

Supporting Information

Pro-apoptotic and size-reducing effects of protein corona-modulating nano-architectures enclosing platinum prodrug in *in vivo* oral carcinoma

Ana Katrina Mapanao^{1,§,§}, Patrizia Sarogni^{1,§}, Melissa Santi^{2,§}, Michele Menicagli³,
Alessandra Gonnelli^{1,4}, Agata Zamborlin^{1,5}, Maria Laura Ermini¹, and Valerio Voliani^{1,*}

1. Center for Nanotechnology Innovation@NEST – Istituto Italiano di Tecnologia, Piazza San Silvestro 12, 56127, Pisa, Italy

2. NEST, Istituto Nanoscienze-CNR and Scuola Normale Superiore, Piazza San Silvestro 12, 56127 Pisa, Italy

3. Fondazione Pisana per la Scienza ONLUS, via Ferruccio Giovannini 13, S. Giuliano Terme 56017, Pisa, Italy

4. Radiation Oncology Unit, Pisa University Hospital, Via Roma 67, 56126, Pisa, Italy

5. NEST – Scuola Normale Superiore, Piazza San Silvestro 12, 56127, Pisa, Italy

§ Current address: Center for Radiopharmaceutical Sciences, Paul Scherrer Institute, 5232 Villigen-PSI, Switzerland

§These authors contributed equally.

*Corresponding author: valerio.voliani@iit.it

Note 1. Synthesis procedures

Synthesis of fluorophore-modified poly(L-lysine) (PL-647)

Poly(L-lysine) hydrobromide (PL; 15-30 kDa) was dissolved in milliQ water to a final concentration of 40 mg/mL. In a microtube, 75 μ L of PL was mixed with 2 μ L of AlexaFluor-647 NHS ester (10 mg/mL in dimethyl sulfoxide; Invitrogen A20006), and 100 μ L of acetate buffer 0.3 M (pH 5.4). The mixture was kept in dark and incubated on a shaker (700 rpm) overnight at room temperature. The product PL-647 was used without further purification.

Synthesis of cisplatin prodrug-modified poly(L-lysine) (PL-cisPt)

The cisplatin prodrug *c,t,c*-[PtCl₂(NH₃)₂(OH)(O₂CCH₂CH₂CO₂H)] was synthesized as described elsewhere [1]. Upon dissolving 12 mg of the prodrug in PBS (1X, pH 7.4, 100 μ L), a mixture of freshly made solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *n*-hydroxysuccinimide (NHS) prepared in PBS (100 μ L) (25 mg EDC/15 mg NHS) was added. The mixture was incubated for 20 min. After which, 750 μ L of aqueous solution of poly(L-lysine) (PL; 15-30 kDa; 30 mg) was added. The mixture was then incubated overnight on a shaker set at 700 rpm, under room temperature. The product (PL-cisPt) was recovered through filtration using Amicon 10 kDa centrifugal filter and washed thrice with milliQ water. Finally, PL-cisPt was resuspended to 1 mL of milliQ water and stored at -20°C until needed.

Synthesis of fluorophore-loaded (NAs-647) or prodrug-loaded nano-architectures (NAs-cisPt)

Gold ultrasmall nanoparticles (USNPs) were synthesized through rapid reduction of gold salt, beginning with 200 μ L of aqueous solution of tetrachloroauric (III) acid (HAuCl₄; Alfa Aesar, ACS 99.99% metal basis; stock: 10 mg/mL) and 10 μ L poly(sodium 4-styrene sulfonate) (PSS; 70 kDa; 30% aqueous solution) being added to 20 mL of milliQ water. Freshly prepared aqueous solution of sodium borohydride (200 μ L of the 8 mg/mL stock) was quickly added to the vigorously stirring solution containing the gold salts. After 2 min of vigorous stirring, the solution was further aged for another 10 min before 177 μ L of PL-647 (for NAs-647) or 165 μ L of PL-cisPt (for NAs-cisPt) was added. Then, the solution was incubated for 20 min. The gold USNP polymeric arrays with the fluorophore or prodrug were collected by centrifugation at 14000 rpm for 5 min. After removing the supernatant, the product was resuspended in 2 mL of milliQ water.

The Stöber process was modified to construct silica shell on the periphery of gold polymeric arrays. Two 50-mL tubes were filled each with 35 mL ethanol and 1.2 mL ammonia solution (Merck, 32%). Once the gold polymeric arrays were ready, 20 μ L tetraethyl orthosilicate and 1 mL of the gold arrays were added on each tube. The mixture was incubated for 3 h at room

temperature under moderate shaking. Then, the resulting nano-architectures (NAs-647 or NAs-cisPt) were collected through a 30-minute centrifugation at 4000 rpm. After discarding the supernatant, the resulting product was added with, sonicated, and resuspended in ethanol. The suspension was spun at 14000 rpm for 3 minutes and the washing was discarded. The product was again added and sonicated with ethanol for another round of washing. After another centrifugation at 14000 rpm for 3 min, the washing was discarded and ethanol was again added and the product was sonicated. Then, short spin (15 s or until rotational speed reaches 14000 rpm) was done to remove larger nanoparticles. The supernatant was separated and spun at 14000 rpm for 3 min to collect the final NAs-647 or NAs-cisPt. After removing the supernatant, the final products (stable for at least 1-year) were resuspended and stored in 1 mL of ethanol.

Synthesis of surface functionalized nano-architecture (NAs-647-Tf2 or NAs-cisPt-Tf2)

The ethanolic suspension of the nanoparticles (1 mL) was mixed with the freshly prepared solution of the linker silane-poly(ethylene glycol)-maleimide dissolved in ethanol (1 mL of the stock 4 mg/mL). The volume was adjusted to 10 mL with ethanol and the mixture was stirred vigorously for 15 min. The nanoparticles were recovered through centrifugation at 14000 rpm for 3 min. After removing the supernatant, the particles were recollected and resuspended in 1.2 mL of N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid (HEPES) buffer (20 mM, pH 7.2). Meanwhile, the Tf2 peptide was prepared as reported in Santi *et al* (2016) [2]. A stock Tf2 solution (2 mg/mL) was prepared by dissolving the lyophilized sample in degassed HEPES buffer. Then, 300 μ L of the peptide solution was added to the buffered solution containing the nanoparticles, resulting in a Tf2 final concentration equal to 0.4 mg/mL. The mixture was stirred for 2 h, added with 500 μ L L-glutathione (GSH; final concentration equal to 200 μ g/mL in HEPES buffer), and stirred for another hour. GSH was added to react with the excess maleimide ends that did not react with the Tf2 peptide. After the cumulative 3-hour incubation, the mixture was spun at 14000 rpm for 3 min to recover the nanoparticles. Finally, the recovered NAs-647-Tf2 or NAs-cisPt-Tf2 was washed twice with ethanol, and stored in ethanol at -20°C.

Table S1. Comparison of the physical-chemical properties of NAs

	Standard NAs	NAs-647	NAs-647-Tf2	NAs-cisPt	NAs-cisPt-Tf2
<i>Average diameter by TEM count (nm)</i>	98.0 ± 19.0	127.6 ± 28.0	113.9 ± 22.7	122.4 ± 16.3	118.6 ± 31
<i>Zeta potential (mV)</i>	-20.6 ± 0.4	-21.3 ± 0.6	-7.2 ± 0.4	-19.6 ± 0.6	-7.01 ± 0.9
<i>Hydrodynamic diameter (nm)</i>	203.1 ± 1.9	207.2 ± 1.2	220.2 ± 30	227.1 ± 0.7	265.3 ± 30.4
<i>Metal loading (%w/w)</i>	Au = 4.5	Au = 9.6	Au = 9.6	Au = 4.9 Pt = 1.4	Au = 4.8 Pt = 0.7
<i>Reference (DOI)</i>	10.1021/acsbm.9b00630	10.1021/acsomega.8b01719	10.1021/acsomega.8b01719	10.3390/cancers12051063	This manuscript

Table S2. Tumor volume data analyses

Metric	Formula	Description/ Note
(1) Volume	$\frac{1}{2} (\text{length} \times \text{width}^2)$	Volume derived from superficial measurements, where length and width correspond to the longer and shorter dimensions, respectively.
(2) Volume fold change	$\frac{\text{Volume}_{(EDD\ 12\ or\ 14)}}{\text{Volume}_{(EDD\ 10)}}$	Volume fold change were measured for each tumor and referred to the respective changes in volume post-treatment (EDD 12 or 14) with respect to pre-treatment volume (EDD 10).
(3) Relative volume fold change ("R-value")	$\frac{\text{Average volume fold change}_{(EDD\ 12\ or\ 14)}}{\text{Average volume fold change of control}_{(EDD\ 12)}}$	This metric compares the change in volume after experimental treatment to the initial effect of the serum-free medium vehicle solution on the tumors treated on EDD 12. The "average volume fold change" refers to the values (metric #2) for each treatment type.

Table S3. Average tumor volumes

	EDD 10 (pretreatment)	EDD 12	EDD 14
Control	5.6 ± 2.2	7.2 ± 2.2	3.7 ± 2.6
cisplatin	5.9 ± 1.4	3.4 ± 0.8	2.5 ± 0.7
NAs-cisPt	9.0 ± 2.2	6.3 ± 1.8	3.7 ± 1.0
NAs-cisPt-Tf2	6.3 ± 2.2	2.5 ± 1.0	1.4 ± 0.5

The data are reported as mean ± standard error of the mean of pooled samples from two independent experiments, with N > 8 eggs per condition.

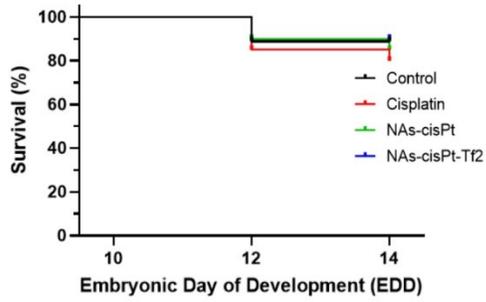
Table S4. Relative volume fold change (R-values) for the different treatments

	EDD 12	EDD 14
Control	1	0.95 ± 0.40
cisplatin	0.55 ± 0.01	0.27 ± 0.09
NAs-cisPt	0.40 ± 0.31	0.25 ± 0.17
NAs-cisPt-Tf2	0.08 ± 0.06	0.05 ± 0.02

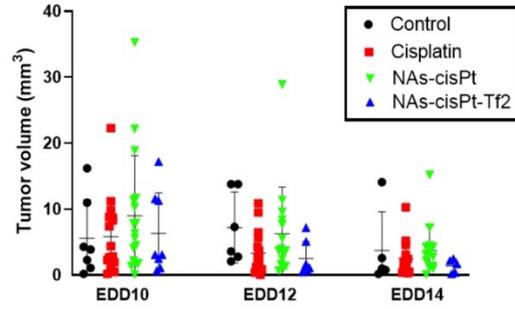
The data are reported as mean ± standard deviation of two independent experiments, with N > 8 eggs per condition, per experiment.

Figure S1

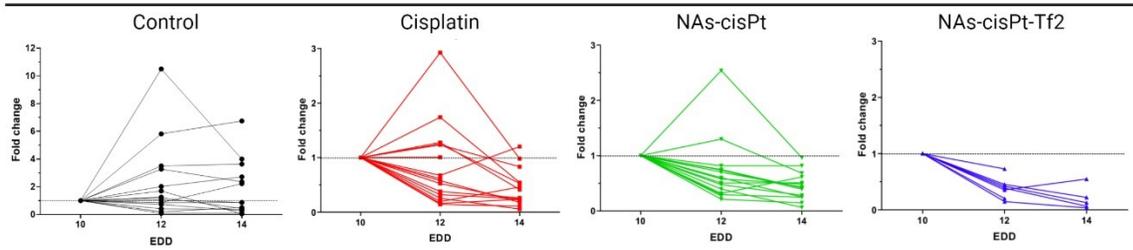
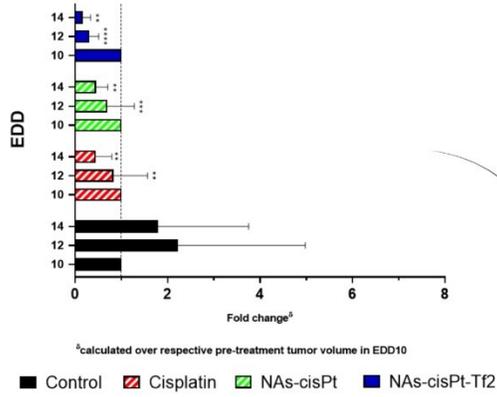
A



B



C



D

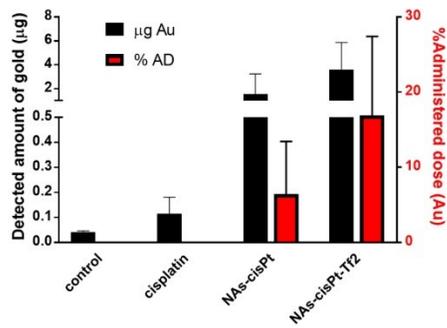


Figure S.1. NAs chemotherapeutic evaluation on SCC-25 CAM tumor models. **(A)** The average survival of the embryos was monitored and reported (Kaplan-Meier) with respect to the initial number of models treated on EDD 10. **(B)** Tumors were monitored until EDD 14 (*i.e.* 4 days post-treatment), and the dimensions were used to calculate the corresponding volumes. The values plotted at EDD 10 correspond to pre-treatment volumes. **(C)** The average tumor volume of a specific treatment condition on EDD12 and 14 was compared to the respective average on EDD10 in order to evaluate the tumor volume fold change (*upper*). Fold change = 1 means no volume change with respect to EDD 10 (pre-treatment). The pooled data are reported as mean + standard deviation of at least 10 tumor models, and two-way ANOVA (Tukey's multiple comparisons test) statistical analysis was performed. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. The individual changes in tumor volume were grouped per treatment. Each dot represents one tumor model, and the lines indicate the respective changes at different EDDs (*bottom*). **(D)** The amount of gold was quantified in the harvested tumors (*black*), and the % administered dose (%AD) was calculated considering the applied amount of gold in the nano-architectures (*red*). Data are reported as mean + standard derivation of at least two tumors per treatment. Two-way ANOVA (Šidák's multiple comparisons test) was performed on %AD (Au) results ($p > 0.1$). Data for cisplatin and NAs-cisPt were reprocessed from Sarogni *et al.* [3]

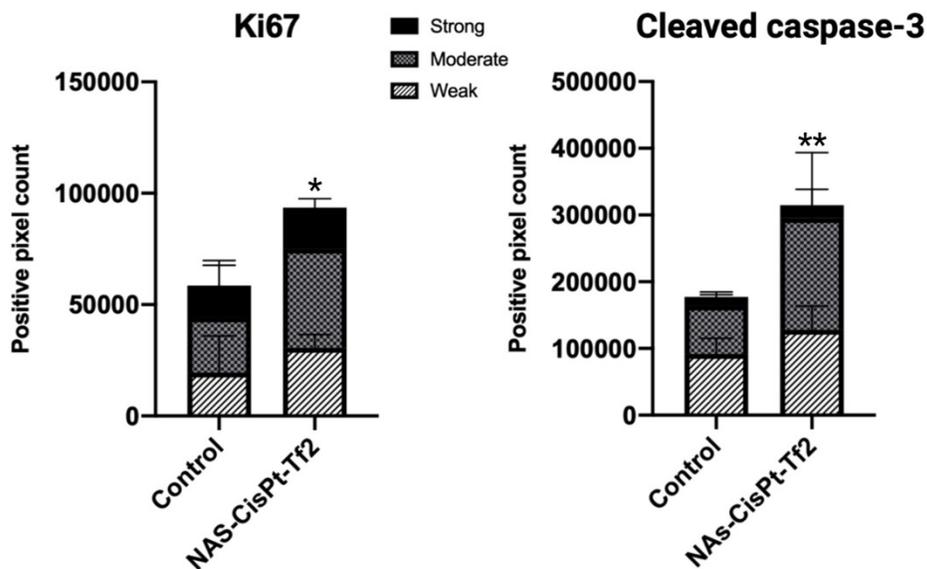


Figure S2

Figure S.2. Evaluation of the proliferation and apoptosis rate. IHC images were further analyzed using Aperio ImageScope software. The algorithm automatically generated a scoring system based on the intensity of positive staining, and classified them into *weak*, *moderate*, or *strong* (brown signal). The data are reported as mean + standard deviation of three areas of the same slide section. Statistical analysis was performed through one-way ANOVA *p-value < 0.05, **p-value < 0.01 (*right*).

References

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<https://doi.org/10.1021/acs.bioconjchem.6b00611>.

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