PAT over F98 and 9L cells using Pt and Au nanoparticles in liposomes

The aim of this proposal was to carry out new therapy strategies *in vitro*, against F98 glioma cells, with different metal compounds (cisplatin, gold nanoparticles, and conjugated nanoparticles) and liposomes as carriers.

Chemical conditions:

- LIPOSOMES were formed by soy licithin with 97 % in phosphatidylcholine (PC) dissolved in chloroform. This liquid was eliminated by rotavaporation obtaining a lipid film that has to be redissoled with a different aqueous condition each time.
- The *Ferrer Farma* CISPLATIN used was the same used in chemotherapy treatments in hospitals.
- Each GOLD NANOPARTICLE was formed by 1,000 gold atoms with a final diameter of 4 nm, and with a sodium citrate as a dissolvent.
- The CONJUGATED NANOPARTICLES were a new generation compounds with a core based on gold nanoparticles of 4 nm identical to the previous one. Some binding molecules let to connect the gold atoms with the platinum ones from the cisplatin. Its total diameter was about 6 nm.

Thanks to the fluorescence experiments carried ou in the ID18F with the conjugated nanoparticles, could observe several signals confirming the presence of these metals in the compounds as well as distinguish their Lγ1 at the work concentration (30 μM).

Metal	Lα1	Lα2	Lβ1	Lβ2	Lγ1
Pt	9,44	9,36	11,07	11,25	12,94
Au	9,71	9,63	11,44	11,58	13,38

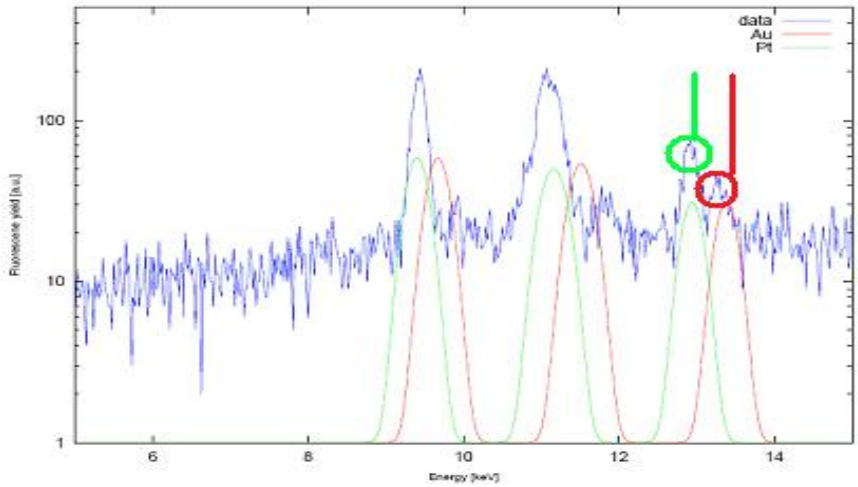


Figura 1. Fluorescence spectrum of conjugated nanoparticles irradiated at 28 keV in the ID18F. In blue, the real data obtained, and in red and green the theorical values.

Both types of nanoparticles were synthesized by the ICN (*Nanotechnology Catalan Institute)* in Cerdanyola del Vallès (Barcelona).

- The F98 rat glioblastoma CELL LINE was obtained from the ID17. F98 cells were cultured in monolayers with DMEM (Gibco, with GlutaMAX I) , fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S). Previously to the confluence cells condition, they were counted and added in F96 microwell plates where the cultures had to rest during 24 hours before the beginning of the experiments.

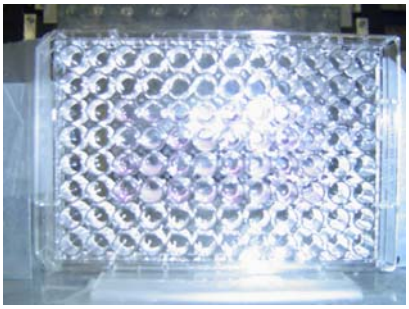


Figure 2. Photography of one F96 microwell plate used in the experiments.

The chemical conditions were always added in the central part of the plates in quadriplicate like it is showed in the next table (***Figure 3**):

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
		control	lipos	Pt	Pt-lip	Au	Au-lip	(Au,Pt)	(Au,Pt)-lip		
		control	lipos	Pt	Pt-lip	Au	Au-lip	(Au,Pt)	(Au,Pt)-lip		
		control	lipos	Pt	Pt-lip	Au	Au-lip	(Au,Pt)	(Au,Pt)-lip		
		control	lipos	Pt	Pt-lip	Au	Au-lip	(Au,Pt)	(Au,Pt)-lip		

* *Control* = cells only, *lipos* = liposomes , *Pt* = cisplatin, *Pt-lip* = liposomes associated to cisplatin, *Au* = gold nanopaticles, *Au-lip* = liposomes associated to gold nanoparticles, *(Au,Pt)* = conjugated nanoparticles, *(Au,Pt)-lip* = liposomes associated to conjugated nanoparticles.

Radiation strategy:

Divide the total dose irradiation in two fractions of 2 Gy each one could decrease the natural resistance of these cells in respect with only one irradiation at 4 Gy. This fact justifies the next schedule in order to irradiate at 80.025 keV each microwell plate.

a) 2 plates in which:
Chemical conditions (6 h) → Irradiation (2 Gy) → (1 h) → Irradiation (2 Gy)
b) 2 plates in which:
Irradiation (2 Gy) → (1 h) → Chemical conditions (6 h) → Irradiation (2 Gy)
c) 2 plates in which:
Chemical conditions (6 h) → Irradiation (4 Gy)
d) 1 plate in order to:
Have a dark control according to the *a)* procedure.

The main work features of the beamline ID17 are exposed in the next figure:

Features	Values
▪ Beam energy	**80,025 keV** (> K-edge of either Pt or Au)
▪ Photon source divergence (V×H)	**0,1 × 3,3 mrad^2**
▪ Photon flux	**10^{14} fotons/s**
▪ Filling mode during the experiment	**7/8 multibunch + 1**
▪ Average intensity in the ring	**200 mA**
▪ Time per plate	**5 min** (2 Gy), **10 min** (4 Gy).

Figure 4. Main radiation parameters used in the medical beamline.

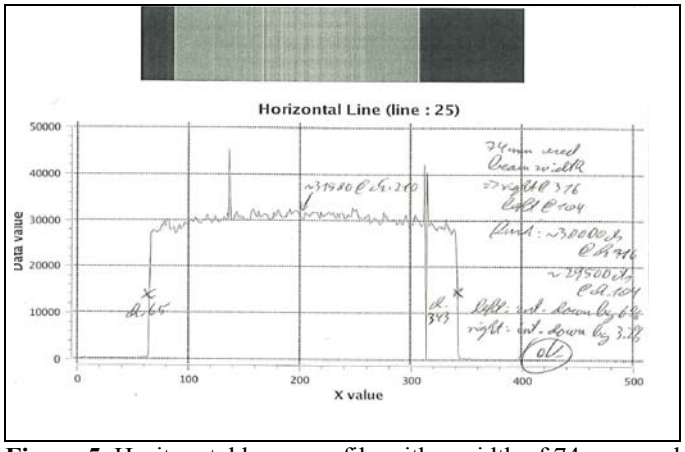


Figure 5. Horitzontal beam profile with a width of 74 mm, and with flunctuations around 3,7 % on the right part, and 6 % on the left one. The wells with controls were in this side.

Experimental procedure and MTT technique:

$2 \cdot 10^4$ cells per microwell were incubated in plates for a span of 24 hours before the experiment. After this period it was possible either to add the chemical conditions, 30 μM each of them, or irradiate the samples according to the procedure of each plate.
All the conditions were in contact with the cells in the microwells during 6 hours.
All the irradiations were carried out without medium and with each plate in a vertical position. After each irradiation cells remained for one hour with medium in the incubator, after this lapse it was possible to continue with the experiment.
Using the MTT (Thiazolyl Blue Tetrazolium Bromide) test is possible to know the effectiveness of the method afterwards each treatment. MTT is a water soluble tetrazolium salt yielding a yellowish solutions when prepared in medium. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes of living cells. This water insoluble formazan can be solubilized using DMSO (Dimethyl Sulfoxide), and this dissolved material may be measured spectrophotometrically yielding absorbance as a function of concentration at a wavelength of 570 nm.

The concentration of the 150 µl of MTT added to each well was 0.5 mg/ml and incubated for 2 hours.

Results and conclusions

- The experiments carried out according to the procedures *a)* and *b)* were not useful as a results of this experiments due to the cells were incubated in the plates for 4 days before treatment instead of 24 hours described on the experimental procedure previously described .
Results of the c and d experimental procedures
- The first plate, which was irradiated only one time at 4 Gy afterwards to take away all the chemical conditions showed before, gave us the next graph:

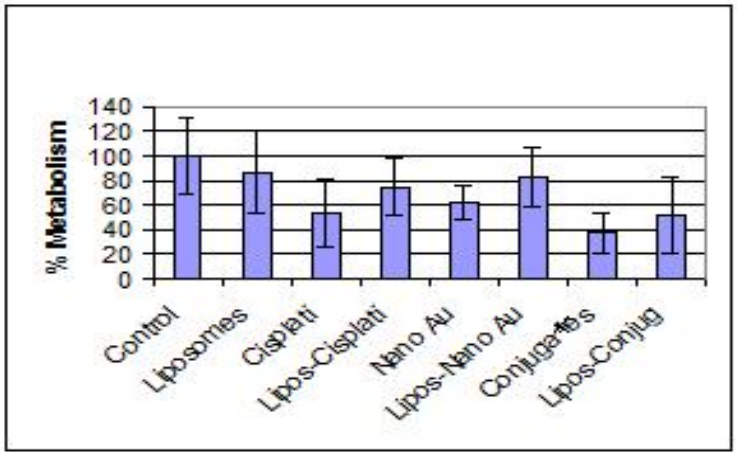
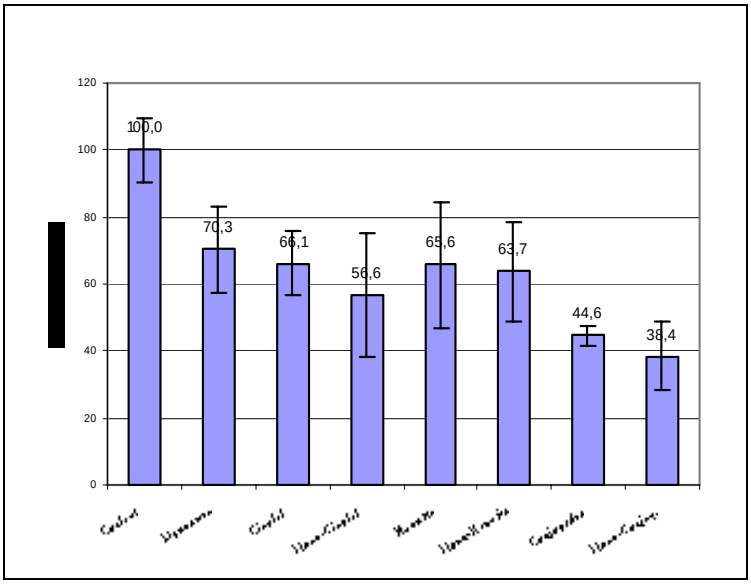


Figure 6. % Metabolism of F98(MTT) vs. each chemical condition for one irradiation at 80.025 keV and 4 Gy.

Free metallic compounds offer similar effectiveness than the same ones associated to liposomes. These results could be related with presence of traces of non reacted MTT in the wells. More accurate repetition of the methodology gave the new results showed in the next graph (**Figure 7**):



The main conclusions of this second experiment at 4 Gy were the followings:

1- Use of the liposomes as a carrier was more effective than using dissolutions of free metals.
2- Conjugated nanoparticles seem induce more cellular damage rather than the rest of delivered drugs assayed.
3- The most effectiveness condition was the association liposomes-conjugated nanoparticles due to their higher concentration of local damages.

- The dark control gave the following results:

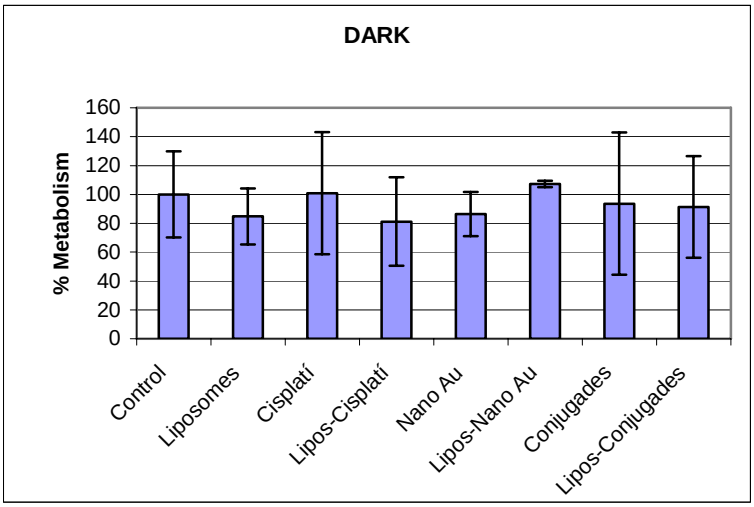


Figure 8. % Metabolism of F98 (MTT) vs. each chemical condition without radiation.

In this experiment, using only chemical therapy, it was possible to observe that all the chemical agents had a very similar effectiveness rate. This fact was understood as a show sign of the high resistance that offer these cells.