Synthesis, characterisation and uptake of lipidic nanocapsules by A549 cells

Introduction and purpose of the visit

In recent years, different colloidal systems such as nanoparticles (NPs) and nanocapsules (NCs) have been reported as potential carriers for drug delivery. Structured NCs are generally described as colloidal systems with a core-shell structure, where the core acts as a liquid reservoir for several molecules or drugs, and the shell as a protective membrane. Their useful properties include biocompatibility and biodegradability, low toxicity, controlled release of drugs and the ability to target specific tissues. In particular, lipid nanocapsules (LNC), consisting of an oil-filled core with a surrounding polymer shell, have special use for encapsulating and delivering hydrophobic drugs.

In order to optimise the efficacy of NP delivery to cells, it is necessary to elucidate the underlying interactions between cells and nanomaterials and the mechanisms by which NPs are internalised by cells, as this will likely determine their ultimate sub-cellular fate and localisation.

It is now understood that once in contact with biological fluids, the NP surface gets covered by a highly specific layer of proteins, forming the so-called NP protein corona. This layer of proteins is stable for times longer than the typical time scale of NP import, and thus can have an impact on the particle uptake and trafficking inside the cells.

The aim of this project was to investigate lipidic NPs uptake mechanisms in a human lung adenocarcinoma epithelial cell line (A549) and the effects of the corona on the NP uptake.

Description of the work carried out during the visit

Two different LNC systems, in which the core was constituted by olive oil and the shell by lecithin and other different biocompatible molecules such as Pluronic® F68 or chitosan, were synthesised—and physico-chemically characterised. Finally, some uptake studies were performed by culturing a lung-carcinoma cell line (A549) with Nile-Red-loaded LNCs. The hydrophobic fluorescent stain Nile Red is a solvatochromic

dye with absorption bands that vary in shape, position, and intensity, depending on the nature of the solvent. Is adecuate to be encapsulated inside LNCs.

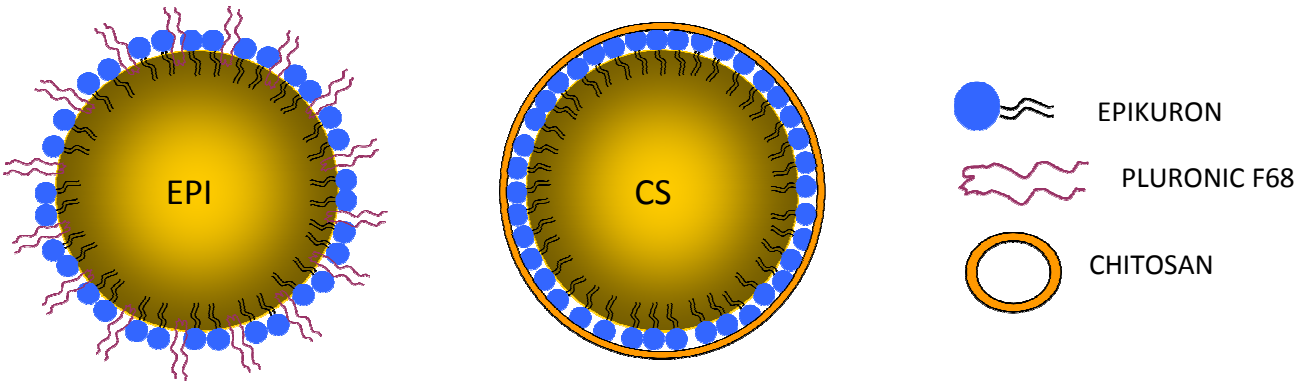
1. Synthesis and characterisation of the LNC

1.1. NPs synthesis

The nanosystems were prepared by a solvent-displacement technique following the procedure of Prego et al.. This is a well-known technique widely reported for the preparation of NCs, where hydrophilic surfactants are usually dissolved in the aqueous phase before emulsion formation. Briefly, an organic phase was prepared containing olive oil and Epikuron 145V dissolved in ethanol and acetone. This organic phase was immediately poured over an aqueous phase containing Pluronic® F68 or chitosan oligomers. The mixture turns immediately milky because of the formation of the nanoemulsion. Then, the organic solvents were evaporated under vacuum to a final volume of 18 mL. All nanosystems presented olive oil in their hydrophobic core and, depending on the composition of the organic and aqueous phases, the final sample showed different interface properties.

The shell of the system negatively charged (referred to as EPI) was constituted by a commercial mixture of phospholipid molecules (Epikuron) and a poloxamer (Pluronic® F68), both acting as colloidal stabilizers. A second system positively charged (referred to as CS) was formulated by substituting the poloxamer by chitosan oligomers.

Nile Red loaded nanocapsules were synthesized to determine their uptake efficiency and mechanisms.



The presence of Nile Red in the oily core of the NPs was previously checked by measurint the fluorescent means. It should be noted that Nile Red presents a very different emission fluorescent spectrum as a function of the medium in which it is dissolved. Figure 1 shows the emission fluorescent spectrum of the different NP systems. For all of them, the spectrum was similar to those found for Nile Red dissolved in olive oil. Comparing these results with the Nile Red emission spectrum dissolved in an aqueous solution, we found that the presence of this fluorescent molecule in the hydrophobic oil core of the LCNs was, therefore, clearly confirmed.

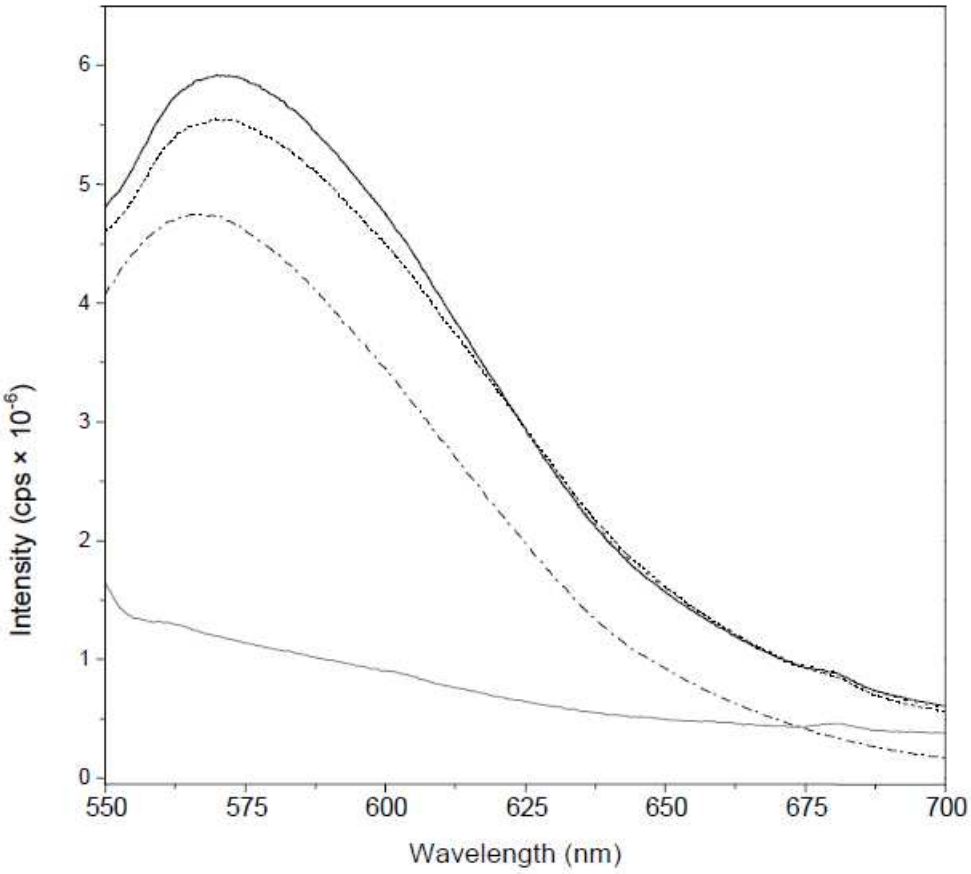


Figure 1. Emission spectrum of Nile Red in olive oil and water, and inside different types of NPs. (black line) EPI; (dashed line) CS; (dash-dot line) olive oil; (gray line) water.

1.2. NPs size and Z-potential

NPs size measurements by dynamic light scattering (DLS) were carried out in water, serum-free minimum essential medium (SF ,medium) and the complete cell culture medium (cMEM), consisting of MEM, supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1% MEM non-essential amino acids. Measurements were performed at 25ºC and 37ºC at different time points (0h, 1h and 24h), using a Malvern Zetasizer Nano ZS90 (Worcestershire, UK). Z-potential (which is a reflection of the charge measured at the material surface, including the most strongly associated

counterions) was measured, on the same instrument, in a solution of NaCl 1mM, cMEM and SF medium.

1.2.1. NPs size

It is reported in literature that upon contact with cMEM, the NP average size increase as the corona forms. However, the data presented in Table 1 do not show significant differences in particle size for the two different media.

EPI particles are very stable because the incorporation of poloxamer into the shell confers a more hydrophilic character ascribed to the PEO fragments of the pluronic F68. These types of polymers, in addition to enhancing the intrinsic colloidal stability of the system, may help to avoid their recognition by the mononuclear phagocyte system (MPS) by repelling plasma proteins. The stability observed at moderate ionic strengths (cMEM and SF medium) is governed by repulsive hydration forces and this process is typical of surfaces with hydrophilic character.

Results showed in Table 2 (CS nanoparticles) are different for the two media and an increase of the particles size at different time points of incubation can be seen. In NaCl, data do not show significant differences in particle size, indicating that CS particles are stable in that solution. On the contrary, in SF medium, CS particles are more unstable. They are more stable in cMEM, perhaps because of the adsorption of proteins (mainly albumin) at hydrophobic surfaces. It bears emphasizing that proteins adsorption is generally favored by hydrophobic interactions between the surface and these macromolecules. In fact, the hydrophobic interaction is one of the key controls in proteins adsorption. It is usually higher for large, soft proteins, such as albumin. This is the reason because the hydrophobic system adsorbs more albumin molecules, giving to this protein stability due to its high charge density at neutral pH and its highly hydrophilic character responsible for hydration forces.

Table 1. Size characterisation of **EPI** NCs in NaCl 1mM, complete MEM (cMEM) and sefum free medium (SF) at 25ºC and 37ºC at three different time points (0, 1 and 24hours).

Temperature	**25ºC**			**37ºC**		
Time	0h	1h	24h	0h	1h	24h
Diameter (nm)SF	158±29	159±33	167±30	179±30	162±27	193±17
PDI	0,102	0,103	0,092	0,073	0,071	0,219
Diameter (nm) cMEM	155±32	157±31	158±28	180±37	157±28	165±35
PDI	0,155	0,143	0,140	0,141	0,124	0,157
Diameter (nm) NaCl	166±28	173±35	174±31	192±39	172±31	176±33
PDI	0,082	0,109	0,102	0,114	0,106	0,102

Table 2. Size characterisation of **CS** NCs in NaCl 1mM, complete MEM (cMEM) and sefum free medium (SF) at 25ºC and 37ºC at three different time points (0, 1 and 24hours).

Temperature	**25ºC**			**37ºC**		
Time	0h	1h	24h	0h	1h	24h
Diameter (nm)SF	193±45	348±189	604±30	213±41	597±55	804±28
PDI	0,155	0,301	0,092	0,127	0,087	0,264
Diameter (nm) cMEM	187±56	190±55	235±113	206±51	251±54	698±33
PDI	0,226	0,231	0,383	0,223	0,275	0,623
Diameter (nm) NaCl	211±64	202±57	190±45	228±50	228±46	210±27
PDI	0,207	0,193	0,148	0,166	0,210	0,224

1.2.2 NPs Zeta Potential

EPI NCs (Table 3) showed typical behavior of colloids with weak acid groups, giving lower Z-potential values at acidic pH values (NaCl) than those found at pH 7.4 (cMEM and SF medium). The surface-charged groups of the EPI NCs come exclusively from the Epikuron molecules, in which phosphatidyl-choline is the major component. Thus, the phosphatidic acid—with a pKa between 3 and 4—is the main charged group in this system. The presence of Pluronic® F68 molecules does not alter the electrical state of the surface, since this poloxamer is a non-ionic surfactant.

Table 3. Z-Potential of **EPI** NCs at 25ºC and 37ºC in NaCl, serum free medium (SF) and complete MEM (cMEM) at different time points.

Temperature	**25ºC**			**37ºC**		
Time	0h	1h	24h	0h	1h	24h
SF	-15,2	-18,1	-17,9	-15,7	-16,4	-16,9
cMEM	-10	-10,4	-12,8	-12	-15,2	-12,8
NaCl	-53,4	-47,9	-45,2			

Table 4. Z-Potential of **CS** NCs at 25ºC and 37ºC in NaCl, serum free medium (SF) and complete MEM (cMEM) at different time points.

Temperature	**25ºC**			**37ºC**		
Time	0h	1h	24h	0h	1h	24h
SF	-8,3	-10,4	-11,6	-5,81	-10,8	-12,9
cMEM	-9	-10,5	-11,3	-10,3	-12,9	-14,6
NaCl	53,1	50	47,1			

The z-potential data of CS particles are showed in table 4. The positive charge of CS NCs in NaCl is provided by the glucosamine groups of chitosan, which presents a weak basic character. At pH 7.4 (cMEM and SF medium), chitosan chains are uncharged, so that the negative z-potential is a reflection of the lecithin phosphatidic groups.

In both kinds of particles the z-potential did not change to lower absolute values in complete medium, as a consequence of the interaction with the serum proteins, as it is reported in literature.

2. Uptake studies

All studies were performed using A549 cells (lung carcinoma), seeded and grown for 24 hours in cMEM, with the medium then being replaced by LNC-containing dispersions. Individual intracellular fluorescence intensity was measured via flow cytometry and averages were calculated over large numbers (typically 10,000) of cells to produce a time-resolved average intracellular fluorescence curve. 1.5 x 10^5 cells were seeded in individual 35mm tissue culture dishes (Greiner Bio-one), and incubated for 24h prior addittion of particles. After 24h, the medium was replaced by the medium containing NPs. Cells were incubated with NPs for a specific time, depending on the experiment, and then the medium was discarded. In the case of export experiments, after exposure to particles during 3 hours, the dispersion was discarded and after 3 washes with DPBS, fresh medium without particles was added to the cells which were incubated for the appropriate times.

After the required incubation time, the medium was removed and the samples were washed thrice with DPBS and harvested with trypsin/EDTA. Cell pellets were then fixed with 4% formalin solution neutral buffered (Sigma) for 20 minutes and re-suspended with constant volumes of DPBS before cell-associated fluorescence acquisition. The results are reported as the mean of the distribution of cells fluorescence intensity, averaged between 3 independent replicas. Error bars indicate the standard deviation among the replicas. The full time curves were performed at least 3 times.

2.1. Kinetics of uptake

Figure 2 shows the flow cytometry results obtained from cells treated with EPI and CS NPs at a concentration of 1x10^{11} nanocapsules/mL in SF medium and cMEM.

Although z-potential and size of LNC do not change to lower absolute values in complete medium, as a consequence of the interaction with the serum proteins, EPI NCs show differences of the kinetics uptake in both media. Nevertheless CS is a hydrophobic system which can absorb more proteins, the uptake in SF medium and cMEM is the same.

Comparing the uptake of both kinds of NPs in SF medium, the fluorescence intensity of cells treated with EPI particles is higher than those treated with CS particles. In this case, size could be a critical factor, because CS particles are much bigger and less stable than EPI. However, in cMEM the uptake of CS NPs is higher than the one of EPI NPs.

The differences between CS and EPI particles are the surface properties (different charge) and the size. CS particles are bigger than EPI and their colloidal stability is less than the one of the EPI.

If fundamental questions, such as the role of the protein corona in the NP uptake, have to be addressed effectively, then such a reliable platform of data will be required. With these results we are not fully able to explain the differences in uptake of both the nanosystems. To understand if the size of CS NPs is playing a key role in their uptake process, we should synthesize lipid NPs with same surface properties and different sizes. Unfortunately, with the protocol of synthesis established for these nanosystems, this is not possible.

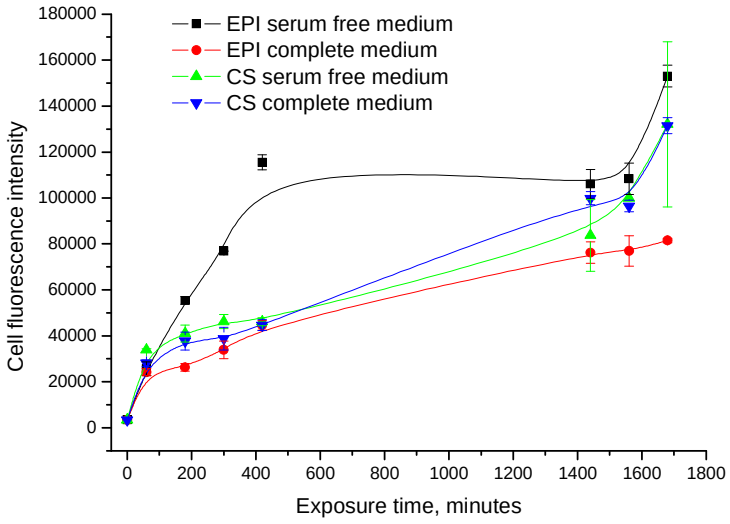


Figure 2. Uptake of lipid NCs by A549 cells after different times of exposure in SF medium and cMEM. Error bars indicate the standard deviation among 3 replicas.

2.2. Kinetics of export

In terms of the export kinetics for cells treated with EPI and CS NCs (Figure 3), a rapid decay of fluorescence over several minutes, followed by a slightly decay process was observed. The curve-trend is the one typical of the export kinetics for cells treated with pure dye.

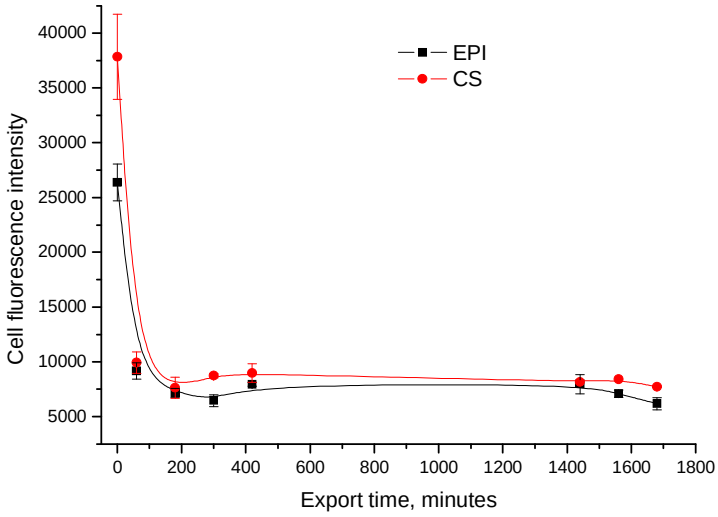


Figure 3. Kinetics of export after 3 hours of uptake followed by removal of the NP-containing medium (cMEM) and replacement with fresh medium (no NPs). Error bars indicate the standard deviation among 3 replicas.

2.3. Uptake energy-dependent study

The impact of energy depletion (pretreatment of the cells with sodium azide or performing the experiment at 4°C) on cellular uptake of the LNCs was used to determine whether the NPs uptake is active (requiring cellular chemical-mechanical energy) or passive (a purely physiochemical diffusive process). For both samples (Figure 4) none impact of energy depletion on cellular uptake was noticed, suggesting that the LNCs uptake is a passive process.

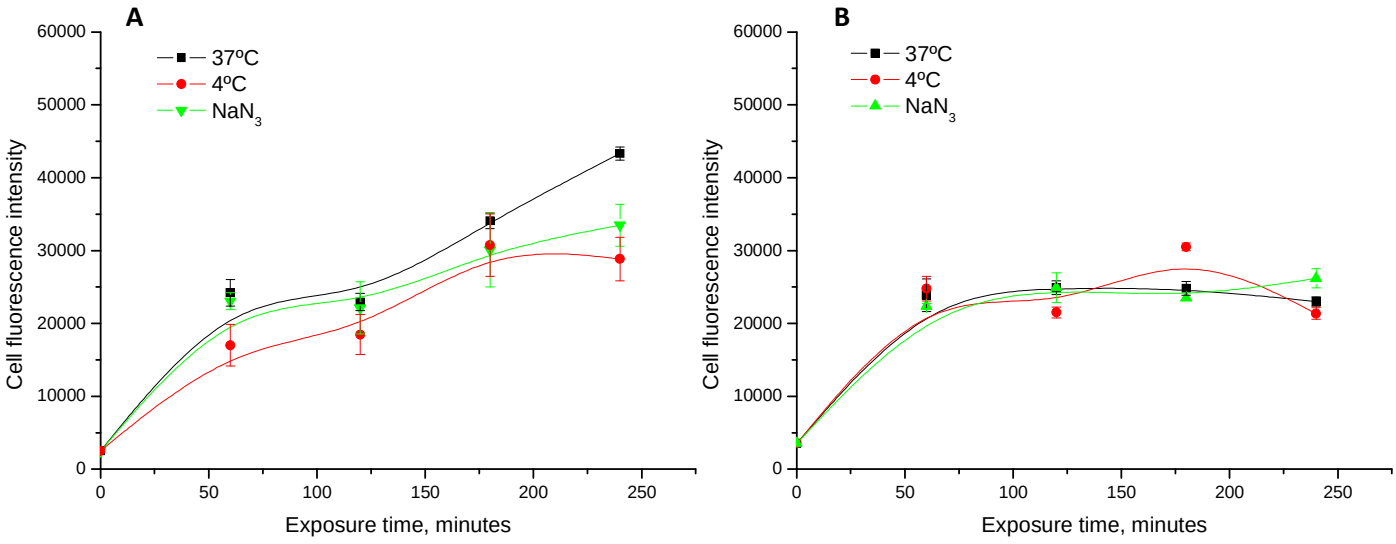


Figure. 4. Energy dependence of uptake of LNCs at a concentration of $1x10^{11}$nanocapsules/mL under normal cell culture conditions (37ºC, cMEM), and in medium containing 5mg/ml NaN3, or at 4ºC. (A) Cells were treated with CS NPs. (B) Cells were treated with EPI NPs.

Confocal microscopy

For confocal imaging, cells were seeded on 15 mm diameter glass coverslips at a density of 1.5 x 10^5 cells per plate and exposed for 7h to $1x10^{11}$ EPI nanocapsules/mL dispersed in cMEM .

Samples were washed with PBS, and then fixed with 4% formalin solution neutral buffered for 20 minutes and incubated for 3 min with DAPI before mounting with MOWIOL on glass-microscopy slides for imaging. The cells were observed using a Carl Zeiss LSM 510 Meta laser scanning confocal microscope (Zeiss, München, Germany) with lasers at 364 nm (DAPI), and 633 nm (Nile Red dye). A full z-stack image was acquired.

An illustrative confocal image is given in Figure 5 (3-dimensional reconstruction of the full z-stack). The fluorescence is spread across the intracellular space and there is a significant association of the dye with the vesicular bodies of the cell.

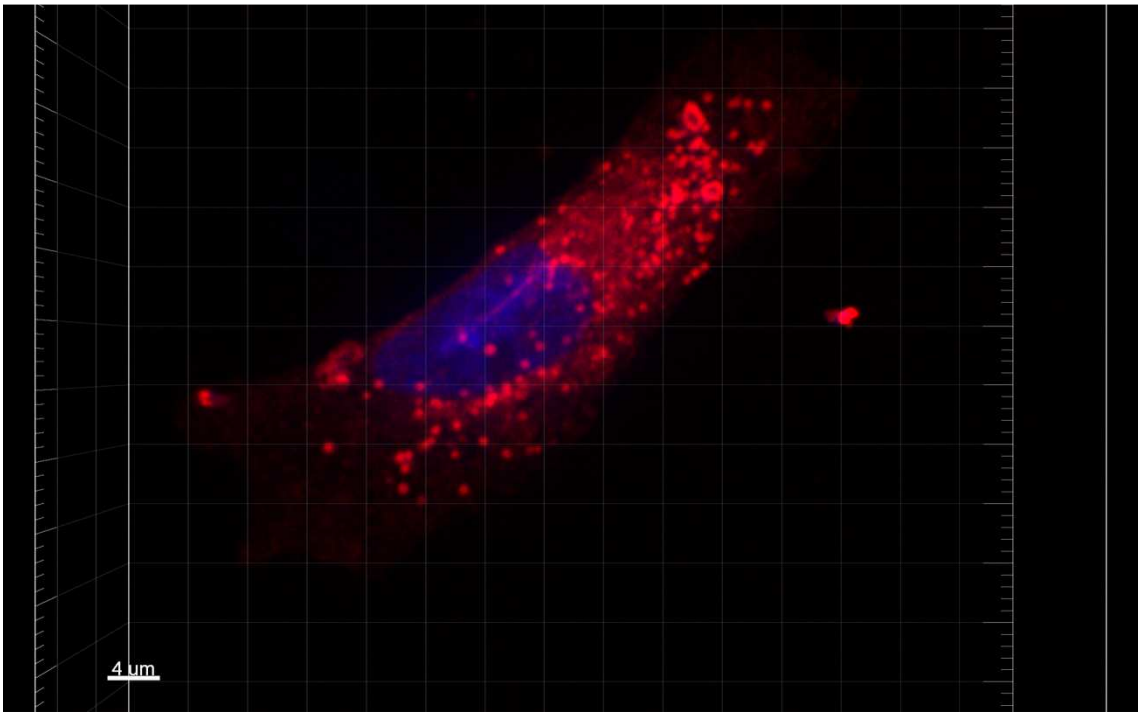


Figure 5. 3D reconstruction confocal image of A549 cell treated with $1x10^{11}$ EPI nanocapsules/mL Nile Red-loaded EPI nanoparticles (red) for 7h. Blue: DAPI stained nuclei.

3. Conclusions

All the obtained results point to the conclusion that the uptake mechanisms of lipid nanocapsules is the fusion with the cell membrane and then the release of the dye inside the cell. The free hydrophobic dye, once inside the cell, stains the vesicular bodies. This hypothesis could also explain the rapid decay of the fluorescence in terms of export kinetics and the non-energy dependence of the uptake rate. Nevertheless, more experiments, such as time-lapse movies recorded by live cell confocal imaging, are necessary in order to confirm these results.

In addittion, to better understand the differences in the uptake of CS particles and EPI particles, more and extensive protein corona and size studies are required.

In terms of clinical applicability, lipid NPs are very promising since they are biocompatible and biodegradable, but CS particles could be not very useful due to their low colloidal stability in biological fluids.

With the pourpose of answering all these questions about the bionano interaction of lipidic NPs, a collaboration with the Centre of Bionano Interaction has been established. New projects that include collaborations between the two participating laboratories are already planned.