairly long half-live in the circulatory system, relevant for the development of drug delivery systems.

Each organ of the mice was withdrawn at 48 h post-injection, and ex vivo fluorescence images were obtained (Figure 8). For both HyA polymer and HyA-AT nanogel strong signals were observed in the lung mostly, but also in the skin and kidney. Weak intensities were observed

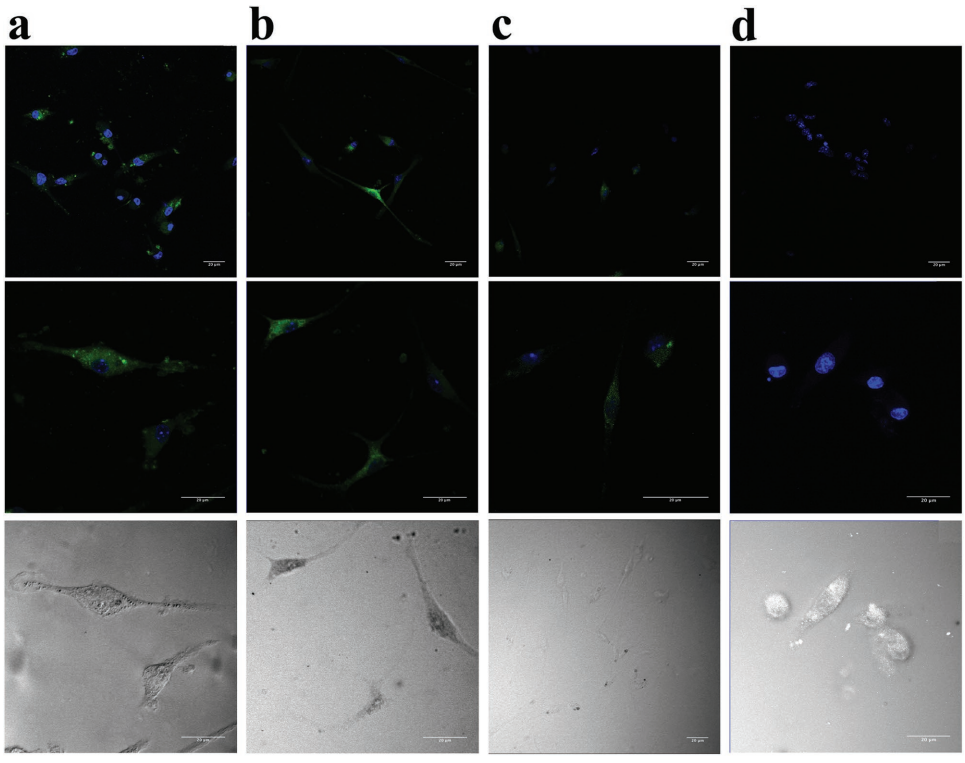

Figure 6. Fluorescence images of murine BMDM obtained by confocal microscopy, incubated for 6 h 0.2 mg mL^{-1} suspension of: a) dextrin nanogel; b) native HyA; c) hyaluronic acid nanogel (HyA-AT); and d) untreated cells, as a control. Cell's nucleus was stained blue with DAPI, 120 ng mL^{-1}. The green fluorescence is due to the fluorescein labeled samples.

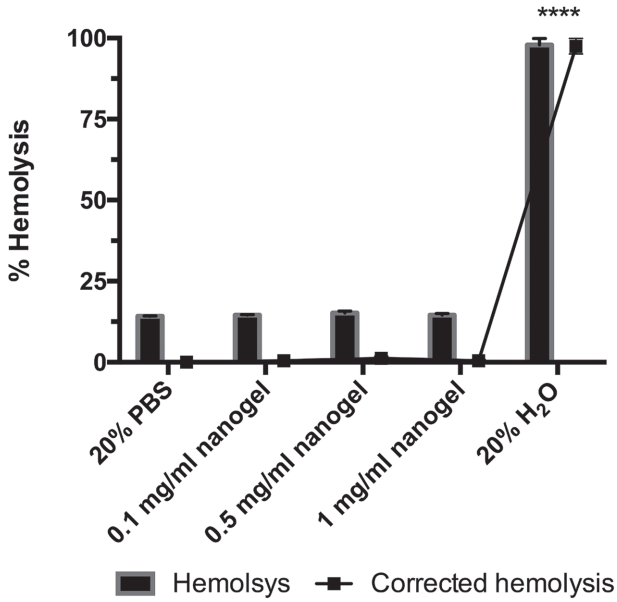

Figure 7. Blood hemolysis index of whole human blood from healthy donors after incubation with 0.1 until 1 mg mL^{-1} HyA-AT nanogel dispersions and 1:5 PBS diluted culture medium and hydrogen peroxide as negative and positive control, respectively.

in heart, spleen and liver, while in the brain no fluorescence signal was detected. This result is consistent with the BMDM internalization studies, which suggest fairly poor recognition of the nanogel, and thus ability to evade the mononuclear phagocytic system. Concerning the observed nanogels accumulation in the lungs, other researchers[28–31] have also reported lung accumulation of nanoparticles, when labeled with Cy5.5 probe. We were able to further clarify the influence of NIR probe – namely Cy5.5 and Alexa Fluor 680 – on the nanogels pharmacokinetics (to be shown elsewhere) and assign the accumulation in the lungs to the probe and not to the nanogel itself.

4. Conclusion

Amphiphilic HyA-AT conjugate was successfully synthetized and self-assembled onto nanostructures with desirable features for drug delivery applications. The engineered nanogel was extensively characterized as for its biocompatibility. Mitochondrial metabolic activity measurements revealed that only for RAW cells challenged with the highest nanogel concentration at the highest incubation time (72 h), a slight reduction on growth rate was observed. However, this effect was not corroborated by membrane integrity evaluation or apoptosis induction. As a matter a fact, in all the cell lines tested and at all the time points it is not perceptible any inhibitory effect. Also, HyA-AT nanogel did not induce the activation of the

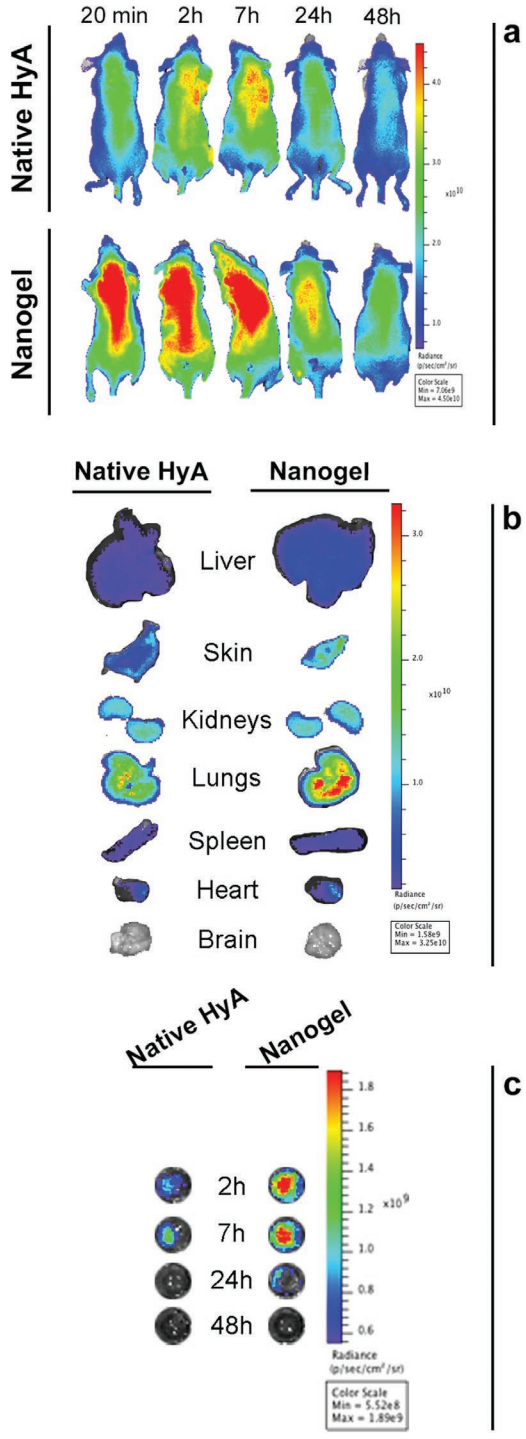

Figure 8. In vivo and ex vivo biodistribution profile of HyA-AT nanogel and native HyA. a) Whole body NIRF images of CD1-Foxn1nu mice treated with native HyA labeled with Cy5.5 hydrazide and HyA-AT nanogel also labeled with the same fluorophore. Top row of animals were administered with native HyA and bottom row with HyA-AT nanogel. b) Ex vivo NIRF images of the organs – Liver, Skin, Kidneys, Lungs, Spleen, Heart and Brain -, 48 h post sample injection. a) Blood sample collected by retro-orbital punction at established time point post sample administration, analyzed in NIRF equipment.

complement system, was poorly recognized and internalized by BMDM and did not cause hemolysis. In vivo biodistribution studies demonstrated the nanogel has a fairly long circulation time, and can be detected in the blood flow up to 48 h. These findings suggest that the nanogel can be a promising drug delivery nanosystem.

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