Supplementary information

Efficient encapsulation of theranostic nanoparticles in cell-derived exosomes: Leveraging the exosomal biogenesis pathway to obtain hollow gold nanoparticle-exosome hybrids

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1. Materials and methods

1.1. Synthesis and characterization of HGNs

All chemicals used in the HGNs synthesis were obtained from Sigma-Aldrich: cobalt (II) chloride hexahydrate, sodium citrate tribasic dehydrate, poly (vinylpyrrolidone) (PVP) Mw=55000 Da, gold (III) chloride hydrate (50 % purity), sodium borohydride, and poly (ethylene glycol)-ether thiol (PEG 1000 Da Mw). HGNs synthesis was scaled from the previously reported protocol reported by Preciado-Flores *et al*.[1] The resulted HGNs were functionalized with monofunctional poly (ethylene glycol)-ether thiol (PEG 1000 Da Mw). After and before the PEGylation step, NPs were characterized by transmission electron microscopy (T20-FEI Tecnai transmission electron microscopy (TEM)), UV-VIS spectroscopy and measurement of surface charge. ImageJ software (NIH-RSB) was employed to determine the average particle diameter of both HGNs and PEG-HGNs, measuring a minimum of 200 particles.

1.2. Cell culture conditions

Metastatic murine skin melanoma cells were provided by cell services from Cancer Research-UK and cultured in Dubecco's modified Eagle's medium (DMEM; Biowest, France) supplemented with 10 % FBS (GIBCO, United States), 1 % penicillin/streptomycin and 1 % amphotericin (Biowest, France), under normoxic conditions. Ultracentrifugated (ULTRACEN medium, 100000 g, 8 h, 4 ºC) serum was used in order to guarantee exosomes free medium.

1.3. Exosome isolation and characterization

B16-F10-exos were isolated following a protocol previously developed in our lab and based on successive ultracentrifugation cycles from cell culture supernatants[2]. First of all, cells were cultured until confluence. In the first step, supernatants were collected and were centrifuged for 20 min at 2000 g at 4 ºC. Secondly, another centrifugation step was carried out for 1 h at 10000 g at 4 ºC to eliminate the microvesicles. To obtain the exosomal fraction, the samples were ultracentrifuged for 2 h at 100000 g at 4 ºC. However, another ultracentrifugation step in the same conditions was

necessary in order to eliminate the surrounded proteins of the exosome. The obtained precipitates were resuspended in PBS and a Pierce BCA protein assay (Thermo Fisher Scientific, United States) was performed in order to estimate the protein content in the exosomes sample. B16-F10-exos size, shape and morphology were characterized by TEM by contrasting them with phosphotungstic acid (3 %). From TEM images, the average diameter of exosomes was obtained using ImageJ software (NIH-RSB). The surface charge of exosomes was also determined at pH= 7 in PBS (Brookhaven 90 plus and ZetaPALS software). To identify specific surface proteins such as CD9, western blotting was carried out. Briefly, 25 μg of B16-F10-exos were precipitated with acetone (1:1 w/w), lysed in Laemmli buffer (Sigma-Aldrich, United States) and boiled at 95 ºC during 5 min. Subsequently, proteins were separated by 12 % SDS-polyacrylamide gel electrophoresis during 2 h and transferred to nitrocellulose membranes at 4ºC during 4 h. The membranes were blocked overnight with non-fat dry milk in tris-buffered saline (TBS) 5 %. After that, blots were incubated with primary antibodies CD9, 1:2000 (Abcam, United Kingdom), TSG101, 1:500 (Abcam, United Kingdom), calnexin, 1:1000 (Abcam, United Kingdom) and α-actin, 1:10000 (Sigma-Aldrich, United States). Membranes were washed three times with TBS-Tween (TBST) followed by the incubation of the secondary antibody (Sigma-Aldrich, United States). Finally, membranes were extensively washed and after the addition of chemiluminescence substrate, imaging was carried out. Cellular lysates from parental cells were used as negative control. Size distribution of exosomes was measured using Nanosight (Malvern Instruments, United Kingdom). Samples were diluted in PBS to optimize the number of particles in the field of view. They were measured at room temperature in triplicate for 60 seconds.

1.4. Physicochemical methods for HGNs-loaded exosomes

HGNs internalization within B16-F10-exos was studied using different physicochemical methods.

Incubation at room temperature. HGNs internalization within exosomes at room temperature was performed by mixing them during 18 h without stirring.

Incubation at room temperature with saponin. It is well known that saponin selectively interacts with membrane cholesterol molecules, forming pores and/or removing cholesterols from the cell membrane.[3] Once B16-F10-exos were mixed with HGNs, saponin was added at 0.2 % and the sample was mixed under stirring during 20 min at room temperature.

Thermal shock I. In this method, the exosome-NPs mixture was frozen at –80 ºC during 30 min and subsequently allowed to defrost. This freeze-thaw cycle was developed three times.

Thermal shock II. A classical strategy for bacterial transformation was applied to the encapsulation of HGNs within exosomes[4]. In this case, the sample was heated at 42 ºC for 30 seconds, followed by incubating it at 4 ºC during 2 minutes.

Sonication. The sonication conditions employed in this method were adapted from Haney *et al.*, employing ultrasounds higher than 20 KHz to generate transitory pores in exosomal membranes.[5] Exosome-NP mixture was sonicated (UW 2200 Bandelin Sonopuls, Germany) in an ice bath at 500 V, 2 kHz during 6 cycles (4 seconds of pulse and 2 seconds of pause).

Electroporation. The conditions previously developed by Hu *et al*. for the electroporation of exosomes to introduce SPIONs were employed when following this method.[6] Briefly, suspended exosomes were electroporated in the presence of HGNs (Genepulser Xcell electroporator, Biorad, United Kingdom) in 4 mm path length electroporation cuvette. A single pulse was applied under a 950 μF of capacitance and infinite resistance.

1.5. Cytotoxicity evaluation

To determine cell viability of B16-F10 cells under the effect of HGNs and PEG-HGNs, the Blue Cell Viability assay® (Promega, United States) was employed as previously reported.[7] The experiment was performed in triplicate.

1.6. PEG-HGNs preincubation in B16-F10 cells and exosPEG-HGNs isolation

The cellular uptake was observed under a confocal microscope (Spectral Confocal Microscope Leica TCA SP2 and ZEISS LSM880 Confocal Laser Scanning Microscope) with a 63x oil immersed N.A.

1.40 objective. Cells were seeded at a density of $2 \cdot 10^4$ cells onto 20 mm cover slips (in a 24-well plate) and cultured for 24 h. The NPs (0.125 mg mL^{-1}) resuspended in DMEM were added to the cells and incubated for 24 h. Then, cells were fixed with para-formaldehyde (PFA) 4 %. Cytoplasmic actin was labeled by staining with phalloidin-Alexa488 (Invitrogen, United States) and Draq-5 was used to observe the nuclei. Reflection of the incident light at 488/490 nm was used to directly visualized HGNs and PEG-HGNs-based agglomerates. Z-stack orthogonal projections were analyzed to determine the presence of NPs inside the cytosol.

Finally, to quantify the amount of gold inside B16-F10 cells, they were seeded onto 6-well plates at a density of 2.5 x 10^5 cells per well and were grown for 24 h. Later, HGNs and PEG-HGNs dispersions (0.125 mg mL^{-1}) were prepared in fresh DMEM and added to the cell cultures for 24, 48 and 72 h. After these time points, cells were harvested and washed twice with PBS (1500 rpm, 5 min). Control samples (cells without treatment) were collected using the same protocol. The cellular pellets obtained were digested with 10 % Aqua regia (HNO_3 + 3HCl) in 1.5 mL of dH_2O. Digestion was performed at room temperature for 1 h. Total amount of gold derived from HGNs and PEG-HGNs was determined by MP-AES (4100 MP-AES, Agilent Technologies, United States). Furthermore, considering NPs as hollow spheres of known diameter and thickness and knowing gold metal density, the gold mass present in each particle (HGH and PEG-HGN) could be estimated, and from this, the number of NPs per cell at 24, 48 and 72 h was calculated after allowing for the contribution of PEG mass, obtained from the thermogravimetric analysis (TGA).[6]

1.7. PEG-HGNs are released through the exosomal secretory route

To evaluate the exosome biogenesis and release pathways, 2 x 105 cells per well were seeded onto a 6-well plate and were grown for 24 hours in complete DMEM medium. Then PEG-HGNs were added at 0.125 mg mL-1 to the cell cultures for another 24 hours. Before purifying exosPEG-HGNs, B16-F10 cells were treated during 2 hours with 2 µM manumycin A (Sigma-Aldrich, United States) in order to suppress exosome secretion. It is reported that manumycin A inhibits endocytosis via

inhibition of Ras signaling in cancer cells.57 Separately, cells were transfected with 250 nM ON-TARGET plus mouse Rab27a siRNA-SMART pool (Dharmacon, United States) using Lipofectamine 2000 (ThermoFisher, United States). Rab27 is a GTPase that mediates the late steps of vesicle exocytosis in cells.58 Thereafter, DMEM medium was replaced by ULTRACEN medium during 48 h for the subsequent exosome isolation procedure. In order to compare the number of exosomes secreted when cells were treated with manumycin A or when they were transfected with Rab27-siRNA, exosPEG-HGNs produced by untreated B16-F10 cells were considered as controls. ExosPEG-HGNs isolated from the different treated cells, were characterized by TEM, WB and BCA as previously described the exosome isolation and characterization section. From TEM images a semiquantitative analysis was performed in order to estimate the number of empty or loaded exosomes produced by treated cells compared with the control cells. In WB, calnexin and TSG101 expression were analysed as shown in the supplementary information section.

1.8. Statistical analysis

All the results expressed as the mean ± the standard deviation were performed in triplicate. The statistical analysis of the data was carried out using the GraphPad Prism 7.04. Significance was determined by one-way analysis of variance (ANOVA) using the Tukey's multiple comparisons test for the encapsulation strategies, cytotoxicity and MP-AES results and the Dunnett's multiple comparison test for the MP-AES data: *$*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$.*

2. Results and discussion

Physicochemical characterization of HGNs

Both HGNs showed a diameter of 36.3 ± 5.4 nm and PEG-HGNs had a PEG shell of around 5 nm (Fig. 1A). As shown in Fig. 1B, both NPs dispersions presented a characteristic surface plasmon resonance peak above 800 nm (NIR region), making them useful for optical hyperthermia applications (this peak is characteristic of spherical PEG-HGNs of approximately 40 nm).

Remarkably, it is crucial to obtain NPs of this size and morphology to guarantee their potential application as optical hyperthermia absorbing moieties in the NIR region.[8] Z-potential measurements gave a negative surface charge in HGNs (-16.38 ± 2.72 mV) and PEG-HGNs (-16.56 ± 2.96 mV) at pH 7.

B16-F10-exos characterization

B16-F10-exos were spherical vesicles with a diameter of 95.1 ± 27.8 nm (Fig 2A) as expected from the literature.[9,10] They presented a double lipidic membrane with a thickness of almost 10 nm (observed in TEM images from Fig 2A). Exosome diameter was also confirmed by NTA, revealing also a diameter of 159.7 ± 57.4 nm (Fig. 2B). Exosomes were negatively charged (zeta potential of -18.56 ± 1.93 mV in 10 mM KCl at pH 7) due to the phospholipid nature of the exosomal membranes.[11] Fig. 2C shows the results of the Western Blot (WB) assays indicating that B16-F10-exos expressed a housekeeping gene (α-actin), an exosomal membrane protein (CD9) and a citoplasmatic exosomal marker (TSG101). On the contrary, when using a non-exosomal protein (calnexin) no band was observed. These results confirm that B16-F10-exos correspond with exosomes rather than with other extracellular vesicles of different nature.[12]

3. References

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SUPPLEMENTARY FIGURES

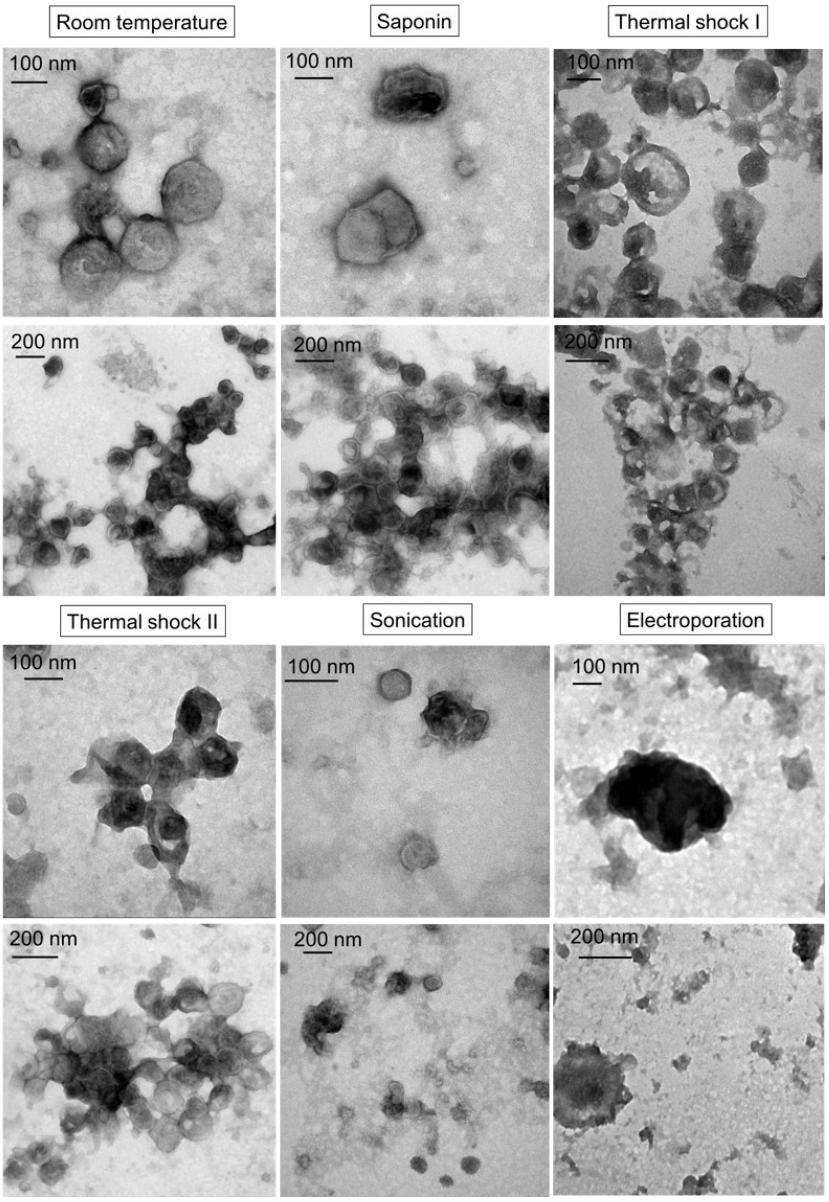
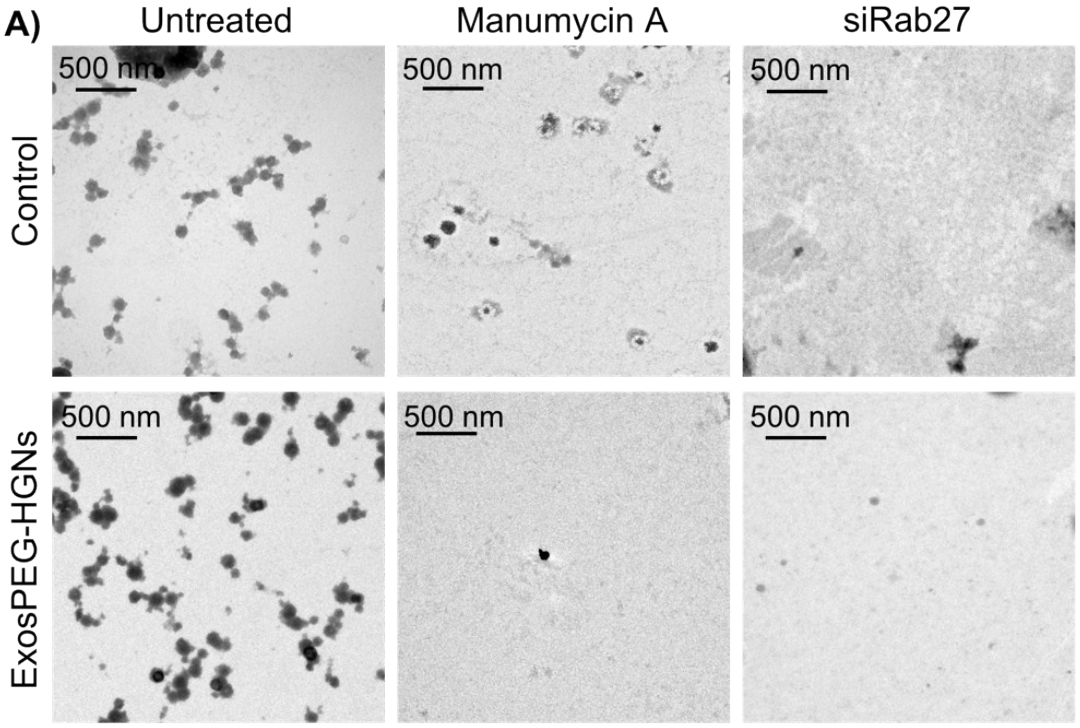


Fig. S1. TEM images of exosomes treated by the different physicochemical approaches in absence of NPs (incubation at room temperature with the presence or absence of saponin, two different thermal shock procedures, sonication and electroporation).



B)

	Untreated	Manumycin A	siRab27
Control	100%	18.75 %	6.25 %
ExosPEG-HGNs	100 %	10.41 %	4.5 %

Fig. S2. A) Representative TEM images obtained from exosomes released by untreated B16-F10 cells, by manumycin A treated cells or by siRab27 transfected cells. B) Semiquantitative analysis of secreted exosomes released by untreated B16-F10 cells, by manumycin A treated cells from TEM images.

SUPPLEMENTARY TABLES

Table S1. Statistical analysis of the encapsulation of NPs within exosomes.

	Summary	Adjusted P Value
Incubation R.T. with saponin (0.2 %) vs. Incubation R.T.	ns	0.9988
Incubation R.T. with saponin (0.2 %) vs. Thermal shock I	ns	0.9241
Incubation R.T. with saponin (0.2 %) vs. Thermal shock II	ns	0.573
Incubation R.T. with saponin (0.2 %) vs. Sonication	ns	0.9645
Incubation R.T. with saponin (0.2 %) vs. Electroporation	*	0.0124
Incubation R.T. with saponin (0.2 %) vs. Incubation with B16-F10cells	****	<0.0001
Incubation R.T. vs. Thermal shock I	ns	0.7093
Incubation R.T. vs. Thermal shock II	ns	0.8343
Incubation R.T. vs. Sonication	ns	0.7993
Incubation R.T. vs. Electroporation	*	0.0296
Incubation R.T. vs. Incubation with B16-F10cells	****	<0.0001
Thermal shock I vs. Thermal shock II	ns	0.1276
Thermal shock I vs. Sonication	ns	>0.9999
Thermal shock I vs. Electroporation	**	0.0018
Thermal shock I vs. Incubation with B16-F10cells	****	<0.0001
Thermal shock II vs. Sonication	ns	0.168
Thermal shock II vs. Electroporation	ns	0.2706
Thermal shock II vs. Incubation with B16-F10cells	****	<0.0001
Sonication vs. Electroporation	**	0.0024
Sonication vs. Incubation with B16-F10cells	****	<0.0001
Electroporation vs. Incubation with B16-F10cells	****	<0.0001

Table S2. Statistical analysis of the cytotoxicity experiment.

HGNs 24 h	**Summary**	**Adjusted P Value**
Control vs. 0.5 mg/mL	****	<0.0001
Control vs. 0.250 mg/mL	**	0.0024
Control vs. 0.125 mg/mL	ns	0.355
Control vs. 0.06 mg/mL	ns	0.8128
Control vs. 0.03 mg/mL	ns	0.917
Control vs. 0.015 mg/mL	ns	0.8727
Control vs. 0.006 mg/mL	ns	0.6999
PEG-HGNs 24 h	**Summary**	**Adjusted P Value**
Control vs. 0.5 mg/mL	ns	0.0813
Control vs. 0.250 mg/mL	ns	0.6189
Control vs. 0.125 mg/mL	ns	0.2749
Control vs. 0.06 mg/mL	ns	0.3113
Control vs. 0.03 mg/mL	ns	0.8098
Control vs. 0.015 mg/mL	ns	0.8757
Control vs. 0.006 mg/mL	ns	0.6958
HGNs 48 h	**Summary**	**Adjusted P Value**
Control vs. 0.5 mg/mL	***	0.0007
Control vs. 0.250 mg/mL	***	0.0005
Control vs. 0.125 mg/mL	ns	0.1041
Control vs. 0.06 mg/mL	ns	0.695
Control vs. 0.03 mg/mL	ns	0.5105
Control vs. 0.015 mg/mL	ns	0.6599
Control vs. 0.006 mg/mL	ns	0.1819
PEG-HGNs 48 h	**Summary**	**Adjusted P Value**
Control vs. 0.5 mg/mL	***	0.0007
Control vs. 0.250 mg/mL	**	0.0049
Control vs. 0.125 mg/mL	ns	0.0824
Control vs. 0.06 mg/mL	ns	0.206
Control vs. 0.03 mg/mL	ns	0.9997
Control vs. 0.015 mg/mL	ns	0.9997
Control vs. 0.006 mg/mL	ns	0.9995
HGNs 72 h	**Summary**	**Adjusted P Value**

Control vs. 0.5 mg/mL	***	0.0007
Control vs. 0.250 mg/mL	**	0.0049
Control vs. 0.125 mg/mL	ns	0.0824
Control vs. 0.06 mg/mL	ns	0.206
Control vs. 0.03 mg/mL	ns	0.9997
Control vs. 0.015 mg/mL	ns	0.9997
Control vs. 0.006 mg/mL	ns	0.9995
PEG-HGNs 72 h	**Summary**	**Adjusted P Value**
Control vs. 0.5 mg/mL	ns	0.622
Control vs. 0.250 mg/mL	ns	0.711
Control vs. 0.125 mg/mL	ns	0.9882
Control vs. 0.06 mg/mL	ns	0.992
Control vs. 0.03 mg/mL	ns	>0.9999
Control vs. 0.015 mg/mL	ns	>0.9999
Control vs. 0.006 mg/mL	ns	0.9995

Table S3. Statistical analysis of the MP-AES experiment. A) Comparison of all the conditions with the untreated cells. B) Comparison of cells treated with one type of NPs at 24, 48 and 72 hwith every other samples treated with these NPs at other time points.

A)	**Summary**	**Adjusted P Value**
Control vs. PEG-HGNs 24 h	****	<0.0001
Control vs. PEG-HGNs 48 h	ns	0.9925
Control vs. PEG-HGNs 72 h	ns	0.8895
Control vs. HGNs 24 h	ns	0.9999
Control vs. HGNs 48 h	ns	0.9999
Control vs. HGNs 72 h	ns	0.9999

B)	**Summary**	**Adjusted P Value**
PEG-HGNs 24 h vs. PEG-HGNs 48 h	**	0.0018
PEG-HGNs 24 h vs. PEG-HGNs 72 h	**	0.0029
PEG-HGNs 48 h vs. PEG-HGNs 72 h	ns	0.9363
	Summary	**Adjusted P Value**
HGNs 24 h vs. HGNs 48 h	ns	0.1067
HGNs 24 h vs. HGNs 72 h	ns	0.6576
HGNs 48 h vs. HGNs 72 h	ns	0.5691

Table S4. Statistical analysis of cell culture temperatures after NIR laser irradiation.

	Summary	Adjusted P Value
0 min	ns	>0.9999
2 min	ns	0.9745
8 min	ns	0.5989
12 min	ns	0.1282
15 min	ns	0.0915
20 min	ns	0.0726
25 min	*	0.0197
30 min	**	0.0037

Table S5. Statistical analysis of exosPEG-HGNs inside cell cytoplasm from confocal images.

	Summary	Adjusted P Value
Control vs. 2 h	ns	0,2762
Control vs. 4 h	ns	0,6152
Control vs. 6 h	***	0,0003
Control vs. 8 h	****	<0,0001
Control vs. 24 h	****	<0,0001
Control vs. 48 h	****	<0,0001