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Bioinspired hyaluronic acid and polyarginine nanoparticles for DACHPt delivery

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(Article begins on next page)

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1                    **Bioinspired hyaluronic acid and polyarginine nanoparticles for DACHPt delivery**

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1 **Abstract**

2 This work provides insights over a novel biodegradable polymeric nanosystem made of hyaluronic  
3 acid and polyarginine for diaminocyclohexane-platinum (DACHPt) encapsulation. Using mild  
4 conditions based on ionic gelation technique, monodispersed blank and DACHPt-loaded nanoparticles  
5 (NP) with a size of around 200 nm and negative  $\zeta$  potential (-35 mV) were obtained. The freeze-  
6 drying process was optimized to improve the stability and shelf-life of the developed nanoparticles.  
7 After reconstitution, nanoparticles maintained their size showing an association efficiency of around  
8 70 % and a high drug loading (8%). *In vitro* cytotoxicity studies revealed that DACHPt-loaded  
9 nanoparticles had a superior anticancer activity compared with oxaliplatin solution. The IC50 was  
10 reduced by a factor of two in HT-29 cells (IC50 39  $\mu$ M vs 74  $\mu$ M, respectively), and resulted almost  
11 1.3 fold lower in B6KPC3 cells (18 $\mu$ M vs 23 $\mu$ M respectively). Whereas toxic effects of both drug and  
12 DACHPt-loaded nanoparticles were comparable in the A549 cell line (IC50 11  $\mu$ M vs 12  $\mu$ M).  
13 DACHPt-loaded nanoparticles were also able to modulate immunogenic cell death (ICD) *in vitro*.  
14 After incubation with B6KPC3 cells, an increase in HMGB1 (high-mobility group box 1) production  
15 associated with ATP release occurred. Then, *in vivo* pharmacokinetic studies were performed after  
16 intravenous injection (IV) of DACHPt-loaded nanoparticles and oxaliplatin solution in healthy mice  
17 (35.9  $\mu$ g of platinum equivalent/mouse). An AUC six times higher (24 h\*mg/L) than the value  
18 obtained following the administration of oxaliplatin solution (3.76 h\*mg/L) was found.  $C_{max}$  was  
19 almost five times higher than the control (11.4 mg/L for NP vs 2.48 mg/L). Moreover, the reduction in  
20 volume of distribution and clearance clearly indicated a more limited tissue distribution. A simulated  
21 repeated IV regimen was performed *in silico* and showed no accumulation of platinum from the  
22 nanoparticles. Overall, the proposed approach discloses a novel nano-oncological treatment based on  
23 platinum derivative with improved antitumor activity *in vitro* and *in vivo* stability as compared to the  
24 free drug.

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## 1 **Introduction**

2 Platinum-based antitumor agents are among the most widely used anticancer drugs for treatment of  
3 lung, colorectal, ovarian, head and neck, breast, and bladder cancers [1,2]. Cisplatin, the first  
4 compound approved by the US Food and Drug Administration (FDA) [3] in 1979, had a profound  
5 impact on cancer management and is now commonly used in medical oncology. However, serious  
6 concerns due to resistance phenomena and dose limiting toxicity hamper its clinic use. To overcome  
7 these limitations, significant efforts have been devoted to develop novel analogues with a better  
8 toxicity profile and improved activity. Modification including the replacement with an oxalate ligand  
9 as leaving group and the diaminocyclohexane (DACH) ligand in the trans-L isomer led to the design  
10 of oxaliplatin (trans-L-diaminocyclohexane oxalate platinum (II)). Oxaliplatin is a prominent third-  
11 generation drug that retains the antitumor properties of cisplatin without its clinical toxicity profile [4].  
12 In addition to DNA damage, oxaliplatin induces ribosome biogenesis stress [5] and helps generating  
13 immune response against tumors by triggering the immunogenic cell death (ICD) [6,7]. Despite these  
14 positive features in terms of efficacy, neurotoxicity with repeated cycles of oxaliplatin administration  
15 [8] and cases of renal failure have been reported [9,10].

16 In this context, nanomedicines hold great promise to improve platinum-based anticancer treatments by  
17 reducing drug toxicity and enhancing therapeutic efficacy by tissue- or cell- targeting. So far, many  
18 efforts have been made to develop nanocarriers entrapping oxaliplatin or its derivatives [1,11–15].  
19 The encapsulation of platinum-based drugs in liposomes showed an improved anticancer activity on  
20 several tumor models in comparison with non-encapsulated drug [16]. MBP-426, a liposomal  
21 formulation carrying oxaliplatin and decorated with transferrin, is currently in Phase II clinical  
22 development in combination with leucovorin and 5-fluorouracil for the treatment of gastric  
23 adenocarcinoma [17]. Polymeric nanoparticles (NP) and micelles were also investigated for platinum  
24 delivery. NP made of N-(2-hydroxypropyl)methacrylamide (HPMA), AP5346 (ProLindac®, Access  
25 Pharmaceuticals, Inc.), conjugated to DACHPt showed a superior activity in terms of tumor growth  
26 inhibition and safety profile [18,19]. Cabral and colleagues developed a co-polymeric micelle  
27 composed of PEG-b-(poly)glutamic acid for cisplatin or (1,2-diaminocyclohexane)platinum(II)  
28 (DACHPt) encapsulation [20,21]. DACHPt micelles exhibited remarkably prolonged blood circulation  
29 and greater accumulation in tumor tissue compared to free oxaliplatin [20].

30 Based on this background, the aim of the present work has been to design and develop a novel NP-  
31 based system made of bioinspired polymers such as hyaluronic acid (HA) and polyarginine (PA) for  
32 DACHPt encapsulation [22]. HA and PA have been chosen due to their non-toxicity, biodegradability  
33 and biocompatibility profile [23]. HA is an anionic polysaccharide widely used in biomedical  
34 applications [24–26]. It is a glycosaminoglycan and an important component of the extracellular  
35 matrix (ECM). Moreover, it can specifically recognize membrane glycoproteins such as CD44  
36 receptors overexpressed by various cancer cells [27,28]. PA is a cationic polyaminoacid consisting in

1 the repetition of 28 to 32 arginine moieties, that can be degraded by peptidases in the body ensuring  
2 high biocompatibility [29]. DACHPt-loaded NP were optimized, physico-chemically characterized  
3 and their cytotoxic activity was assessed *in vitro* against pancreatic (B6KPC3), lung (A549) and colon  
4 (HT-29) cancer cells lines. The ability of DACHPt-loaded NP to induce ICD by quantifying release of  
5 alarmins such as HMGB1 (High-mobility group box 1) and ATP were also evaluated. The uptake  
6 mechanism of these nanosystems by cancer cells was also evaluated in correlation with CD44  
7 expression. Finally, the *in vivo* behaviour of the developed NP was assessed in a pharmacokinetic  
8 study following intravenous administration (IV) in healthy mice.

## 9 10 **Materials and methods**

### 11 **Chemicals**

12 Dichloro(1,2-diaminocyclohexane)platinum(II) (DACHPt-Cl<sub>2</sub>, *M<sub>w</sub>* = 380.17 Da), AgNO<sub>3</sub> (*M<sub>w</sub>* =  
13 169.97 Da), mannitol (*M<sub>w</sub>* = 182 Da), N1 medium supplement 100X (0.5 mg/mL recombinant human  
14 insulin, 0.5 mg/mL, human transferrin - partially iron-saturated-, 0.5 µg/mL sodium selenite,  
15 1.6mg/mL putrescine, and 0.73 µg/mL progesterone; Sigma-Aldrich) and Dulbecco's Phosphate  
16 Buffer Saline (PBS) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Poly-L-  
17 Arginine (PA) hydrochloride (*M<sub>w</sub>* = 5800 Da) was purchased from Alamanda® Polymers (Huntsville,  
18 Alabama, USA). Low molecular weight hyaluronic acid (HA) (*M<sub>w</sub>* ≈ 20 kDa) was purchased from  
19 Lifecore® Biomedical (Chaska, Minnesota, USA). MTS cell titer 96® Aqueous One was provided by  
20 Promega (Charbonnières-les-Bains, France). Oxaliplatin solution (Hospira®, Meudon, France) is a  
21 kind gift from CHU Angers, France. Alexa Fluor 647 carboxylic acid, TRIS (triethylammonium) salt  
22 (0.8 mg/mL) was purchased from Invitrogen (Merelbeke, Belgium). Deionized water (MilliQ water)  
23 was obtained from a Milli-Q plus system (Merck-Millipore, Darmstadt, Germany).

### 24 25 **Preparation of DACHPt**

26 DACHPt-Cl<sub>2</sub> was suspended in MilliQ water and mixed with silver nitrate (AgNO<sub>3</sub>) to form an  
27 aqueous complex ([AgNO<sub>3</sub>]/[DACHPt]=1) [12]. The solution was kept in the dark under magnetic  
28 stirring (500 rpm) at room temperature (22°C) for 24h. Silver chloride precipitates were found after  
29 reaction and eliminated by centrifugation at 2,000 g for 20 min at room temperature (22°C). The  
30 DACHPt solution was recovered from the supernatant, filtered through a 0.22 µm filter and stored at  
31 +4°C.

### 32 33 **Preparation of polyarginine hydroxide**

34 Polyarginine hydroxide (PA-OH) solution was prepared from PA-Cl using an Amberlite® IRA 900 Cl  
35 ion exchange resin (Sigma Aldrich, Saint Quentin Fallavier, France). Briefly, 3 mL of NaOH (1 M)  
36 were added to a column containing 1 mL of wet resin. After 30 min, the column was rinsed with  
37 MilliQ water to reach a final pH of 7. Then, PA-Cl solution (50 mg/mL) was added on the top of the

1 column and PA-OH was recovered. The column was rinsed with MilliQ until the solution reached the  
2 desired concentration (12.5 mg/mL) and pH around 11. PA-OH stock solution was then stored at  
3 +4°C.

#### 4 5 **Design and development of blank, fluorescent and DACHPt-loaded hyaluronic acid-polyarginine** 6 **nanoparticles**

7 Blank nanoparticles (NP) made of HA and PA-OH were obtained using the ionic gelation technique  
8 [30,31]. All the solutions were filtered through a 0.22  $\mu\text{m}$  filter prior to their use. To obtain NP, 500  
9  $\mu\text{L}$  of HA solution (concentration ranging from 0.5 to 12 mg/mL, pH = 5) were added to 500  $\mu\text{L}$  of  
10 PA-OH solution (concentration 2 mg/mL, pH = 11) and left under magnetic stirring for 5 minutes. To  
11 prepare DACHPt-loaded NP, 500  $\mu\text{L}$  of DACHPt (1.5 mg/mL) and 500  $\mu\text{L}$  of PA-OH solution (2  
12 mg/mL) were mixed at stirred at room temperature. Then, 500  $\mu\text{L}$  of HA solution (concentration  
13 ranging from 0.5 to 12 mg/mL) were poured over the DACHPt and PA-OH solution, mixed and left  
14 under magnetic stirring for 5 min.

15 Alexa Fluor 647 carboxylic acid was used to label blank HA-PA NP. Prior to obtain NP, 80  $\mu\text{L}$  of PA-  
16 OH solution (2.5 mg/mL) and 120  $\mu\text{L}$  of Alexa Fluor 647 carboxylic acid, TRIS (triethylammonium)  
17 salt (0.8 mg/mL) were mixed in a glass amber vial with a magnetic stirrer. Then, 100  $\mu\text{L}$  of HA  
18 solution (9 mg/mL) was added to the mix and the dispersion was kept under magnetic stirring for 10  
19 minutes. To isolate the NP, 300  $\mu\text{L}$  of the nanocarrier dispersion was transferred to an eppendorf  
20 microtube containing 20  $\mu\text{L}$  of glycerol and centrifuged at 16,000 g for 30 min at 25°C (Hettich Mikro  
21 220, Sérésin-du-Rhône, France). Subsequently, the supernatant was discarded and the pellet was  
22 resuspended in distilled water through vigorous vortexing.

#### 23 24 **Physicochemical characterization of nanoparticles**

25 Particle size (z-average) of blank and DACHPt-loaded NP was determined by Dynamic Light  
26 Scattering (DLS). Samples were diluted to an appropriate concentration in deionized water and each  
27 analysis was carried out at 25°C with an angle detection of 173°. The zeta potential values were  
28 calculated from the mean electrophoretic mobility values, as determined by Laser Doppler  
29 Anemometry (LDA). For LDA measurements, samples were diluted in NaCl solution (1 mM) and  
30 placed in an electrophoretic cell. DLS and LDA analyses were performed on three independently  
31 prepared samples using Malvern Zetasizer® apparatus DTS 1060 (Nano Series ZS, Malvern  
32 Instruments S.A., Worcestershire, UK). Transmission electron microscopy (TEM) was performed with  
33 a Philips CM120 microscope at “Centre Technologique des Microstructures” (CT $\mu$ ) at the University  
34 of Lyon 1 (Villeurbanne, France). A small drop of suspension (5  $\mu\text{L}$ ) was deposited on a  
35 carbon/formvar microscope grid (Delta Microscopies, Saint-Ybars, France), stained with a 1% w/w  
36 sodium silicotungstate aqueous solution, and slowly dried in open air. The dry samples were observed

1 by TEM under 120 kV acceleration voltage. The osmolality of the formulations was measured using a  
2 Wescor Vapro 5520 Vapor Pressure Osmometer (ELITECH group, Signes, France).

3

#### 4 **Freeze-drying of blank and DACHPt-loaded nanoparticles**

5 Blank and loaded NP were freeze-dried using an ALPHA 1-4 LSC (CHRIST) freeze dryer equipped  
6 with RZ6 Vacubrand pomp (Fisher Scientific, Illkirch, France). The bulking agent mannitol was  
7 prepared at 10 % w/v and used to dissolve HA and dilute PA solutions before formulation. The final  
8 mannitol concentration in the formulations was around 6% w/v. Size (z-average), polydispersity, zeta  
9 potential, osmolality, drug loading and entrapment efficiency were evaluated after resuspension of the  
10 freeze-dried powders in MilliQ water.

11

#### 12 **Encapsulation efficiency and loading capacity of DACHPt-loaded nanoparticles**

13 The encapsulation efficiency (EE) was determined indirectly by measuring the unbound DACHPt in  
14 the aqueous phase. DACHPt-loaded NP were centrifuged at 7,000 g for 30 min at 20°C (Hettich Mikro  
15 220, Sérésin-du-Rhône, France) using Amicon Ultra-filter (Cut-off 30kDa, Merck Millipore, Cork,  
16 Ireland). Supernatants were recovered and analyzed by inductively coupled plasma optical emission  
17 spectrometry (ICP-OES). The total amount of drug was estimated from a fresh aliquot of the NP  
18 formulation. From the EE values, the drug loading (DL) of DACHPt-loaded NPs was calculated using  
19 the following Equation 1 and 2 respectively:

20

$$21 \quad EE (\%) = \frac{\text{Total drug} - \text{Free drug in supernatant}}{\text{Total drug}} \times 100 \quad (1)$$

22

$$23 \quad DL (\%) = \frac{\text{Mass of encapsulated drug}}{\text{Mass of the nanoparticles}} \times 100 \quad (2)$$

24

25 All measurements were performed in triplicate using DACHPt aqueous solution as control.

26

#### 27 **Platinum quantification using inductively coupled plasma optical emission spectrometry**

28 ICP-OES measurements were performed on a Thermo Scientific iCAP 7000 Plus Series ICP-OES  
29 (ThermoFischer Scientific, Les Ulis, France). Samples were assayed in axial mode, using a  
30 plasmaflow of 12 mL/min and nebulizer flow of 0.5 mL/min, in three replicates. Prior to  
31 measurement, the samples were diluted in 1% v/v HNO<sub>3</sub>. All the standard solutions were daily  
32 prepared by diluting the stock solution in 1% v/v HNO<sub>3</sub>. Elemental certified stock solutions of 1000  
33 mg/L containing Pt (Merck, Darmstadt, Germany) were used for calibration prior to element analysis  
34 by ICP-OES (1–50 mg/L). Emission wave-length  $\lambda_{Pt}$  = 214.4 nm was monitored for Pt, and was based  
35 on the best correlation coefficient of the calibration curve. Instrumental limits of detection (LDs) and

1 quantification (LQs) obtained by ICP-OES were calculated following the  $3\sigma$  and  $10\sigma$  criteria,  
2 respectively. Methodological LDs obtained was 0.4  $\mu\text{g/L}$  by ICP-OES.

#### 4 **Single particle tracking of fluorescent NP (f-NP) in distilled water and human serum**

5 Fluorescence Single particle tracking (fSPT) was used to characterize the diffusion of NP in distilled  
6 water and serum. fSPT made use of an iXon ultra EMCCD camera (Andor Technology, Belfast, UK)  
7 and swept-field confocal (SFC) microscope (Nikon eclipse Ti, Tokyo, Japan) equipped with an MLC  
8 400 B laser (Agilent technologies, Santa Clara, California, USA) to obtain movies of single  
9 fluorescent labelled particles. fSPT measurements on f-NP in biological fluid (90% in volume) were  
10 performed as described below. Firstly, the nanoformulations were diluted 15 times in distilled water.  
11 Then 5  $\mu\text{L}$  of the prepared dilution was added to 45  $\mu\text{L}$  of buffer or human serum (90% vol.) and  
12 incubated for 1 h at 37°C. Then, 7  $\mu\text{L}$  of the sample was mounted on a microscope slide in the middle  
13 of a secure-seal adhesive spacer (8 wells, 9 mm diameter, 0.12 mm deep, Invitrogen, Merelbeke,  
14 Belgium). The slide was covered by a cover slip (24  $\times$  50 mm) in order to avoid evaporation of the  
15 sample and allow for free diffusion entirely. Subsequently, the slide was placed on the swept field  
16 confocal microscope and movies were recorded focused at about 5  $\mu\text{m}$  above the bottom of the  
17 microscope slide. Videos were recorded at room temperature (22.5°C) with the NIS Elements software  
18 (Nikon, Tokyo, Japan) driving the EMCCD camera and a swept-field confocal microscope equipped  
19 with a CFI Plan Apo VC 100 $\times$  NA1.4 oil immersion objective lens (Nikon, Tokyo, Japan). Analysis of  
20 the videos was performed using in-house developed software. Human serum was obtained from a  
21 healthy donor as described before [32]. Analyzing movies obtained in fSPT with in-house image  
22 processing software [33] as performed in this work led to a distribution of diffusion coefficients,  
23 which was converted into size distribution using the Stokes-Einstein equation taking into account the  
24 viscosity of the biofluids at which the experiment was performed.

#### 26 ***In vitro* experiments**

##### 27 **Cell lines**

28 B6KPC3 murine pancreatic ductal adenocarcinoma cancer cell line was obtained from the Department  
29 of Oncology and Surgical Sciences of Padova University (Italy). B6KPC3 cells were isolated from  
30 transgenic mice KPC that spontaneously develop pancreatic ductal adenocarcinoma. B6KPC3 cells  
31 medium was composed of Dulbecco's Modified Eagle Medium, DMEM (Lonza, Verviers, Belgium)  
32 supplemented with 10% fetal bovine serum (FBS), L-glutamine 2 mM, HEPES buffer 10 mM, 1% of  
33 antibiotics (10,000 units penicillin, 10 mg streptomycin, 25  $\mu\text{g}$  of amphotericin B/mL solubilized in  
34 appropriate citrate buffer) and  $\beta$ -mercaptoethanol 20  $\mu\text{M}$  (Sigma-Aldrich, Saint-Quentin-Fallavier,  
35 France). Human lung alveolar carcinoma (A549) were provided by the Cell Bank of the IZSLER  
36 (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Brescia, Italy), these  
37 cells were grown in Ham's F12 medium supplemented with 10% FBS, 1 mM glutamine and

1 antibiotics. Human colorectal adenocarcinoma (HT-29) cells were also provided by the Cell Bank of  
2 the IZSLER (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Brescia,  
3 Italy), and were grown in DMEM High (glucose 4.5 g/l) supplemented with 10% FBS, 2 mM  
4 glutamine and antibiotics (10,000 units penicillin, 10 mg streptomycin, 25 µg of amphotericin B/mL  
5 solubilized in appropriate citrate buffer). Trypsin EDTA (0.05%) was provided by Gibco (Fisher  
6 Scientific France, Illkirch, France). All cells were incubated at 37°C at 5% CO<sub>2</sub>; after thawing, all the  
7 cells were used for less than ten passages.

### 9 **Viability studies on B6KPC3, A549 and HT-29 cell lines**

10 B6KPC3 cells (3.5×10<sup>3</sup> per well), A549 cells (3.5×10<sup>3</sup> per well) and HT-29 cells (7.5×10<sup>3</sup> per well)  
11 were plated into 96-wells plates for 48 h and incubated at 37°C in air controlled atmosphere (5% CO<sub>2</sub>)  
12 with medium containing serum previously described. The medium was removed and cells were treated  
13 for 24 h with increasing concentrations of DACHPt-loaded NP and oxaliplatin solution. Blank NP  
14 were also used as control. All the systems were treated with drugs and nanoparticles diluted with  
15 serum free N1 supplemented medium. Serum free N1 supplemented medium was prepared with  
16 DMEM with amino acids, 1% antibiotics (10,000 units penicillin, 10 mg streptomycin, 25 µg  
17 amphotericin B/mL solubilized in an appropriate citrate buffer), 1% HEPES buffer, 1% NEAA (Non  
18 Essential Amino Acid) 100X, 1% sodium pyruvate, 1% N1 medium supplement 100X [34,35].

19 Cell viability reduction assay was performed using CellTiter 96® AQueous One Solution cell  
20 proliferation assay kit containing a tetrazolium compound[3-(4,5-dimethylthiazol-2-yl)-5-(3-  
21 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]. Following 24 h of  
22 incubation, the medium was removed and cells were incubated with 200 µL of a 1:10 diluted MTS  
23 solution. After 2 h at 37°C, the absorbance at 492 nm was recorded using a Microplater Reader  
24 (Multiskan Ascent®, Labsystem, Cergy-Pontoise, France for B6KPC3 cells and EnSpire® Multimode  
25 Plate Reader, Perkin Elmer, Boston, MA, USA for A549 and HT-29 cells). Cell viability (CV)  
26 percentage was evaluated through the following equation 3, with *Absorbance control* well, the  
27 absorbance value of untreated cells.

$$29 \quad CV (\%) = \frac{\text{Absorbance treated well}}{\text{Absorbance control well}} \times 100 \quad (3)$$

### 31 **CD44 surface receptors assessment on B6KPC3 cell line.**

32 B6KPC3 cells were treated with trypsin EDTA for 2 min at 37°C, collected with DMEM 10% FBS  
33 and counted. B6KPC3 cells were divided in polypropylene tubes at the density of 5×10<sup>5</sup> cells per tube,  
34 centrifuged, washed and resuspended in 100 µL of PBS. Test antibody anti-human/mouse CD44  
35 eFluor® 450 clone IM7 Invitrogen and control antibody eBioscience™ Mo IgG2b k Isotype were  
36 provided by eBioscience (ThermoFisher Scientific, Illkirch, France). The antibodies were diluted at a

1 1:10 ratio, PBS was used as a negative control. Tubes were completed with PBS, incubated 20 min at  
2 room temperature. Then, cells were centrifuged, the supernatant discarded and the pellets were  
3 reconstituted in PBS and analyzed using a FACS Canto II (BD Biosciences, Le Pont-de-Clay, France).

#### 4 5 **Evaluation of HMGB1 secretion and ATP release upon incubation of DACHPt-loaded** 6 **nanoparticles with B6KPC3 cells**

7 B6KPC3 cells were plated and treated as described above, with increasing concentrations of DACHPt-  
8 loaded NP, oxaliplatin and DACHPt water solution during 24 h. Blank NP were also tested as a  
9 control. The release of damage associated molecular patterns, i.e. HMGB1 and ATP, was assessed [6]  
10 in cell culture supernatant using an anti-HMGB1 enzyme-linked immunosorbent assay (ELISA) [36]  
11 (IBL International, Hamburg, Germany) and ATP assay [37] (Roche Diagnostics GmbH, Mannheim,  
12 Germany), respectively, both were used according to the manufacturer's instructions.

#### 13 14 **Uptake studies of fluorescent nanoparticles by B6KPC3 cells**

15 B6KPC3 cells were seeded in a 24-well plate at cell density of  $5 \times 10^5$  cells per well with 500  $\mu\text{L}$  of a  
16 complete culture medium. Cells were allowed to grow in an incubator at  $37^\circ\text{C}$  in a humidified  
17 atmosphere with 5%  $\text{CO}_2$ . For uptake experiments in biofluid, f-NP (labelled with Alexa-Fluor 647)  
18 were dispersed in 90% vol. of human serum and incubated at  $37^\circ\text{C}$  for 1 h prior to adding the  
19 formulation to the cells. Subsequently, cells were washed with PBS and incubated in Opti-MEM with  
20 f-NP, suspended in biofluid (90% vol.), for 24 and 48h at  $37^\circ\text{C}$ . At the end of incubation time, cells  
21 were washed twice with PBS, then trypsinized and analysed by Cytoflex (Beckman Coulter, Suarlée,  
22 Belgium).

#### 23 24 ***In vivo* experiments**

##### 25 **Animals**

26 Eight-week-old female C57BL/6 mice were purchased from Charles River Laboratories Inc. (Calco,  
27 Italy). All the mice were maintained under specific pathogen-free conditions in the animal facility of  
28 the Istituto Oncologico Veneto (Padua, Italy). All animal experiments were approved by the local  
29 animal ethics committee (CEASA nr 23bis) at the University of Padua and were executed in  
30 accordance with governing Italian law and EU directives and guidelines. All the animal experiments  
31 were in accordance with the Amsterdam Protocol on animal protection and welfare: the mice were  
32 monitored daily.

##### 33 **Pharmacokinetic evaluation of DACHPt-loaded NP following intravenous injection**

34 Healthy mice were intravenously (IV) injected with 200  $\mu\text{L}$  of DACHPt-loaded NP at the dose of 35.9  
35  $\mu\text{g}$  of platinum equivalent per mouse. Oxaliplatin and DACHPt aqueous solution were used as  
36 controls. At specific time points (15 min, 30 min, 1 h, 1.5 h, 3 h, 5 h, 8 h and 12 h), 200  $\mu\text{L}$  of blood

1 were withdrawn and separated from plasma. Pt content was detected using ICP-OES (ICP-OES ICAP  
2 7200 duo Thermo Scientific). A first compartmental PK analysis was done in Phoenix (Pharsight -  
3 a Certara™ L.P. software 1998-2014, Build 6.4.0.78) using WinNonlin 6.4, Connect 1.4. A first-  
4 order two-compartment model with bolus administration, expressed in terms of clearance (CL) and  
5 distribution volume (V) was applied to fit the data, using  $1/y_{\text{hat}}^2$  as weighting function. The model was  
6 either fitted using average data or individual data, and results were comparable in each of the cases.

## 7 8 ***In silico* experiments**

### 9 **Simulation of repeated administration**

10 A multiple administration regimen was simulated using WinNonlin 6.4, Connect 1.4. 8 cycles of 3.5  
11 mg/kg of oxaliplatin solution or DACHPt-loaded NP [38]. The average body weight was 25 g for a  
12 mouse and the absolute dose of 87.5 µg equivalent platinum per mouse. The regimen schedule was: IV  
13 infusion (2 h) performed on days 1, 4, 8, 11, 15, 18, 22 and 25, a total of two doses per week for 4  
14 weeks.

### 15 16 **Statistical analysis**

17 Statistical tests were performed using R (R: A language and environment for statistical computing. R  
18 Foundation for Statistical Computing, Vienna, Austria and RStudio (RStudio: Integrated Development  
19 for R. RStudio, Inc., Boston, MA, USA). Graphical representation was performed using GraphPad  
20 Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).  
21 Normality of the distribution was assessed with Shapiro-Wilk test. When the normality of the  
22 distribution was off, non-parametric Kruskal and Wallis tests were performed and the error adjusted  
23 with Dunn tests, the type one error rate was taken as  $\alpha = 5\%$  (\*  $p < 0.05$ ).

## 24 25 **Results**

### 26 **Development and physicochemical characterization of blank and DACHPt-loaded hyaluronic 27 acid-polyarginine nanoparticles**

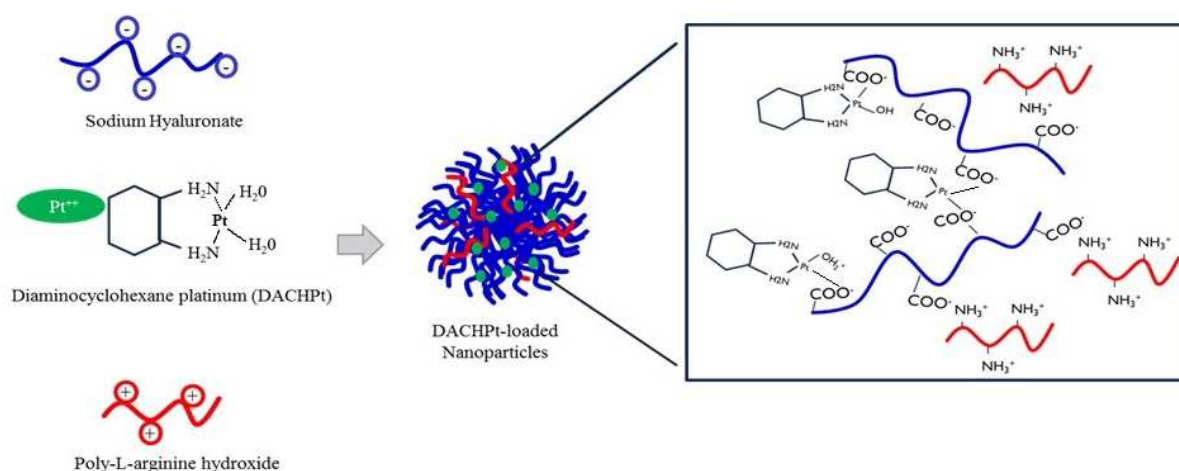
28 NP were prepared by polyelectrolyte complexation of negatively charged HA and positively charged  
29 PA using the ionic gelation technique [23,39]. In absence of active ingredient, the electrostatic  
30 interactions between the carboxylic groups of HA and guanidine groups of PA-Cl led to the formation  
31 of the NP. All the formulations were obtained at pH 7.4 to ensure the interaction of the opposite  
32 charged polymers. Different polymer ratios were tested to set up the optimal conditions in order to  
33 obtain stable, monodispersed and negatively charged NP (**Table 1**). Size (z-average) of blank NP was  
34 around 200 nm, and the polydispersity index indicated a mono-modal distribution  $<0.2$ . The  
35  $\zeta$  potential was negative for all the formulations, around -35 mV.

1 **Table 1** - Physicochemical properties of blank HA-PA nanoparticles prepared with different mass  
 2 ratios of [HA]/[PA] (n=3, PDI: Polydispersity Index)

Mass ratio (w/w) HA/PA	Mole ratio HA/PA	z-average (nm)	PDI	ζ Potential (mV)
2.4	8.3	203 ± 8	0.2	-39 ± 9
2.8	9.7	224 ± 36	0.18	-32 ± 3
3.2	11.0	220 ± 10	0.16	-35 ± 2
3.6	12.4	203 ± 3	0.16	-37 ± 2
4	13.8	227 ± 20	0.2	-40 ± 8
4.5	15.5	206 ± 6	0.16	-33 ± 3

4  
 5 To activate DACHPt and to increase its solubility in water, DACHPt-Cl<sub>2</sub> was pretreated with AgNO<sub>3</sub>  
 6 [(AgNO<sub>3</sub>)/(DACHPt) = 1]. The aqueous DACHPt was obtained with a reaction yield of around 70%  
 7 confirmed by the ICP-OES. Then, DACHPt-loaded NP were formulated by mixing DACHPt solution  
 8 with the PA-OH solution before adding HA solution (**Figure 1**).

9 PA-OH was used instead of PA-Cl to produce nanoparticles because chloride ions strongly interfere  
 10 with the association of DACHPt to the nanosystem reverting the reaction of drug activation leading to  
 11 the precipitation of DACHPt-Cl<sub>2</sub>. The driving force for DACHPt association to NP was the formation  
 12 of the metal-polymer complex between platinum and the carboxylic group of the HA in absence of  
 13 chloride ions, while PA-OH was needed to form stable nanosystems. PA-OH, the cationic  
 14 polyaminoacid, is a crosslinking agent that allows the formation of stable complexes with high drug  
 15 encapsulation efficiency as previously reported [39].



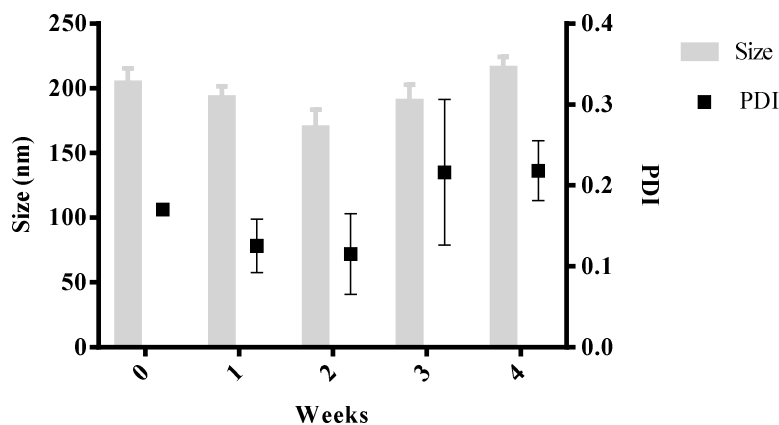
18  
 19 **Figure 1** - Schematic representation of DACHPt-loaded NP. .

1 DACHPt-loaded NP showed a size (z-average) distribution between 100 and 170 nm for all the  
2 [HA]/[PA] ratios screened (0.2 to 4.8 w/w) (**Table 2**). The presence of the cationic drug DACHPt led  
3 to the formation of smaller particles as compared to the blank systems. The polydispersity index (PDI)  
4 was in an acceptable range (<0.2) for all the formulations tested indicating that the populations  
5 obtained were homogeneous and monodispersed. The  $\zeta$  potential varied according to the [HA]/[PA]  
6 ratio. When the [HA]/[PA] mass ratio was below 0.2, the surface of the systems was mainly composed  
7 of positively charged PA, while, above this ratio, the organization was reversed and HA chains were  
8 exposed on the surface leading to a negative surface potential. The stability of DACHPt-loaded NP  
9 during storage was assessed following freeze-drying and resuspension in MilliQ water. The  
10 cryoprotectant agent selected was mannitol to help the dispersion after reconstitution in water. As  
11 shown in **Table 2**, after resuspension, sizes (z-average) of all the NP increased and were probably due  
12 to the aggregation of nanoparticles during freeze-drying. The encapsulation efficiency of DACHPt to  
13 different formulations assessed using ICP-OES, was around 70% for all the systems, and the drug  
14 loading was around 8%. Moreover, the presence of mannitol allowed to obtain an osmolality of around  
15 260 mOsm/kg, a value compatible with an intravenous injection [40].  
16

**Table 2** - Physicochemical properties of DACHPt-loaded NP prepared with different mass ratios of [HA]/[PA] before and after freeze-drying (n>5, PDI: Polydispersity Index, EE: entrapment efficiency).

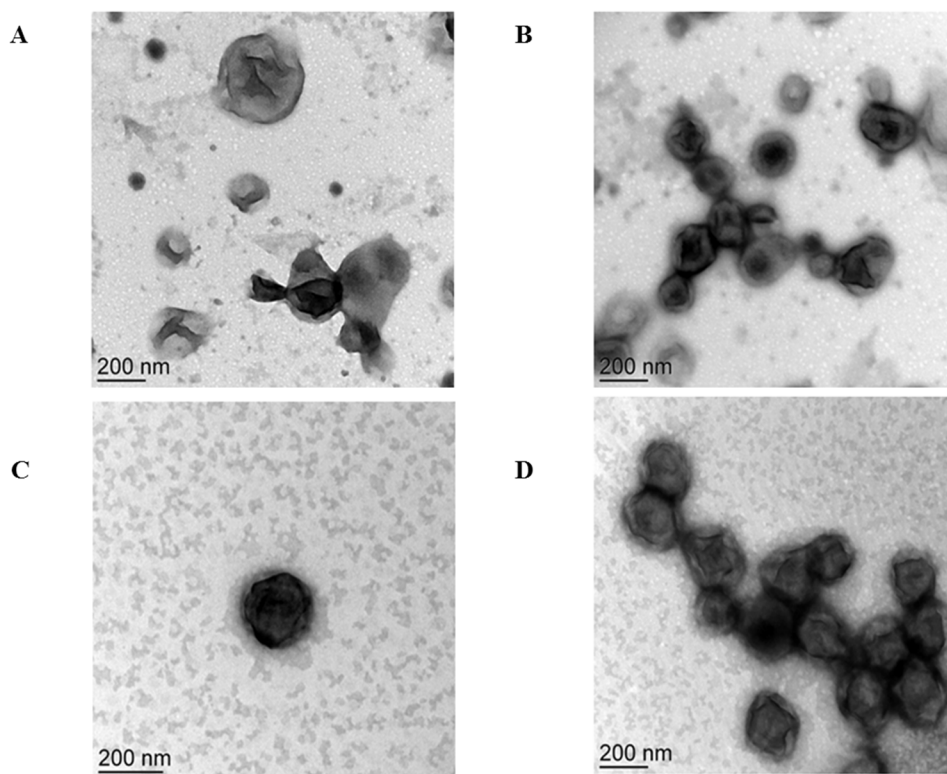
Before freeze-drying						After freeze-drying						
Mass ratio (w/w) HA/PA	Molar ratio HA/PA	Charge ratio HA/PA	z-average (nm)	PDI	ζ Potential (mV)	z-average (nm)	PDI	ζ Potential (mV)	Entrapment Efficiency (EE%)	Drug loading (% w/w)	Osmolality (mOsm/kg)	pH
0.2	0.7	1.1	105 ± 3	<0.1	+22 ± 3	NA	NA	NA	NA	NA	NA	NA
1.6	5.5	8.7	130 ± 2	<0.1	-27 ± 2	244 ± 4	<0.1	-42 ± 1	54 ± 3	11 ± 0.7	260 ± 14	5.7
2.8	9.7	15.3	153 ± 2	<0.1	-45 ± 2	235 ± 2	0.12	-46 ± 1	66 ± 4	9 ± 0.6	263 ± 2	5.7
3.6	12.4	19.7	177 ± 3	<0.1	-38 ± 1	242 ± 15	0.10	-44 ± 4	68 ± 0.3	8 ± 0.0	266 ± 9	5.6
4	13.8	21.8	160 ± 1	<0.1	-45 ± 1	241 ± 10	0.11	-41 ± 3	72 ± 2	8 ± 0.2	276 ± 16	5.7
4.5	15.5	24.6	161 ± 4	<0.1	-42 ± 4	238 ± 10	0.11	-46 ± 3	70 ± 3	7 ± 0.3	267 ± 14	5.7
4.8	16.6	26.2	164 ± 3	<0.1	-47 ± 6	236 ± 8	0.10	-40 ± 6	70 ± 1	7 ± 0.1	274 ± 5	5.7

1 Then, the stability of the systems after reconstitution was studied. The formulation prepared at the  
2 HA/PA ratio of 4.5 remained stable without significant change in terms of size (z-average) and PDI  
3 for 4 weeks after reconstitution (**Figure 2**). This result indicated that the selected formulation could  
4 have a strong potential as drug delivery system and was retained for further studies.  
5



6  
7 **Figure 2** – Size (z-average) and PDI of freeze-dried DACHPt-loaded nanoparticles (polymer mass  
8 ratio [HA]/[PA] = 4.5) after reconstitution (mean  $\pm$  SD, n=3).

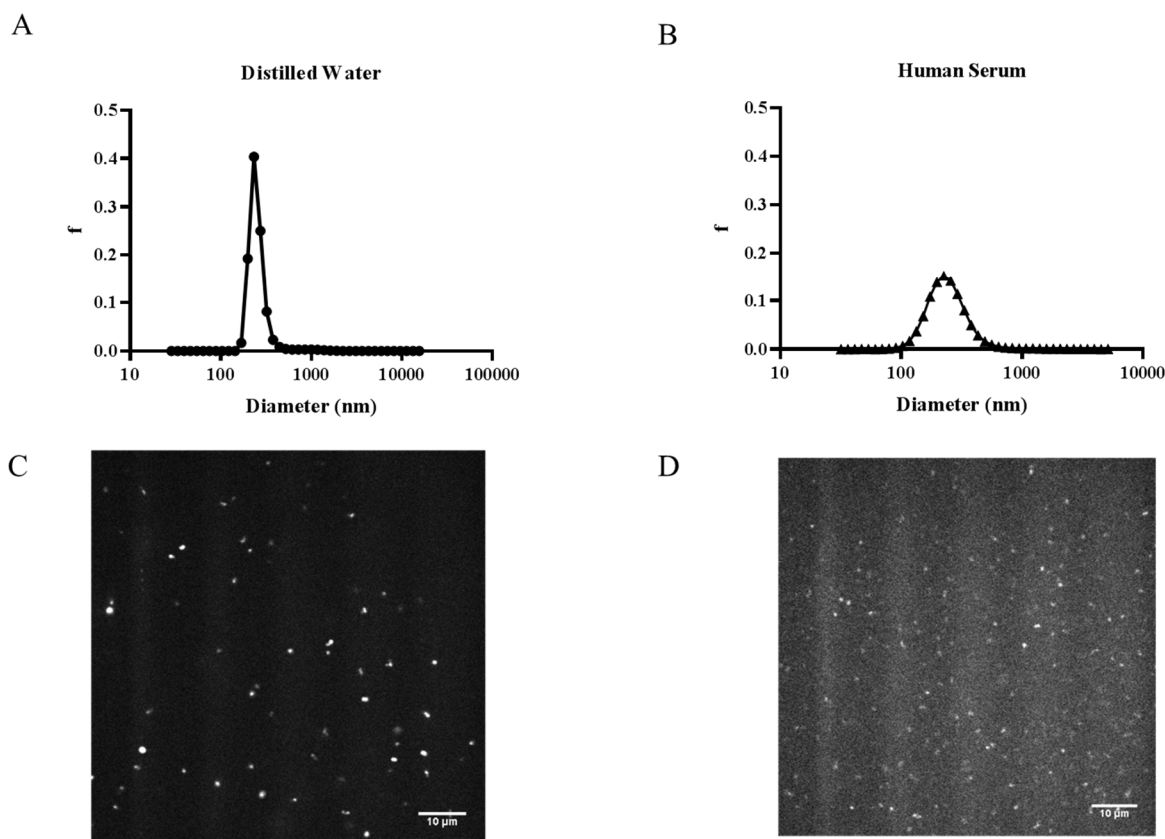
9  
10 To analyze the morphological structure of blank and DACHPt-loaded NP, transmission electron  
11 microscopy (TEM) analyses were performed. The images obtained after freeze-drying of the NP and  
12 resuspension in water are showed in **Figure 3**. Both blank and DACHPt-loaded NP formed well-  
13 defined and homogeneous structures with spherical shape. The sizes measured confirmed those  
14 obtained by DLS analysis (around 250 nm). Following reconstitution in water, some aggregates  
15 appeared, confirming the increase in size observed by DLS after freeze-drying.



1  
2 **Figure 3** - TEM of DACHPt-loaded nanoparticles after freeze-drying (A and B) and blank  
3 nanoparticles (C and D). (Scale bar is 200 nm and operating voltage = 120 kV).

4  
5 Once the system was optimized, single particle tracking analysis was performed to assess the stability  
6 of the NP upon incubation in human serum. **Figure 4** shows that when fluorescent NP (f-NP) were  
7 diluted in MilliQ water, an average size of 266 nm was detected. This result was consistent with the  
8 DLS values and was also close to the values obtained by TEM observations. Also, after incubation in  
9 90% volume of human serum for 1 h at 37°C, NP showed a narrow size distribution with an average  
10 size of 275 nm. The results demonstrated the colloidal stability of NP under biological conditions  
11 which is pivotal for biomedical applications.

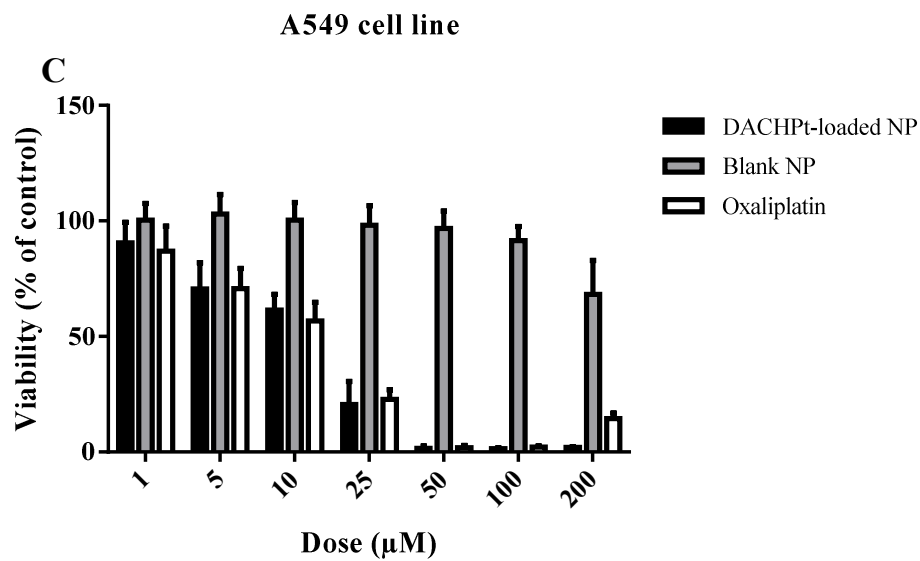
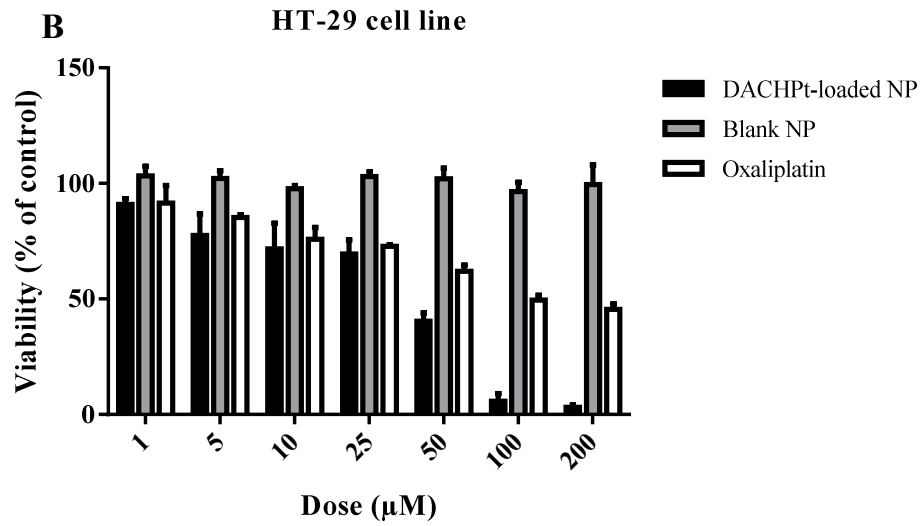
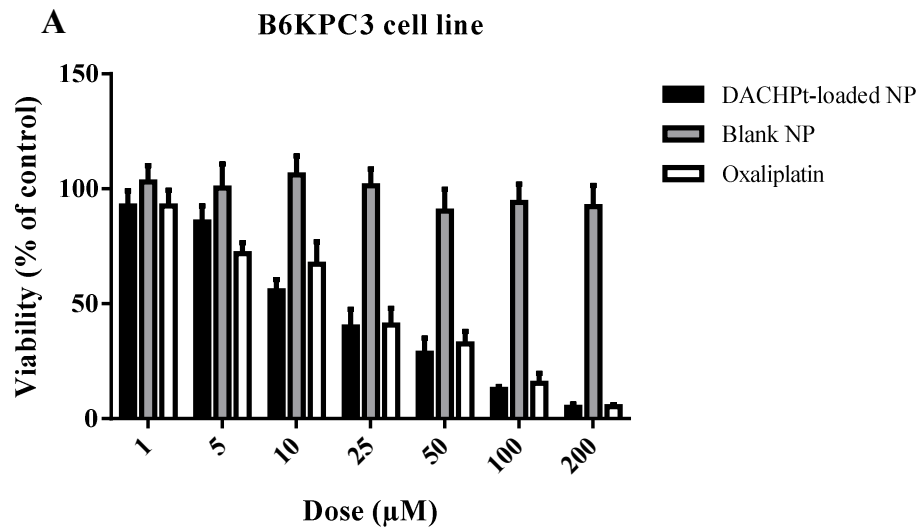
12  
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1  
2  
3 **Figure 4** – Single particle tracking size measurements of fluorescent NP (f-NP) after incubation  
4 during 1 h in distilled water (A) and human serum (B). Snapshots of the movies showing f-NP  
5 dispersed in distilled water (C) and in human serum (D).

6  
7 ***In vitro* cytotoxic evaluation of DACHPt-NP**

8 To assess the efficacy in viability reduction of DACHPt-loaded NP on different tumor cell lines, MTS  
9 test was used. B6KPC3, HT29 and A549 cells were treated with DACHPt-loaded NP, and oxaliplatin  
10 at drug concentrations ranging from 1 μM to 200 μM during 24 h (**Figure 5**). Blank NP, at the same  
11 concentrations in HA and PA as DACHPt-loaded NP were tested to assess the biocompatibility of the  
12 nanocarrier. *In vitro* cytotoxicity studies (**Table 4**) revealed that DACHPt-loaded NP were able to  
13 reduce cell viability for all the cancer cell lines tested. In the case of B6KPC3 cells, IC<sub>50</sub> of DACHPt-  
14 loaded NP was 1.3 times lower (18 vs 23 μM) in comparison to the oxaliplatin solution. In the case of  
15 HT-29, the IC<sub>50</sub> was reduced by a factor of 2 as compared to the reference oxaliplatin solution (IC<sub>50</sub>  
16 of 39 vs 74 μM, respectively), while NP toxicity was comparable with the oxaliplatin reference  
17 solution in A549 cell lines (IC<sub>50</sub> 11 and 12 μM, respectively). Blank NP did not interfere with cell  
18 viability at all the concentrations tested.



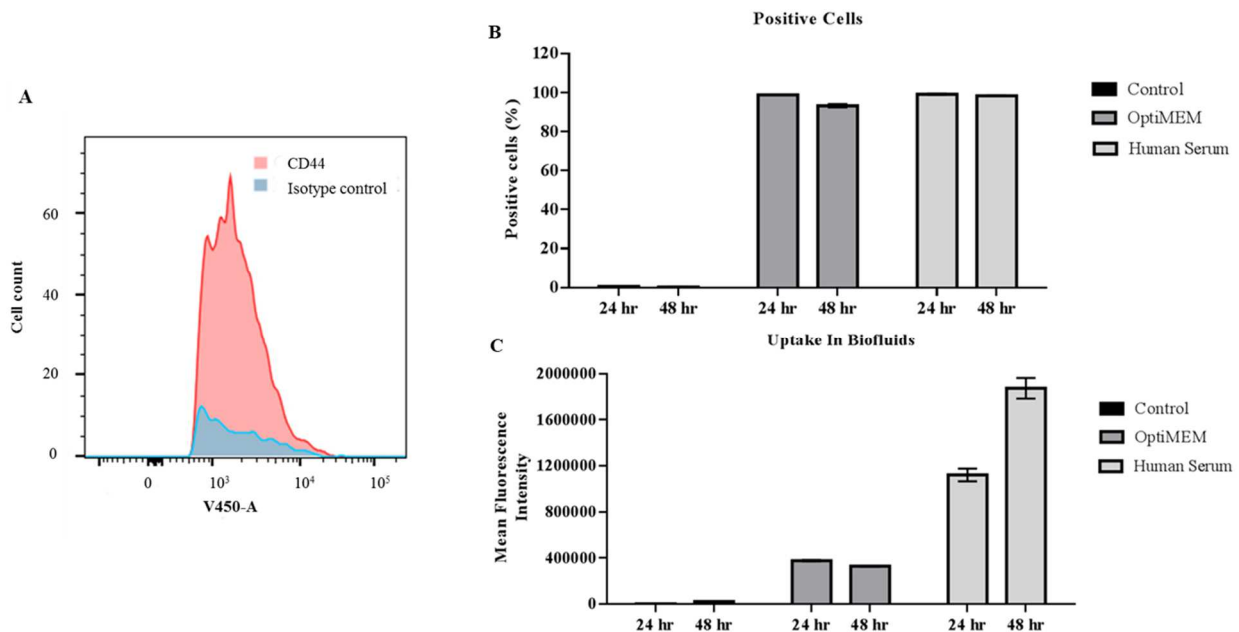
1 **Figure 5** - Viability assessment performed on B6KPC3 (A), HT-29 (B) and A549 (C) cells after 24 h  
2 of incubation at 37°C with increasing concentrations (0 - 200 µM) of oxaliplatin, blank NP and  
3 DACHPt-loaded NP for 24 h (Mean ± SD, n=6 (A) & n=3 (B and C)). (A boxplot representation of  
4 B6KPC3 cells plot can be found in *supplementary data*).

1 **Table 4** – Calculated IC50 for B6KPC3, HT-29 and A549 cells lines after 24h of incubation at 37°C  
 2 with increasing concentrations (0 - 200 μM) of DACHPt-loaded nanoparticles, oxaliplatin and  
 3 DACHPt solutions. (Mean ± SD, n=6 (B6KPC3) & n=3 (HT-29 and A549))

Cell line	Oxaliplatin	DACHPt-loaded NP
B6KPC3	23 μM ± 1.0	18 μM ± 1.1
HT-29	74 μM ± 2.9	39 μM ± 2.3
A549	11 μM ± 2.2	12 μM ± 1.2

4  
 5 **Assessment of CD44 expression at B6KPC3 cell surface and cellular uptake of fluorescent**  
 6 **nanoparticles in biofluids**

7 Cell internalization of HA decorated systems can occur through CD44 binding as demonstrated for  
 8 colon and lung cancer cell lines [41,42]. To assess if CD44 was also expressed on B6KPC3 cells and  
 9 hence could trigger NP internalization, cytometry analysis was performed. An isotype control was  
 10 used to assess non-specific binding. The CD44 appeared to be expressed at the surface of the B6KPC3  
 11 cells (**Figure 6A**). This result indicated that HA decorated nanosystems could be efficient to target  
 12 these cells.



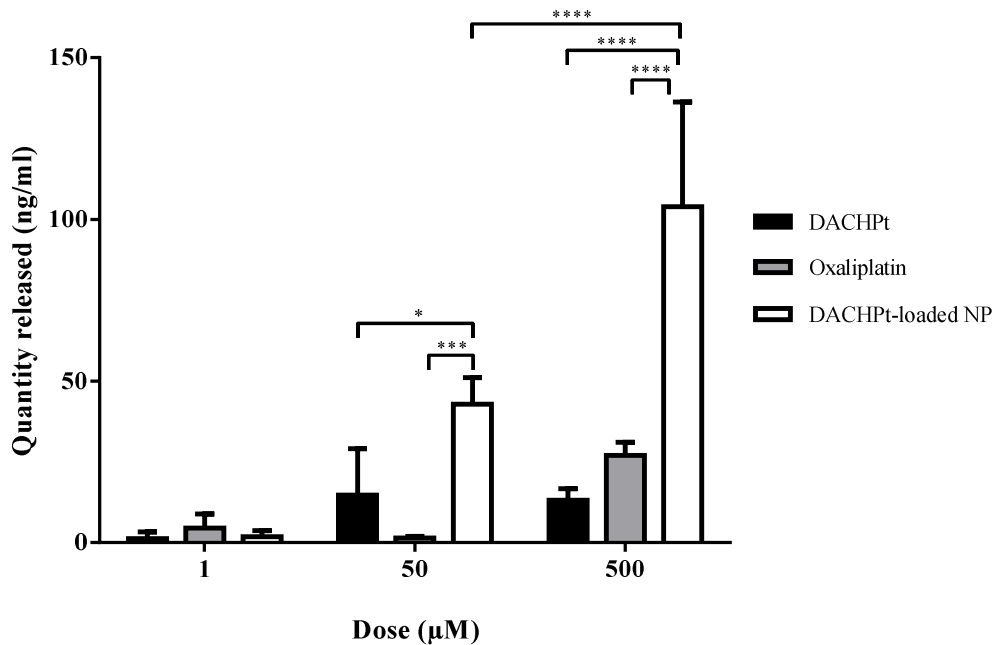
13 **Figure 6** – (A) Histogram for CD44 expression at the surface of B6KPC3 cells (red), 1.10<sup>5</sup> cells were  
 14 gated per condition. An isotype control (blue) was used to assess the non-specific binding. The  
 15 binding is expressed as a cell count according to the fluorescence intensity obtained with the selected  
 16 laser (V450-A). (B and C) Uptake studies of f-NP by B6KPC3 cells in culture media (control),  
 17 OptiMEM and human serum. (Mean ± SD, n=3)

1 The uptake of f-NP was studied using B6KPC3 cells in presence of biofluids such as serum-free  
 2 medium (OptiMEM) and human serum. **Figure 6B and 6C** revealed that f-NP were highly uptaken by  
 3 B6KPC3 cells in presence of 90% volume of biofluids. All the cells displayed a positive fluorescence  
 4 signal indicating that 100% of the cells internalized the labelled NP in the OptiMEM and human  
 5 serum at 24 h. The cellular uptake increased in human serum compared to OptiMEM. After 48 h, the  
 6 cellular uptake declined in OptiMEM while mean fluorescence intensity remained stable following  
 7 incubation in human serum suggesting that the f-NP were more uptaken by B6KPC3 in media with  
 8 human serum.

9

10 **Improved ability of DACHPt-loaded NP to induce HMGB1 secretion *in vitro***

11 Recently, it has been reported that oxaliplatin can induce the immunogenic cell death (ICD) of tumor  
 12 cells [6,43]. It was suggested that nanoparticles loaded with DACHPt may impact ICD due to their  
 13 proven advantages in delivery the chemotherapeutic drug to cancer cells. Major signs of ICD are the  
 14 extracellular release of adenosine triphosphate (ATP), and the passive release of chromatin-binding  
 15 protein high mobility group B1 (HMGB1). Therefore, the ability of DACHPt-loaded NP, oxaliplatin  
 16 and DACHPt to induce HGMB1 release after 24 h of incubation with B6KPC3 cells was evaluated  
 17 (**Figure 7**). At low drug concentration (1  $\mu$ M), no HMGB1 release was observed for all the treatments.  
 18 Besides, at high concentration (50 and 500  $\mu$ M of DACHPt) loaded NP significantly increased  
 19 HMGB1 release from B6KPC3 cells. In the case of ATP release, no differences were found among the  
 20 different conditions tested (data not shown).



21

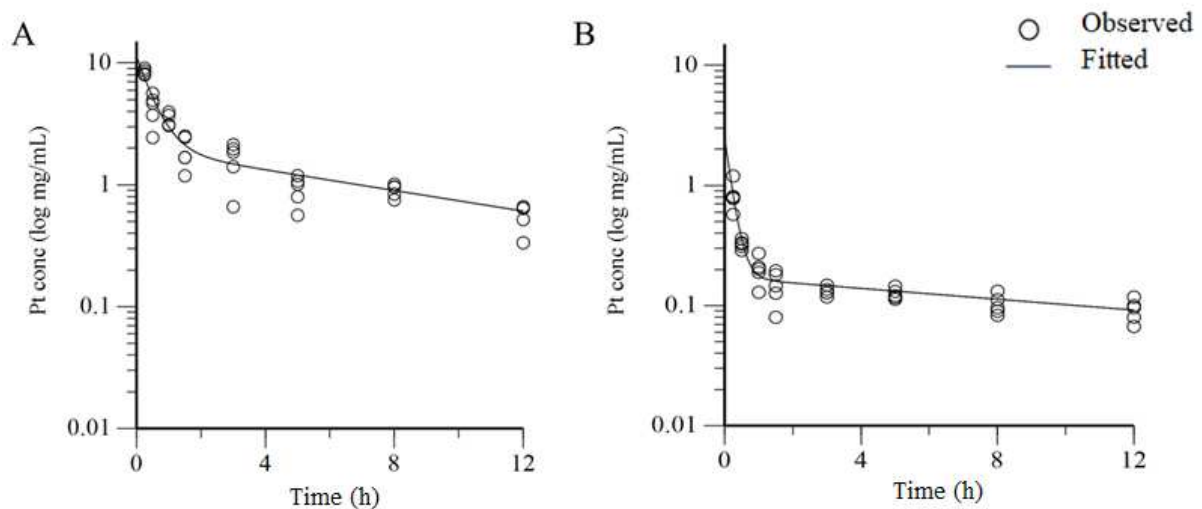
22 **Figure 7** – HMGB1 release by B6KPC3 cells after 24 h of treatment with DACHPt-loaded NP,  
 23 oxaliplatin or DACHPt water solution. (Mean  $\pm$  SD, n=3)

24

## 1 *In vivo* experiments: pharmacokinetic studies in healthy mice

2 In order to study the pharmacokinetic behavior of DACHPt-loaded NP, healthy mice were  
3 intravenously injected (IV) with DACHPt-loaded NP. Oxaliplatin solution at the same dose was used  
4 as a control. Platinum (Pt) and DACHPt concentrations were quantified using ICP-OES as described  
5 above. Pt concentration derived from oxaliplatin solution and DACHPt concentration derived from  
6 DACHPt-loaded NP were plotted as a function of time (**Figure 8**) and data were analyzed using a two-  
7 compartmental model. As shown by the plasma level-time curves, free oxaliplatin or encapsulated  
8 DACHPt showed a biphasic pattern, which resulted from the extensive distribution of platinum  
9 derivatives into different tissues, followed by their terminal elimination from the body.

10



11

12 **Figure 8** - Plasma concentration-time curves of Pt derivatives after IV bolus injection of 35.9  $\mu\text{g}$   
13 DACHPt-loaded NP (A) or oxaliplatin solution (B) to mice. The red circles show the observed plasma  
14 concentrations in the individuals, while the blue line represents the best fit through the data.  
15 Parameters obtained from the fitted curves are displayed in **Table 5**.

16

17 As reported in **Table 5**, the Area Under the Curve (AUC) following the administration of DACHPt-  
18 NP was 24  $\text{h}\cdot\text{mg/L}$ , six times higher than the value obtained following the administration of  
19 oxaliplatin solution, which was 3.76  $\text{h}\cdot\text{mg/L}$ . The  $C_{\text{max}}$ , defined as the maximal drug concentration  
20 detected in the blood at time 0h was 11.4  $\text{mg/L}$  for NP and 2.48  $\text{mg/L}$  for oxaliplatin solution, almost  
21 five times higher than the control drug.

22 The distribution half-life (alpha half-life) indicated that when the drug was entrapped into NP, it was  
23 distributed slower than oxaliplatin (0.370 h for NP vs 0.135 h for oxaliplatin solution). However, the  
24 elimination rate (beta half-life) was more rapid for NP in comparison to oxaliplatin solution (6.49 h for  
25 NP vs 13.6 h for oxaliplatin solution). The results showed that the Pt concentration in plasma started at  
26 a lower level and initially decreased much more rapidly for the oxaliplatin solution as compared to the  
27 NP.

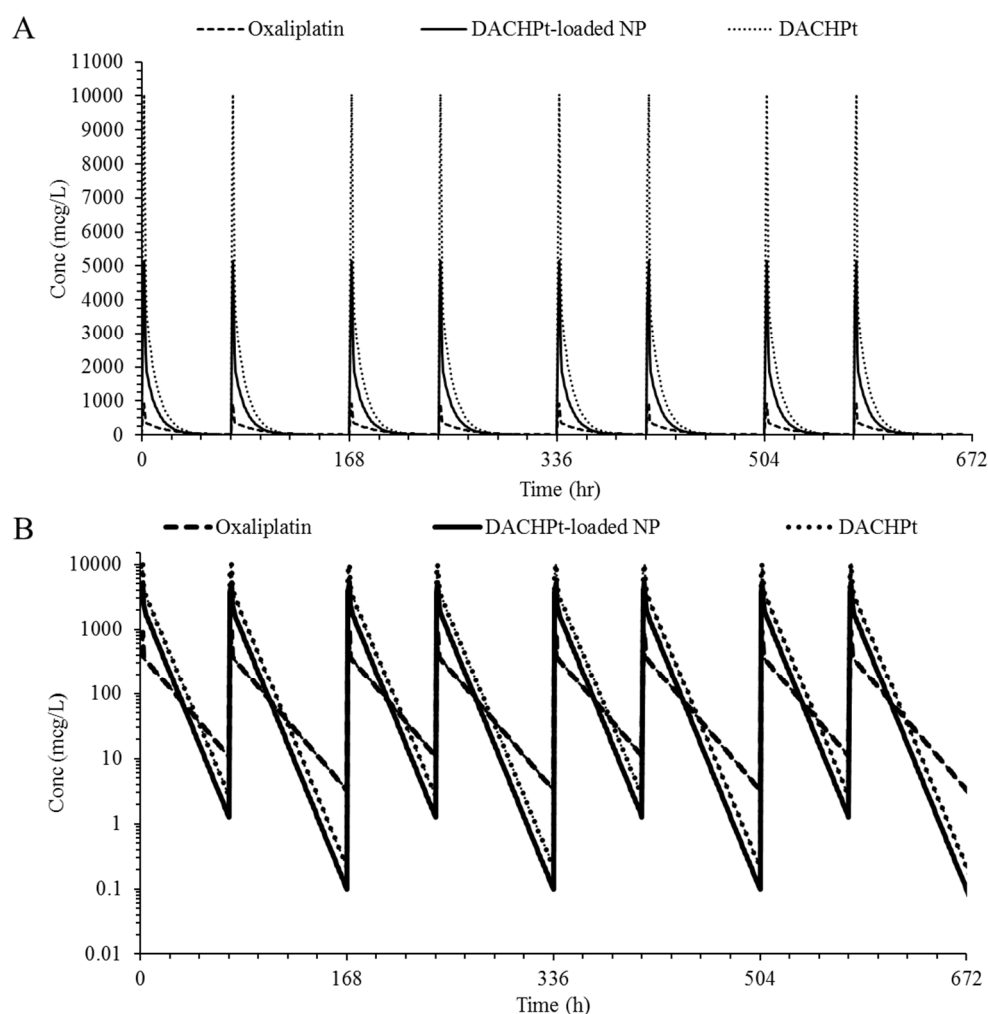
1  $K_{12}$  (rate constant of distribution from central to peripheral compartment) was higher for oxaliplatin  
 2 solution as compared to the one obtained with DACHPt NP (4.14 vs 1.08 h<sup>-1</sup>).  $K_{21}$  (rate constant of  
 3 distribution from peripheral compartment to central compartment) was similar for oxaliplatin and  
 4 DACHPt NP (0.398 and 0.418 h<sup>-1</sup>). Volume of distribution of the central and peripheral compartment  
 5 ( $V_c$  0.003 and  $V_p$  0.008 L for the NP vs  $V_c$  0.014 and  $V_p$  0.150 L for oxaliplatin solution) as well as  
 6 the volume of distribution at steady state ( $V_{ss}$ ) of the NP were lower with respect to the oxaliplatin  
 7 solution (0.011 vs 0.165 L). Plasma clearance differed by a factor of five, being 0.002 and 0.010 L/h  
 8 for NP and oxaliplatin solution, respectively.

9  
 10 **Table 5** - Pharmacokinetic parameters obtained following IV bolus injection of an equivalent dose of  
 11 a 35 µg Pt/mouse of DACHPt-loaded NP or oxaliplatin solution.

Parameters	Units	DACHPt-loaded NP	Oxaliplatin
		Calculated values	
AUC	h*mg/L	23.8 ± 1.52	3.76 ± 0.52
Alpha half-life	h	0.370 ± 0.075	0.135 ± 0.023
Beta half-life	h	6.49 ± 1.08	13.60 ± 2.89
$K_{12}$	1/h	1.08 ± 0.26	4.14 ± 0.72
$K_{21}$	1/h	0.418 ± 0.088	0.398 ± 0.054
$C_{max}$	mg/L	11.4 ± 1.82	2.48 ± 0.68
AUMC	h*h*mg/L	178 ± 37.8	65.1 ± 23.8
$V_{ss}$	L	0.011 ± 0.001	0.165 ± 0.018
$V_c$	L	0.003 ± 0.0005	0.014 ± 0.004
CL	L/h	0.002 ± 0.0001	0.010 ± 0.001
$V_p$	L	0.008 ± 0.001	0.15 ± 0.02

12  
 13 Then, in order to study the maintenance of drug therapy, a repeated administration regimen was  
 14 simulated. The repeated administration schedule was intended to minimize side effects while  
 15 maintaining therapeutic drug concentration in plasma. In order to simulate an *in vivo* multiple  
 16 administration regimen, a protocol constituting of 8 cycles of 3.5 mg/kg of oxaliplatin solution or  
 17 DACHPt-loaded NP was established [44]. In **Figure 9**, concentrations of Pt from oxaliplatin solution  
 18 or from DACHPt-loaded NP as well as DACHPt, were represented as a function of time as linear scale  
 19 (y-axis), or semilog scale (y-axis).

20  
 21  
 22



1  
2  
3 **Figure 9** - Plasma concentration-time curves of platinum derivatives after 2 h of IV infusion of  
4 multiple administrations (twice a week for 4 weeks) of 3.5 mg/kg of oxaliplatin solution, DACHPt-  
5 loaded NP and DACHPt solution (continuous line DACHPt-loaded nanoparticles, dotted lines:  
6 Oxaliplatin and DACHPt solution respectively) to mice. (B) Plasma concentration-time curves  
7 (concentration is plotted on a log scale) of Pt derivatives after 2 h of repeated IV infusion of 3.5 mg/kg  
8 of oxaliplatin solution, DACHPt-loaded NP and DACHPt (continuous line DACHPt-loaded  
9 nanoparticles, dotted lines: oxaliplatin and DACHPt solution respectively) solution to mice.

10  
11 From **Figure 9** it can be noticed that there was no accumulation of Pt with the selected administration  
12 regimen and the drug was always eliminated before the next administration. Plasma drug  
13 concentration fluctuations were observed for all the treatments tested. Finally, steady state was rapidly  
14 reached for all the compounds with two injections per week during four weeks.

15  
16  
17

## 1 Discussion

2 Chemotherapy regimens using platinum drugs are highly effective and widely used for the treatment  
3 of several malignancies. However, the improvement of specific delivery to tumors is required to  
4 increase therapeutic drug levels in pathological sites and to reduce side effects associated with  
5 unspecific drug distribution [45–47]. In this field, polymer nanosystems made of bioinspired materials  
6 represent an attractive strategy due to their biocompatibility, tunable design, possibility to load a wide  
7 range of molecules and ability to selectively accumulate in solid tumors [48]. The aim of this work has  
8 been to develop and characterize nanoparticles made of HA and PA as a versatile platform for the  
9 encapsulation of hydrophilic actives, as platinum derivatives. To reach this objective, an exhaustive  
10 physico-chemical characterization of the nanosystem has been developed aimed at selecting the  
11 suitable parameters to obtain stable NP. Moreover, to increase their shelf-life and to facilitate their  
12 handling and storage, a freeze-drying process was investigated.

13 NP were obtained using the ionic gelation technique which is based on the electrostatic interactions  
14 between the positively charged primary amino groups of PA and the negatively charged carboxylic  
15 groups of HA. The optimal blank formulation in terms of stability had a HA/PA mass ratio of 4.5,  
16 yielded particles with an average size of 206 nm, a  $PDI \leq 0.2$  and a negative zeta potential (-35 mV).  
17 In the case DACHPt-loaded NP, the modification of polyarginine chloride was required to obtain  
18 chlorine free PA and hence facilitate the encapsulation of the drug in the nanosystems. Finally,  
19 particles with an average size of 161 nm, a  $PDI \leq 0.2$  and a negative zeta potential (-42 mV) were  
20 obtained. The reduction in size of drug-loaded nanoparticles, already observed with other actives, is  
21 related to the attractive forces that modulates electrostatic interactions within the complexes in  
22 presence of activated DACHPt [39]. This process allowed the formation of nanoparticles in a fast and  
23 simple process paving the way for a future scale up of the technology, with no need for chemical  
24 synthesis.

25 To stabilize the system during freeze-drying and to obtain a fluffy white cake easily resuspendable in  
26 water, mannitol was used as a cryoprotectant and bulking agent. Following resuspension, the  
27 physicochemical characteristics of NP in terms of size, PDI and drug loading were maintained. NP  
28 showed an average size of 238 nm and a  $PDI \leq 0.12$ , this slight increase in size measured in DLS  
29 might be due to some NP aggregation as observed in TEM image, while, the entrapment efficiency  
30 was above 70%. To assess the integrity of NP in complex media such as human plasma, SPT has been  
31 used to monitor the size of nanosystems in undiluted biofluids [33,49,50]. fSPT gave comparable size  
32 distributions to DLS for NP assayed in colloidal suspension (around 200 nm). Upon 48h of incubation  
33 NP maintained their size in human plasma suggesting *in vivo* stability of the carriers.

34 Then *in vitro* experiments were carried out to evaluate the anticancer activity of DACHPt-loaded NP  
35 in colon (HT-29), lung (A549) and pancreatic (B6KPC3) cancer cell lines following 24h of incubation.  
36 In HT-29 cells, the IC<sub>50</sub> of DACHPt-loaded NP was 2 times lower in comparison to the value  
37 obtained with oxaliplatin (39  $\mu$ M and 74  $\mu$ M). In B6KPC3 cells, the IC<sub>50</sub> value was also lower than

1 oxaliplatin solution (18  $\mu$ M and 23  $\mu$ M), whereas DACHPt-loaded NP and oxaliplatin had comparable  
2 toxicity in A549 cell line (12  $\mu$ M and 11  $\mu$ M). At all the concentrations tested, blank NP did not affect  
3 cell viability highlighting their biocompatibility. These results suggest that HA-PA NP is an effective  
4 carrier in enhancing anticancer activity of associated DACHPt. Besides, for a short incubation time  
5 (24 h), nanocarriers encapsulating drug showed higher *in vitro* cytotoxicity when compared to the free  
6 drug. Also, Tsai *et al.* showed that copolymer polyglutamic acid-b-D- $\alpha$ -tocopheryl polyethylene  
7 glycol 1000 succinate nanoparticles worked more effectively than oxaliplatin did on A549 cells at 24 h  
8 [51]. While, Murakami, *et al.* observed that DACHPt-loaded micelles made of polyglutamic acid and  
9 PEG displayed a value of IC<sub>50</sub> against HT29 cells lower than that of oxaliplatin by a factor of 4.7  
10 following 48 h of treatment [15]. In the case of micelles made of dendritic block copolymer poly-  
11 (amidoamine)-polyglutamic acid-b-polyethylene glycol (PAM-PG<sub>lub</sub>-PEG) and loaded with DACHPt  
12 the enhancement of cytotoxicity was reached only after 72 h of incubation [52]. This reduction in cell  
13 viability could be ascribed to an enhanced internalization of NP via CD44/HA interaction. HA is  
14 known to be a ligand of different CD44 isoforms overexpressed by a large panel of cancer cell lines  
15 including pancreatic, lung and colon cancer cell lines [53,54]. The involvement of CD44 in the  
16 progression of lung and colon cancer, has been largely described, while there were only a few reports  
17 regarding the interaction of CD44 in pancreatic cancer cell lines [55]. The interactions between the  
18 polymer free in solution, grafted to liposomes or coated in NP, can create stronger binding to CD44  
19 receptors. Longer HA chains have multiple interactions with CD44 molecules, leading to an increase  
20 in affinity, and in enhancement of HA internalization into cells. However, long HA chains are rapidly  
21 cleared from the circulation by the hyaluronan receptor for endocytosis (HARE) which is present on  
22 liver sinusoidal endothelial cell [55]. In this study, a low molecular weight HA (20 kDa) was selected,  
23 the HA was long enough to bind to CD44 but too short to bind to the HARE receptor. By means of  
24 FACS analysis, it was disclosed that B6KPC3 expressed CD44 receptor and the internalization by  
25 these cells could be driven by receptor recognition. Internalization experiments in B6KPC3 cells  
26 confirm that cells treated with f-NP highly expressed fluorescence; therefore, they internalized f-NP in  
27 a high amount.

28 In addition to a direct cytotoxic effect, DACHPt-loaded NP induced signals related with ICD. ICD is a  
29 form of cell death that triggers a T cell-dependent immune response depending on a temporal  
30 sequence emission of damage-associated molecular patterns (DAMPs) such as ATP, calreticulin  
31 (CTR) and high mobility group box 1 (HMGB1) [56,57]. The emission of those DAMPs enhances the  
32 immunogenicity of dying cells leading to an anticancer immunity responses [58]. It has been reported  
33 the efficacy of oxaliplatin based treatments inducing increased HMGB1 release and ATP secretion in  
34 pancreatic cells Panc-1 and Pan02 cells after 48 h of exposure [7]. Here it was demonstrated that  
35 DACHPt-loaded nanoparticles can induce ICD also on B6KPC3 enhancing HMGB1 release and ATP  
36 release after 24 h of incubation. Altogether, these results indicated that NP encapsulation is a powerful  
37 method to increase DAMPs exposure improving drug cytotoxicity *in vitro*.

1 Finally, DACHPt-loaded NP and oxaliplatin solution were injected intravenously and the plasma  
2 concentrations quantified to determine the pharmacokinetic behavior of the formulations. The PK of  
3 platinum in plasma ultrafiltrate after oxaliplatin administration is characterized by a short initial  
4 distribution phase and a long terminal elimination phase. In clinical studies, it was observed that at the  
5 end of a 2 h oxaliplatin infusion, only 15% of the administered Pt was present in the systemic  
6 circulation [59]. As reported in the literature, once injected, Pt binds irreversibly to plasma proteins  
7 (predominantly serum albumin) and erythrocytes. The erythrocytes did not serve as a reservoir for Pt  
8 in the systemic circulation, and accumulation of Pt in blood cells is not considered to be of clinical  
9 relevance [59].

10 On the contrary, following the administration of DACHPt-loaded NP, AUC and  $C_{max}$  values were 6  
11 and 5 times higher respectively, than the values obtained following the administration of oxaliplatin  
12 solution. The alpha and beta half-lives indicated that DACHPt-loaded NP were distributed more  
13 slowly (0.37 h vs 0.135 h for oxaliplatin) but eliminated more rapidly than the reference oxaliplatin  
14 solution (6.49 h vs 13.60 h for NP and oxaliplatin solution, respectively).

15 So far, a significant effort have been spent on developing long circulating nanoparticle formulation  
16 able to enhance the plasmatic half-life of the associated drugs [12–14]. Prolonged circulation could  
17 also mean slow tissue distribution of the nanoparticles (including to the target tissue) and very slow  
18 drug release. A right balance between long circulating properties, distribution and elimination rates is  
19 hence needed to avoid side effects and indiscriminate accumulation [60]. In the case of nanoparticles,  
20 the achievement of higher exposures during and immediately post dosing is evident. Then, the drug  
21 concentration decreased rapidly for both oxaliplatin solution and DACHPt-loaded NP.

22 From a simulated multiple administration regimen of DACHPt-loaded NP, there was no accumulation  
23 of Pt and steady state was rapidly reached for all the moieties plotted. Generally, in a repeated  
24 administration schedule, accumulation can occur when the drug is administered before the previous  
25 dose is completely eliminated. Then, the amount of drug in the body will progressively rise. From  
26 Figure 9 it can be observed that the drug was always eliminated without relevant accumulation.  
27 However, in the case of DACHPt-loaded NP, a higher exposure was maintained for at least two days,  
28 while in the case of oxaliplatin solution the amount of the drug detected in the blood was quite low  
29 even at the starting point. Globally, the results presented here are associated with a high stability,  
30 slower *in vivo* release and clearance of the nanoparticles as compared to the drug alone.

## 31 32 **Conclusion**

33 This present work discloses a novel nanosystem made of HA-PA NP for the encapsulation of  
34 DACHPt. This system was stable in human serum, could be lyophilized, and after reconstitution in  
35 water the physicochemical properties as size, polydispersity, and osmolality were still compatible with  
36 IV injection. The anticancer activity *in vitro* both in terms of cytotoxic activity and ICD inducer, of  
37 DACHPt was enhanced by its encapsulation in the HA-PA nanoparticles, while blank nanosystems did

1 not show any toxicity. Moreover, due to the HA coating, it could be suggested that nanoparticles were  
2 internalized via CD44 in B6KPC3 cell. *In vivo* pharmacokinetic studies showed that following IV  
3 injection of DACHPt-loaded NP a slower drug release, associated to a lower elimination and  
4 distribution of the drug as compared to oxaliplatin solution, occurred. These observations suggested  
5 that tunable nanomedicines based on bioinspired polymers have the potential to enhance drug efficacy  
6 and be more effective than oxaliplatin alone.

7

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## 11 **Declaration of interest**

12 The authors declare no conflict of interest

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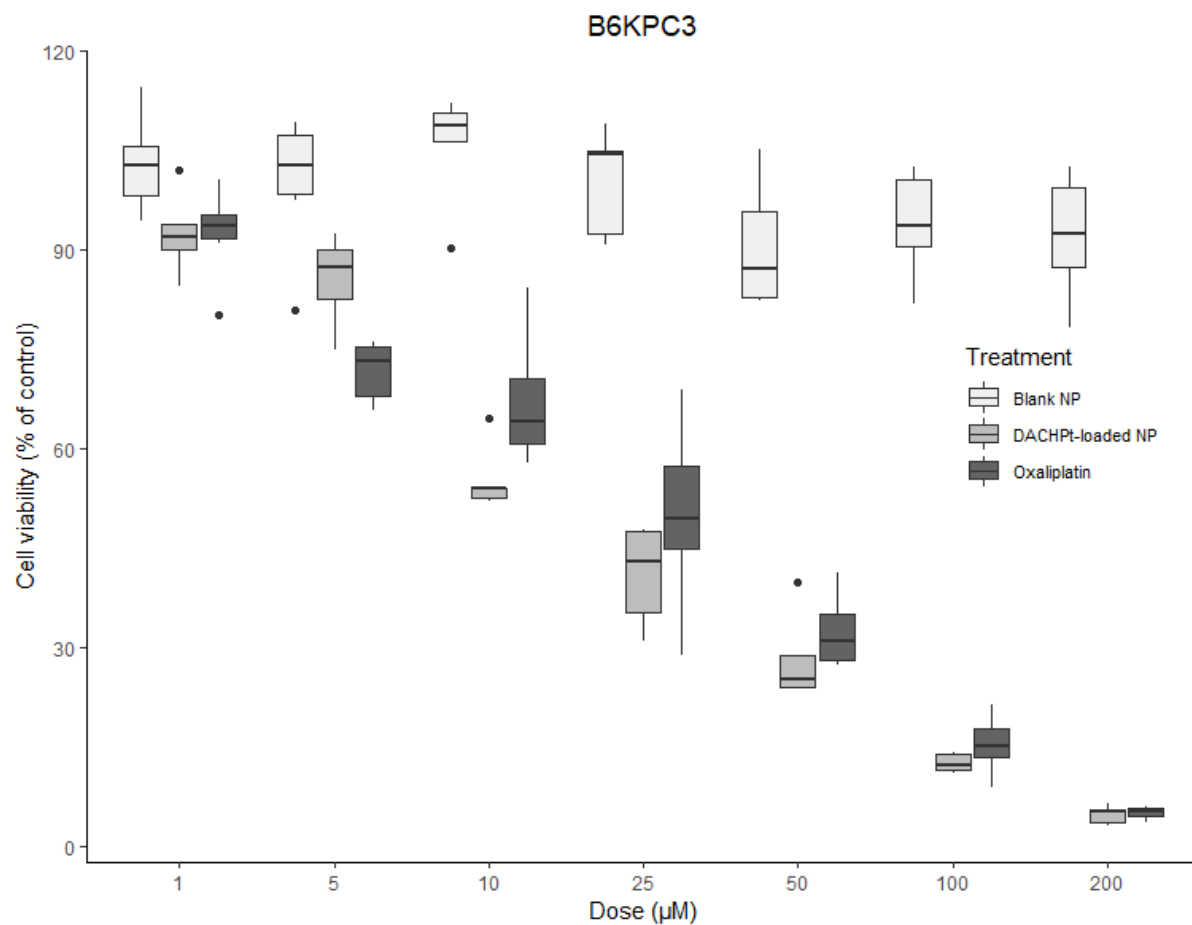
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26

27

1 *Supplementary data*



2

3 **Figure 1** - Viability assessment performed on B6KPC3 cells after 24h of incubation at 37°C  
4 with increasing concentrations (0 - 200 µM) of oxaliplatin, blank NP and DACHPt-loaded NP  
5 for 24h (n=6).

