Diameter-dependent release of a cisplatin pro-drug from small and large functionalized carbon nanotubes

Laura Muzi[1,2], Cécilia Ménard-Moyon[1], Julie Russier[1], Jian Li[3], Chee Fei Chin[4], Wee Han Ang[4,5], Giorgia Pastorin[3,5*], Gianfranco Risuleo[2*], Alberto Bianco[1*]

Electronic Supplementary Information

General information

All reagents and solvents were purchased from different commercial suppliers and used as received. The cyanine 5 derivative **5** was provided by Dr. Anthony Romieu.[S1] The Boc-monoprotected diamine derivative **2** was synthesized according to the protocol described in ref. S2. The L-CNTs **S1** were prepared according to the procedure reported in reference S3.

When stated, suspensions of CNTs were sonicated either in a water bath (Transsonics Digitals Elma, 20 W, 40 kHz) or by using tip sonication (Vibra-Cel Ultrasonic Processor). Dialysis was performed using Spectra/Por® dialysis membrane, molecular weight cut-off (MWCO): 12-14,000 Da. The Kaiser test was performed according to a procedure described in reference S4.

Preparation of oxidized S-CNTs 1

The pristine purified S-CNTs (0.5 g) were dispersed in a mixture of sulfuric acid 98% (56 mL) and nitric acid 65% (19 mL). The suspension was sonicated in a water bath (20 W, 40 kHz) for 24 h. The temperature of the water bath was regularly cooled down by adding ice. The temperature did not exceed 35 °C. Deionized water was then carefully added to the dispersion cooled down in an ice bath. The suspension was filtered (Omnipore® membrane filtration from Millipore, 0.45 μm), the solid was dispersed in deionized water and filtered again. This sequence was repeated until the pH of the filtrate was neutral. The oxidized S-CNTs **1** were dried under vacuum.

Preparation of S-CNTs 3

The oxidized S-CNTs **1** (102 mg) were dispersed in oxalyl chloride (22 mL) using water bath sonication for 10 min. The suspension was heated at reflux under argon for 19 h. The excess of

oxalyl chloride was evaporated under vacuum. The resulting acyl chloride CNTs were dispersed in dry THF (13 mL). *N*,*N*-diisopropylethylamine (1.3 mL) and a solution of Boc-monoprotected diamine derivative **2** (612 mg) in dry THF (13 mL) were added. The dispersion was sonicated in a water bath for 2 min and heated at reflux for 32 h. The suspension was filtered using 0.45 µm filtration membrane. The solid recovered on the filter was dispersed in DMF (300 mL), sonicated for 10 min in a water bath, and filtered again. This sequence was repeated with methanol and dichloromethane. The resulting solid was dried under vacuum to give S-CNTs **3**.

Preparation of S-CNTs 4

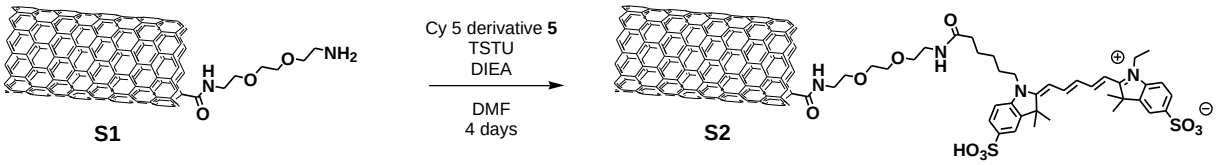
S-CNTs **3** (95 mg) were dispersed in 1,4-dioxane (60 mL). HCl 4 M in 1,4-dioxane (30 mL) was added. The dispersion was sonicated in a water bath for 5 min and stirred for 17 h. The suspension was filtered using 0.45 µm filtration membrane. The solid recovered on the filter was dispersed in MeOH (200 mL), sonicated for 10 min in a water bath, and filtered again. This sequence was repeated with methanol and dichloromethane. The resulting solid was dispersed in deionized water and dialyzed against water for 2 days using 12-14,000 Da MWCO dialysis membrane. The suspension was lyophilized to give S-CNTs **4**.

Preparation of S-CNTs 6

To a solution of Cy 5 derivative **5** (5 mg) in anhydrous DMF (2.5 mL) were added DIEA (5 µL) and a solution of TSTU (2.75 mg) in anhydrous DMF (2.5 mL). The reaction mixture was stirred for 3 h in the dark. Then, DIEA (20 µL) and a suspension of S-CNTs **4** (11 mg) in anhydrous DMF (3 mL) were added to the reaction mixture. The dispersion was sonicated in a water bath for 5min and stirred in the dark for 40 h. The suspension was filtered using 0.45 µm filtration membrane. The solid recovered on the filter was dispersed in DMF (150 mL), sonicated for 5 min in a water bath, and filtered again. This sequence was repeated with DMF, twice with methanol and once with dichloromethane. The resulting solid was dried under vacuum to give S-CNTs **6**.

Preparation of L-CNTs S2

The protocol for the preparation of S-CNTs **6** was used to prepare L-CNTs **S2** from L-CNTs **S1**.



Scheme S1 Functionalization of amino-L-CNTs with cyanine 5.

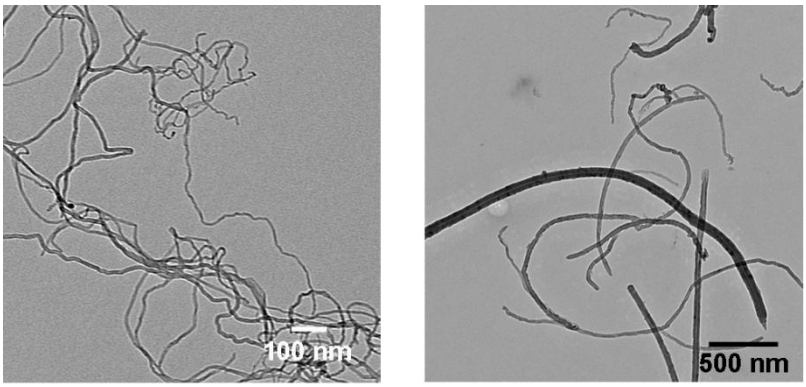


Fig. S1 TEM of pristine purified S-CNTs (left) and L-CNTs (right).

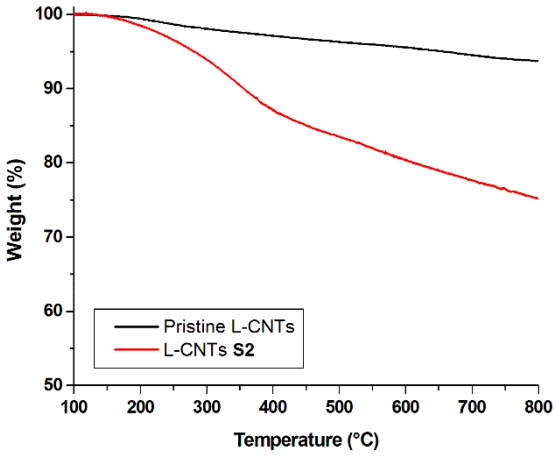


Fig. S2 TGA of pristine L-CNTs and L-CNTs **S2** under N_2.

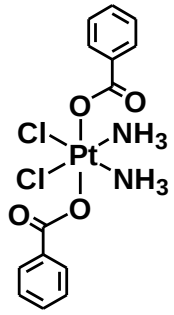


Fig. S3 Structure of platinum(IV) complex **S3**.

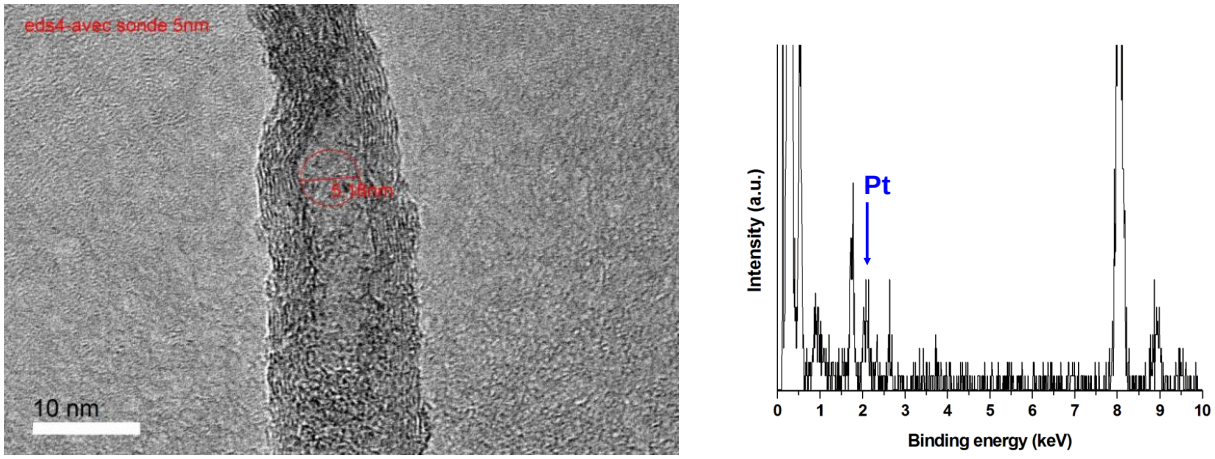


Fig. S4 HR-TEM of Pt(IV)@S-CNTs **6** and EDX spectrum taken in the cavity. Scale bar corresponds to 10 nm.

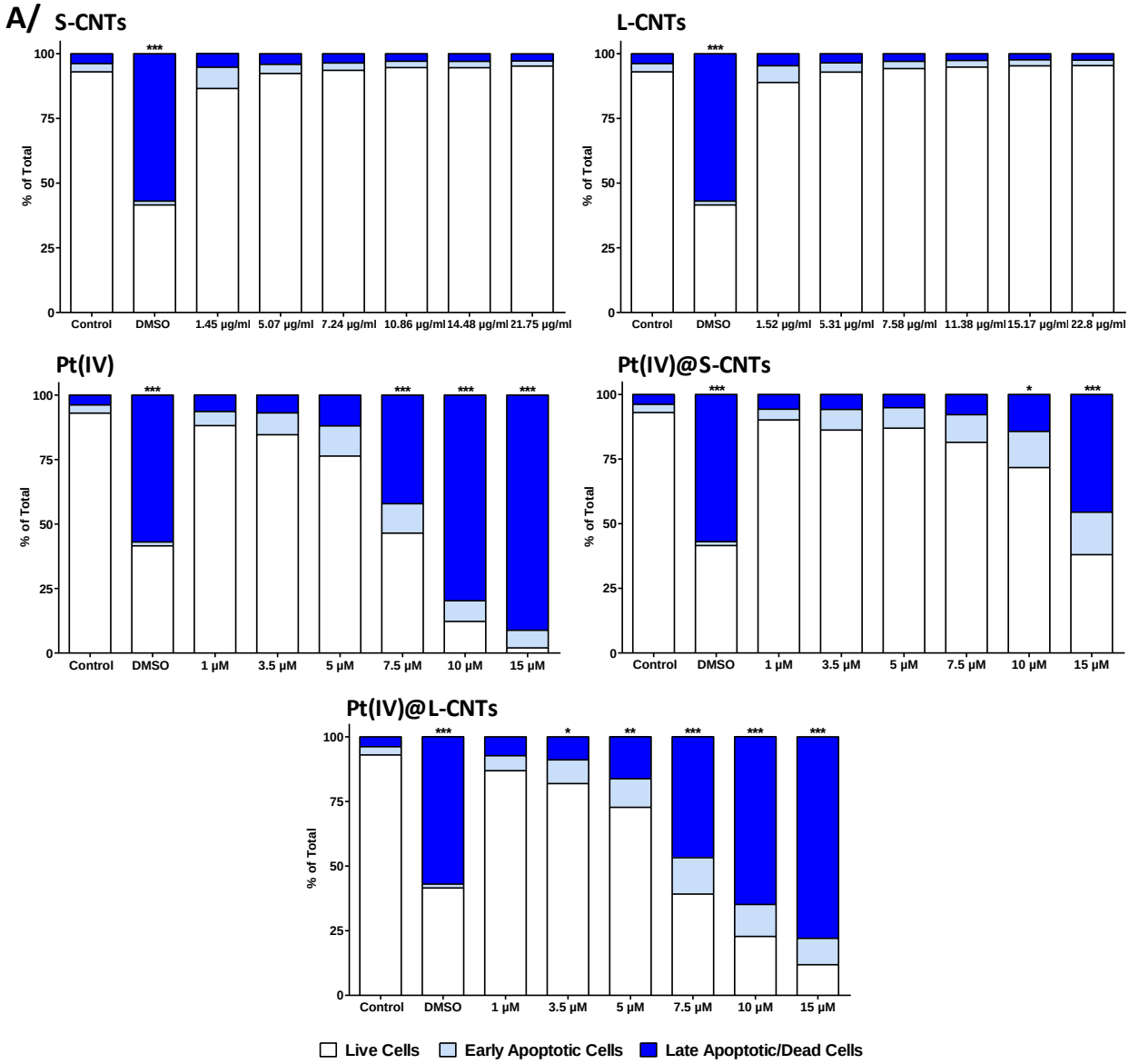
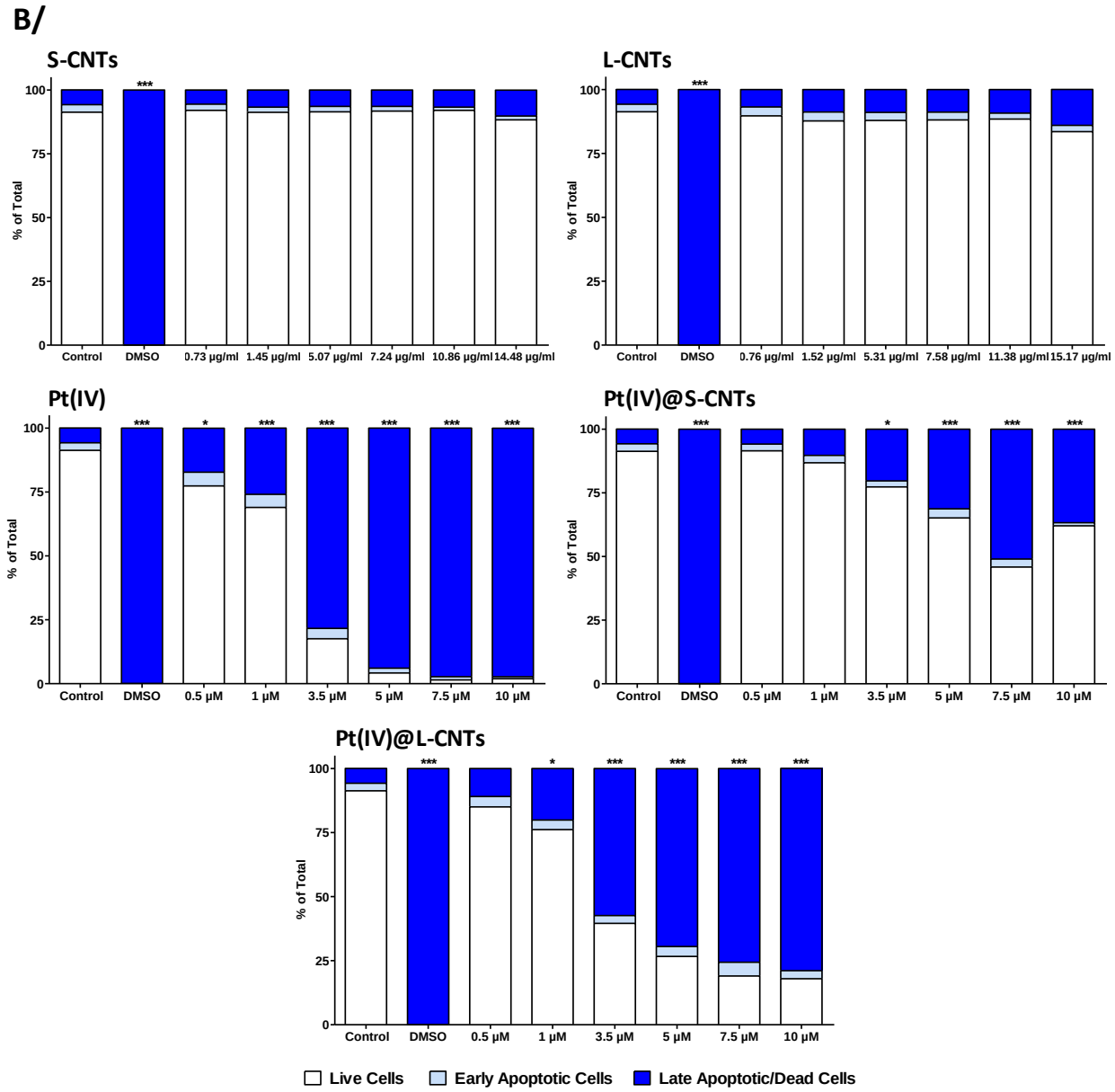


Fig. S5 (A) Flow cytometry analysis of cellular viability in HeLa cells exposed to different concentrations of S-CNTs, L-CNTs, Pt(IV)@S-CNTs and Pt(IV)@L-CNTs for 24 h. Two-ways ANOVA followed by Bonferroni's post-test was performed to determine the statistical differences *versus* control cells (*p<0.05; **p<0.01; ***p<0.001).



(B) Flow cytometry analysis of cellular viability in RAW 264.7 cells exposed to different concentrations of S-CNTs, L-CNTs, Pt(IV)@S-CNTs and Pt(IV)@L-CNTs for 24 h. Two-ways ANOVA followed by Bonferroni's post-test was performed to determine the statistical differences *versus* control cells (*p<0.05; **p<0.01; ***p<0.001).

Cells were treated with different concentrations of the complexes during 24 h. CNT concentrations were back-calculated from Pt(IV) loading inside CNTs. The concentrations of the encapsulated drug are reported in the graph as µM. In the case of the empty control, the corresponding CNT concentrations are reported as µg/ml. In the case of HeLa cells (Fig. S5A), a concentration-dependent toxicity was observed with both CNT samples and also the free drug. Furthermore, there was no significant difference comparing the free drug to Pt(IV)@L-CNTs in all the concentration range. On the contrary, both the free drug and the larger CNTs were more cytotoxic compared to

the smaller ones. It has to be noticed that the observed toxicity on HeLa cells was only due to Pt(IV)@CNT hybrids since the empty CNTs caused no alteration of HeLa cell viability at the same corresponding concentrations.

Same dose-dependent cytotoxicity but at lower concentrations was observed in RAW 264.7 cells (Fig. S5B). Moreover the free drug was significantly more effective compared to both L- (starting from 3.5 μM) and S- (starting from 1 μM) Pt(IV)@CNTs. Large CNTs were again more effective compared to the smaller ones as observed in HeLa cells.

As well as HeLa cells, the viability of RAW 264.7 macrophages was not affected by the control empty CNTs.

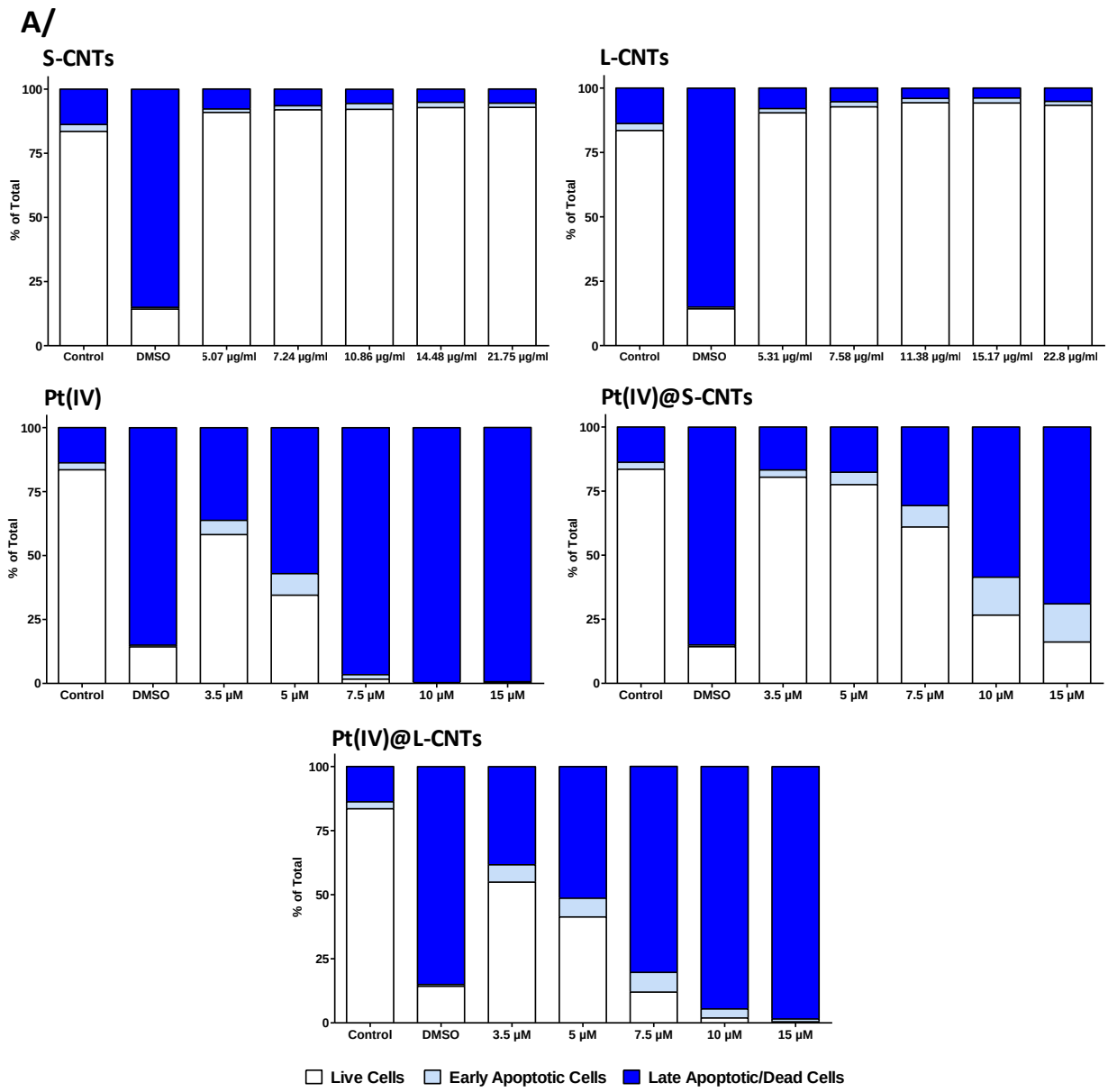
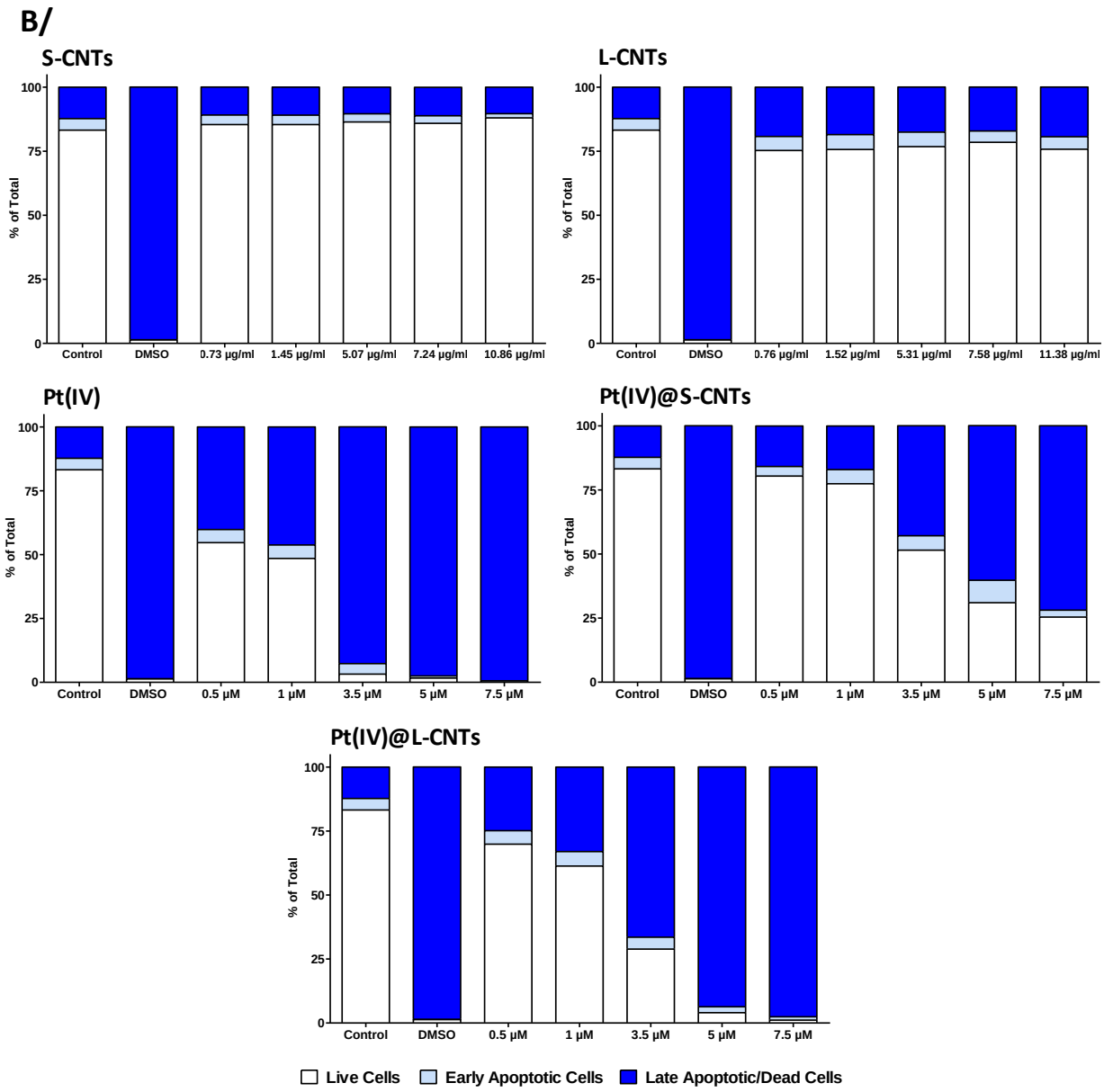


Fig. S6 (A) Flow cytometry analysis of cellular viability in HeLa cells exposed to different concentrations of S-CNTs, L-CNTs, Pt(IV)@S-CNTs and Pt(IV)@L-CNTs for 48 h.



(B) Flow cytometry analysis of cellular viability in RAW 264.7 cells exposed to different concentrations of S-CNTs, L-CNTs, Pt(IV)@S-CNTs and Pt(IV)@L-CNTs for 48h.

When a 48 hour CNT exposure was performed on both HeLa (Fig. S6A) and RAW 264.7 (Fig. S6B) cells, similar results to the 24 hour experiments were obtained.

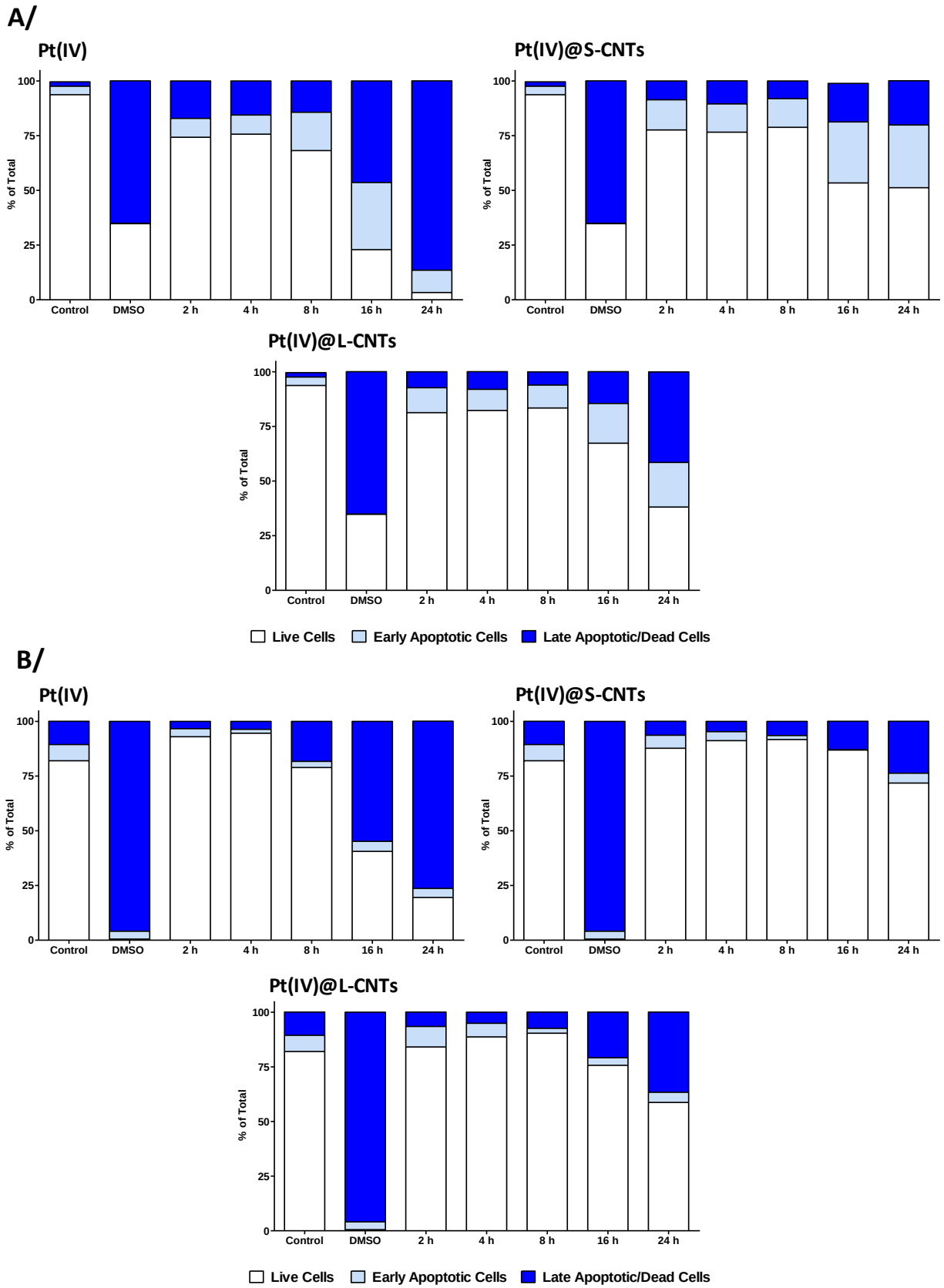


Fig. S7 Flow cytometry analysis of cellular viability in HeLa cells (A) and RAW 264.7 cells (B) exposed to 10 µM or 5 µM Pt(IV), respectively of Pt(IV)@CNTs for different times.

For the time course experiment, cells were exposed to both Pt(IV)@CNTs and the free drug for different times (from 2 to 24 hours). In this context, either in the case of HeLa (Fig. S7A) and RAW 264.7 (Fig. S7B), the free drug displayed a general higher cytotoxicity compared to Pt(IV)@CNTs.

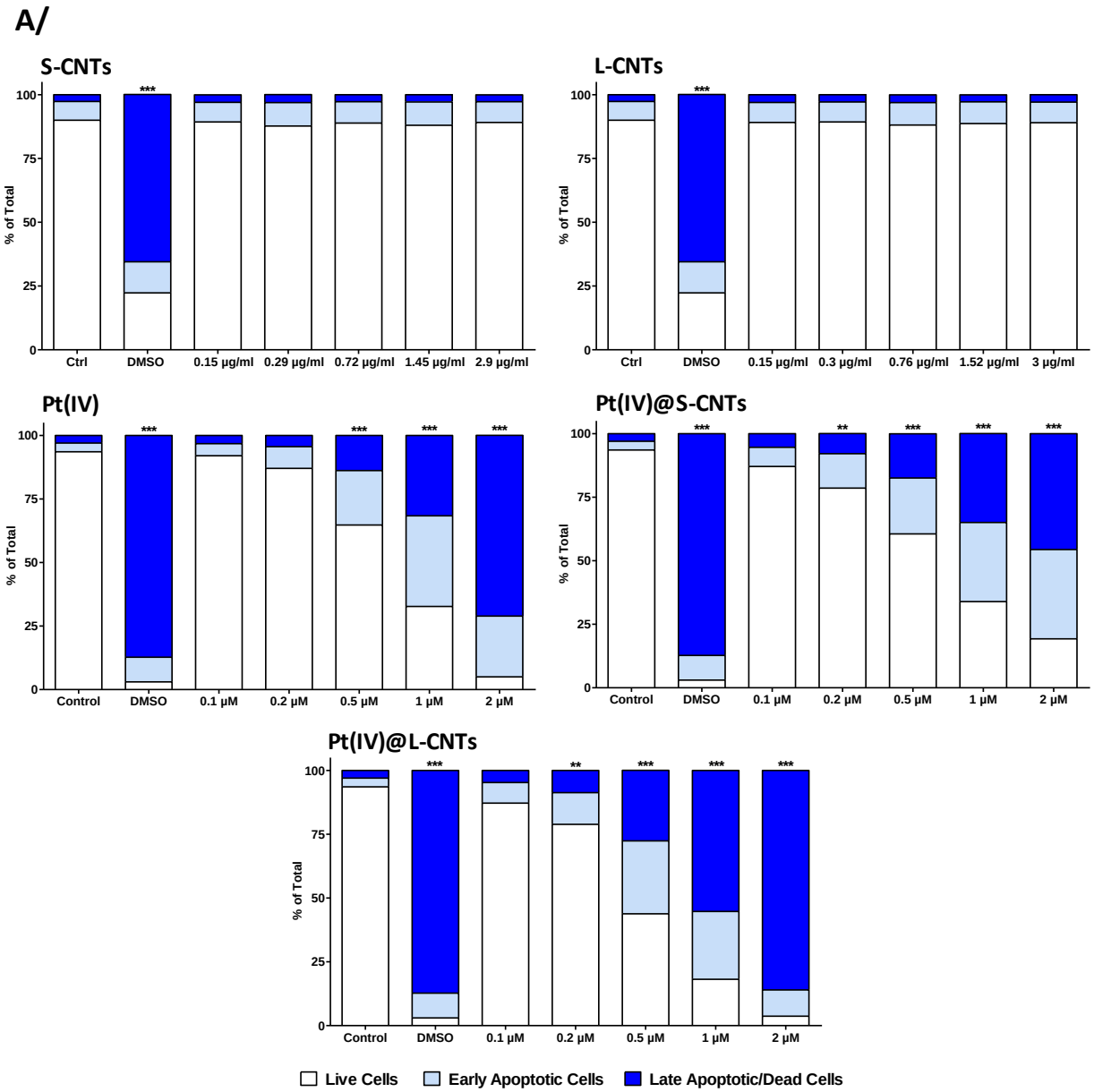
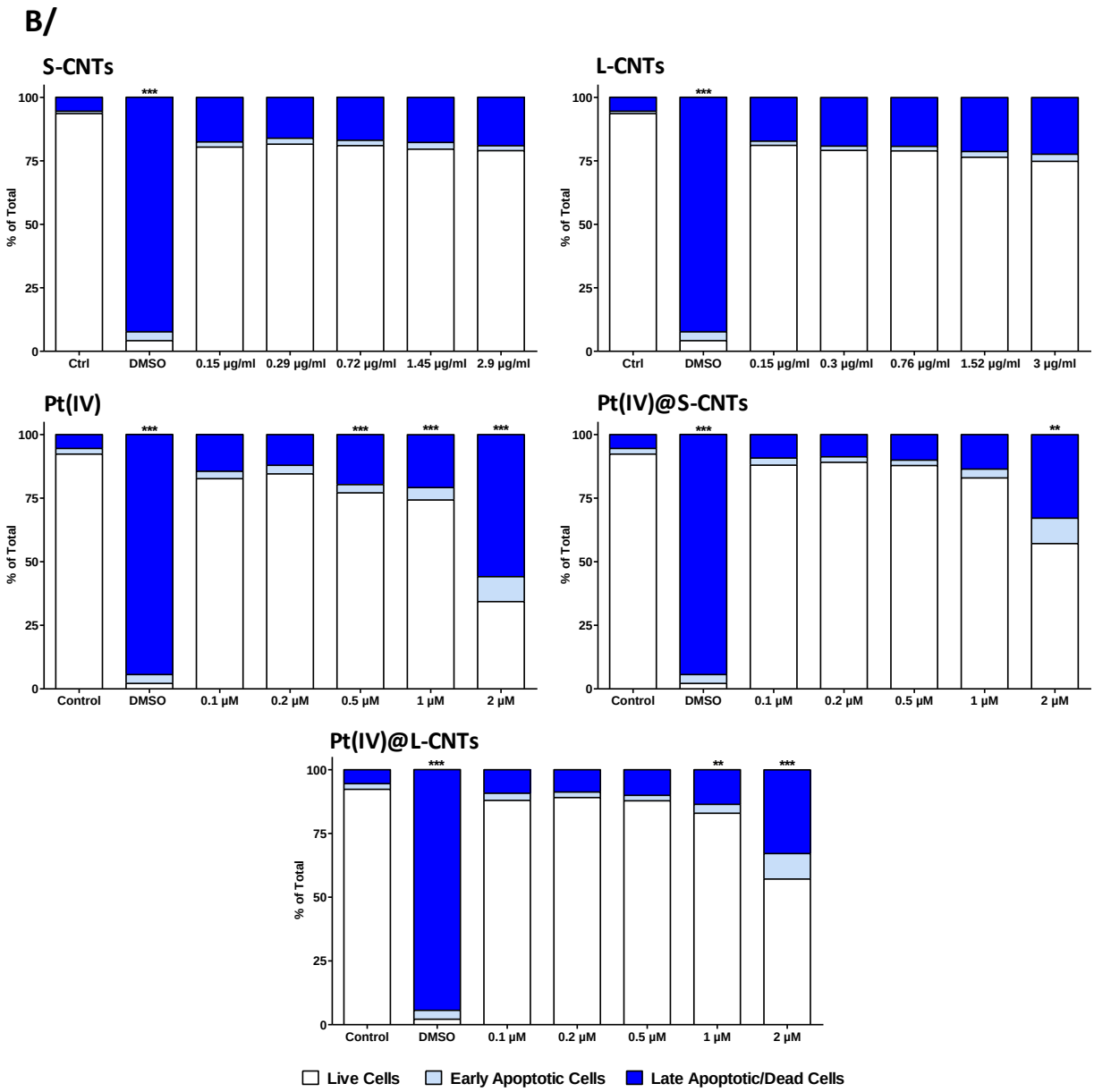


Fig. S8 (A) Flow cytometry analysis of cellular viability in HeLa cells exposed to different concentrations of S-CNTs, L-CNTs, Pt(IV)@S-CNTs and Pt(IV)@L-CNTs for 6 h and allowed to grow for further 72 h. Two-ways ANOVA followed by Bonferroni's post-test was performed to determine the statistical differences *versus* control cells (*p<0.05; **p<0.01; ***p<0.001).



(B) Flow cytometry analysis of cellular viability in RAW 264.7 cells exposed to different concentrations of S-CNTs, L-CNTs, Pt(IV)@S-CNTs and Pt(IV)@L-CNTs for 6 h and allowed to grow for further 72 h. Two-ways ANOVA followed by Bonferroni's post-test was performed to determine the statistical differences *versus* control cells (*p<0.05; **p<0.01; ***p<0.001).

In this case, Pt(IV)@S-CNTs cytotoxicity on HeLa cells (Fig. S8A) was comparable to the free drug although they were poorly effective after 24 hours even at higher concentrations. On the other hand, the larger CNTs induced significantly higher cellular mortality in comparison to both the free drug (from 0.5 to 1 µM) and the smaller ones (from 0.5 to 2 µM).

While in the first experiment RAW 264.7 cell line was more sensitive compared to HeLa to both the free drug and Pt(IV)@CNTs, in this case, the effect was negligible in comparison to HeLa response (Fig. S8B). Moreover, in all the concentration range, the free drug was more cytotoxic compared to the drug-filled CNTs and there was no difference between the effect of the two Pt(IV)@CNTs.

As well as the first cell viability experiments, no contribution from the empty CNTs on cell mortality for both cell lines was observed.

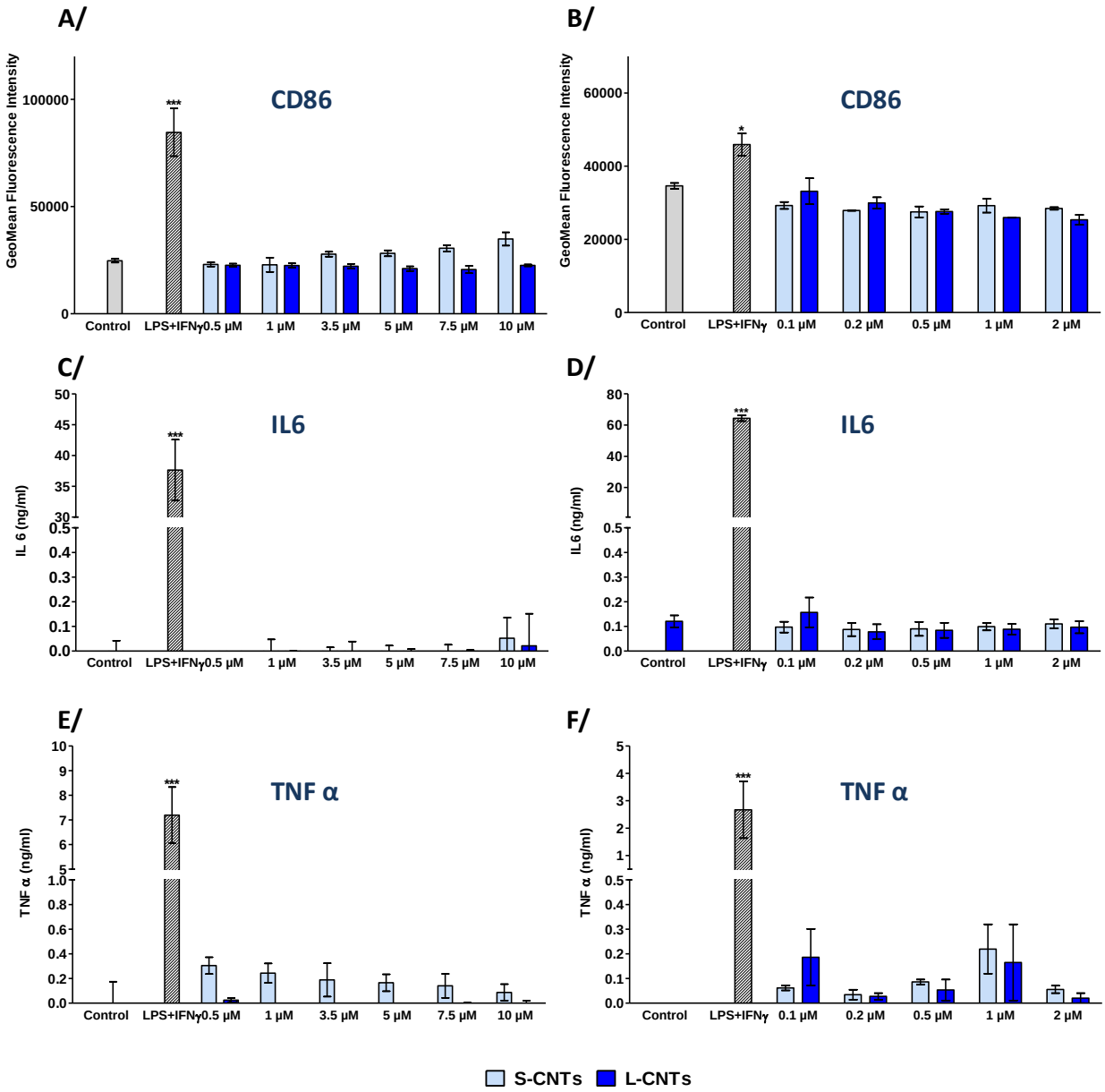


Fig. S9 Flow cytometry analysis of CD86 expression in RAW 264.7 exposed to different concentrations of S-CNTs and L-CNTs for 24 h (A) or 6 h and allowed to grow for further 72 h (B). IL6 and TNF α production by RAW 264.7 cells after exposure to S-CNTs and L-CNTs for 24 h (C and E) or after cells were allowed to grow for 72 h after a 6 h exposure to CNTs (D and F). Two-ways ANOVA followed by Bonferroni's post-

test was performed to determine the statistical differences versus control cells and to compare the two Pt(IV)@CNT samples to each other and to Pt(IV) (*p<0.05; **p<0.01; ***p<0.001).

RAW 264.7 macrophages were exposed to the empty S-CNTs and L-CNTs as controls, in order to avoid a contribution of the free materials on macrophage activation. As it is possible to see in Fig. S9, no increase in CD86 expression compared to the control was observed after cells were exposed to the materials for 24 hours (A) or 72 hours after the end of 6 hour incubation with CNTs (B). Moreover, no pro-inflammatory cytokines were detected in the cell supernatants after 24 hours (C and E) and after the cells were allowed to grow for 72 hours after the end of 6 hour exposure to empty CNTs (D and F).

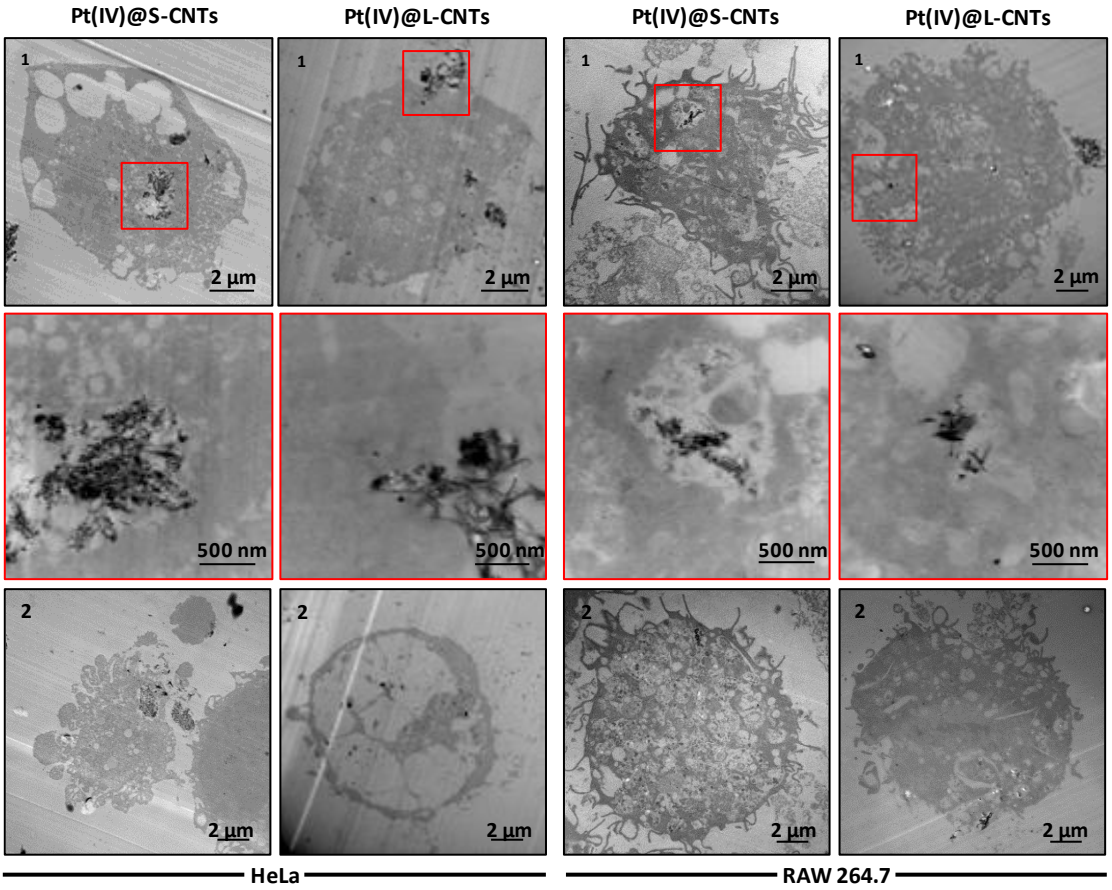


Fig. S10 TEM images of HeLa and RAW 264.7 incubated with Pt(IV)@S-CNTs and Pt(IV)@L-CNTs (10 µg/ml) for 24 h. The doted areas are enlarged in the respective bottom pictures.

Both CNT types were found inside the human (Fig. S10, left panel) and murine cells (Fig. S10, right panel) by TEM observations thus confirming their cellular uptake. Going more in details, a CNT packaging inside vesicles can be observed in all the cases. This suggests an endocytic or phagocytic internalization pathway in the case of HeLa and RAW 264.7, respectively. After an

incubation of 24 h with Pt(IV)@CNTs, a strong impairment of the general cellular structure due to the transported drug effect could be observed.

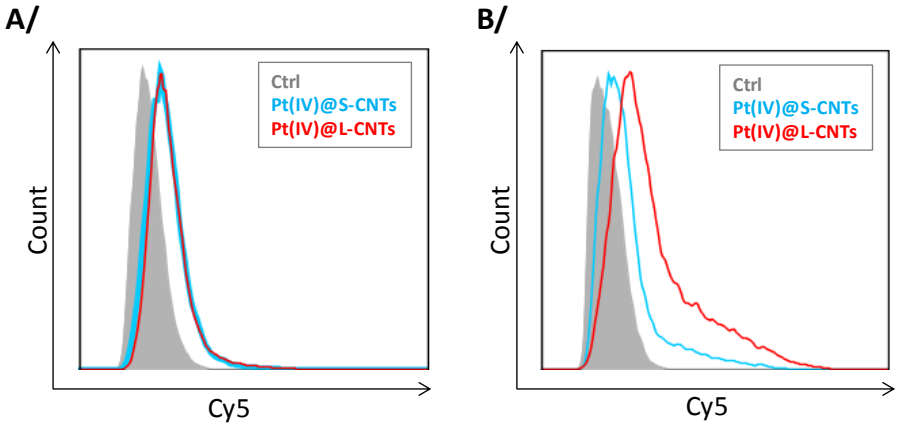


Fig. S11 Flow cytometry analysis of Pt(IV)@CNTs (10 µg/ml) HeLa (A) or RAW 264.7 (B) cellular uptake. The histogram is relative to one of three representative experiments.

The cellular uptake of Pt(IV)@CNTs was followed by flow cytometry thanks to the functionalization with the Cy5 fluorophore. For this purpose, cells were exposed for 6 hours to 10 µg/ml of CNTs. Cells were then detached, and immediately analyzed. The results shown in Fig. S11A (HeLa) and B (RAW 264.7) are relative to one representative experiment (n=3). A fluorescence shift of the cell population (corresponding to the Cy5 on the CNT surface) from the control could be observed in all the cases, thus confirming their intracellular uptake. We can assert that only a negligible amount of fluorescence could be derived from the CNTs interacting with the cellular surface as the samples were extensively washed before the analysis.

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S2 G. Pastorin, W. Wu, S. Wieckowski, J.-P. Briand, K. Kostarelos, M. Prato, A. Bianco, *Chem. Commun.*, 2006, 1182-1184.

S3 J. Li, A. Pant, C. F. Chin, W. H. Ang, C. Ménard-Moyon, T. R. Nayak, D. Gibson, S. Ramaprabhu, T. Panczyk, A. Bianco, G. Pastorin, *Nanomedicine*, 2014, **10**, 1465–1475.

S4 C. Ménard-Moyon, C. Fabbro, M. Prato, A. Bianco, *Chem. Eur. J.*, 2011, **17**, 3222-3227.