Viral mimetic engineered particles (VMP) for cytoplasmic cargo delivery

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Introduction

The ideal delivery vehicle needs: i) to have an appropriate packaging size for its cargo, ii) to escape the immune system, iii) to have the highest specificity for the target cell, and iv) to efficiently deliver cargo.

During years scientists have been developing different strategies based on **carrier systems**. Among the non viral vectors, exosomes and liposomes, among others, enter into cells by membrane fusion (no-specific system) and although they are not recognised by the immune system, their efficiency of transfecting host cells is relatively low. **Viral vectors**, on the contrary, have the ability to perform specific receptor mediated endocytosis and also, once they are inside cells, are able to scape the lysosomes, delivering their genetic load into the cytoplasm. However, they can cause an immune response (Hartman, *et al*., 2007), provoke an insertional mutagenesis and can only deliver nucleic acids (Seow, *et al*., 2009) but not proteins or drugs. Recent advances in gene editing technologies urge the development of **new targeted delivery systems** that **can carry *ad hoc* combinations of protein and DNA.**

In this study we have investigated the biosynthetic interaction of a new **viral mimetic engineered particle** for cytoplasmic cargo delivery. My aims were to identify the cell-entry mechanisms and the intracellular destiny of the particles, focusing on the lysosomal escape step.

The particles were designed reproducing the morphology of typical eukaryotic viruses, often round shapes displaying spicules on their surface that serve as ligands for receptor mediated endocytosis. As a proof of concept, these structures have a 500 nm solid silica cores, future multi-purpose delivery capsules. To imitate the morphology of the virus, the silica spheres were covered with carbon nanotubes to mimic the viral spicules (Fig. 1-A). Since the carbon nanotube surface is very high (1 gr=1200m2), this nanotube shell offers countless possibilities, including customization with many different ligands to interact with specific cell surface receptors. As a control we used uncoated silica spheres (Fig. 1-B).

Material and Methods

- Virus mimetic particles (VMP) were synthesized at the laboratory of Dr. Correa, CINBIO University of Vigo (Fig.1)
- Functionalization of particles: by mild sonication in standard tissue culture medium containing serum
- Cell survival and cycle analysis: Trypan Blue assay to determine necrotic cells and flow cytometry (Hoescht) to determine apoptotic cells and the cell cycle.
- Uptake of particles:
  - Dyes: Phalloidin 647 (1:1000) to stain actin, Hoescht (1:4000) for the nuclei, Acridine Orange (1:2000), Lysotracker® (1:10) for endosomes/lysosomes staining respectively
  - Confocal microscopy: Nikon A1R. High resolution single plane intracellular confocal microscopy images were taken with a Nikon 100x lens 1.49 N.A. All the images are pseudo-colored.
- Quantification of particles was carried out with the *Image J* free software.
- Transmission electron microscopy: 1% glutaraldehyde in 0.12M phosphate buffer followed by Aradite embedding
- Statistical analysis: Student´s *t* test analysis. Significance was given by *=p<0,005; **=p<0,001; ***=p<0'0005

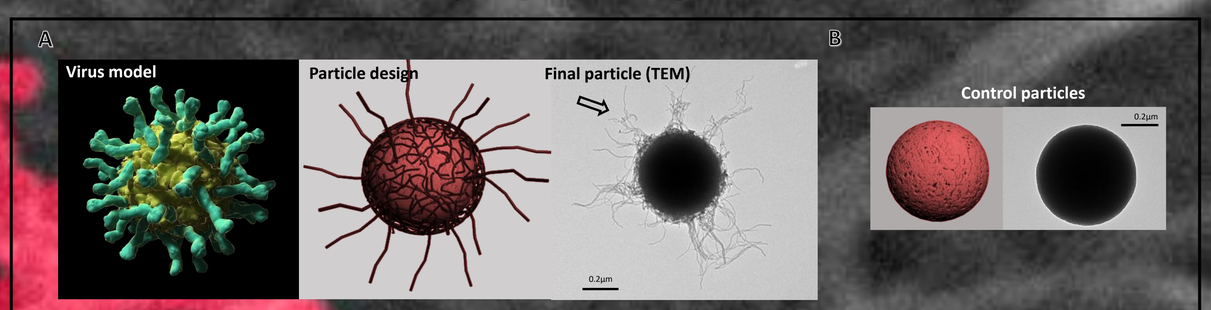

Figure 1. **A) (*Left*) Viral model (poliovirus) , (*middle*) diagram of the engineered VMP, (*right*) Transmission Electron Microscopy image of the synthetized VMP (the black arrow shows the carbon nanotubes); B) Uncoated control particles (no carbon nanotubes).**

VMP do not trigger cytotoxic effects on HeLa cells

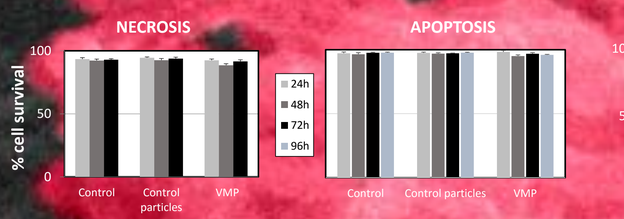

Figure 2. **(left) HeLa cells exposed to VMP particles do not display cell permeabilization typical of necrosis compared to controls. (right) Flow cytometry determination of apoptosis in control cells and cultures exposed to the VMP. These results correspond to 3 replicate experiments.**

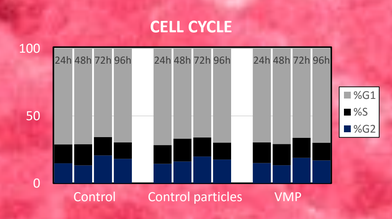

Figure 3. **Flow cytometric analysis of the cell cycle in control cultures and Hela cells exposed to VMP at 24/48/72 and 96 h.**

Cells have mainly two different ways to die; necrosis (cell membrane rupture) and apoptosis (programmed death). The administration of CNT (50µg/mL) themselves have a cytotoxic (Garcia Hevia, *et al.,* 2015), anti-proliferative (Garcia Hevia, *et al.,* 2016) and anti-migratory (Garcia Hevia, *et al.,* 2015) effect. So, we analysed the cytotoxicity of these VMP surrounded by CNTs. Cell counting experiments (Trypan Blue assay and flow cytometry) reveal that VMP have no cytotoxic effects. As we can see in the first two diagrams, there is no more necrotic cells neither apoptotic cells (Fig. 2) than in the control. Moreover, the different phases of the cell cycle are not altered (Fig. 3).

Carbon nanotubes enhance VMP entry

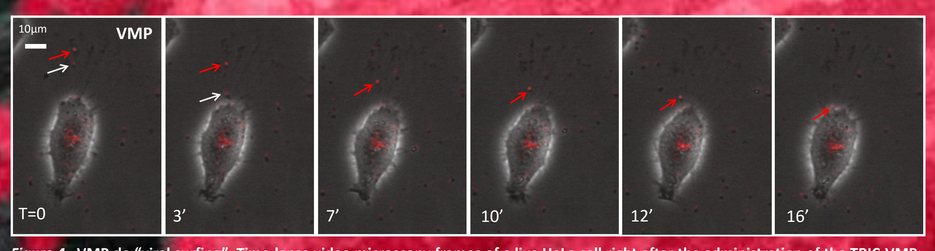

Figure 4. **VMP do "viral surfing". Time-lapse video microscopy frames of a live HeLa cell right after the administration of the TRIC-VMP. Red arrows mark how the particles is surfing along the filopodia of the cell.**

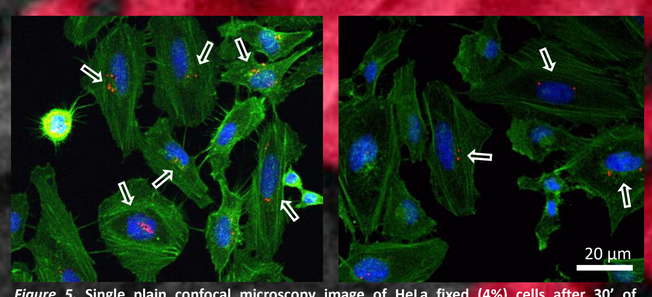

Figure 5. **Single plain confocal microscopy image of HeLa fixed (4%) cells after 30' of exposure of (left) VMP and (right) silica particles. White arrows point out particles inside cells.**

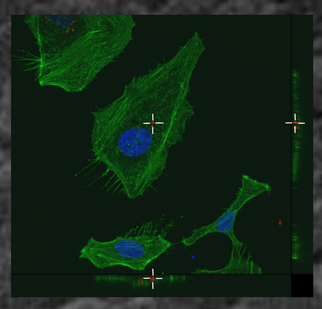

Figure 6. **Single plain confocal microscopy image of a VMP out of a Z-series. Lateral cellular Z projections demonstrating the localization of a single particle (in the white cross). The VMP can be unequivocally localized inside the cell after an overnight of exposure (actin in green channel; DNA blue channel).**

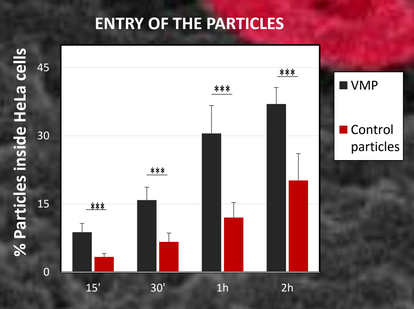

Figure 7. **Percentage of the different particles after 15 min, 30 min, 1 h and 2 h of exposure. These percentages have been calculated taking as a 100% the total overnight intracellular particle load for each culture respectively.**

The VMPs surf along the filopodia (Fig. 4) of the cells as exosomes or viruses do (Heusermann, *et al.,* 2015, Lehmann, *et al.,* 2005). VMP are able to enter faster and in a higher concentration (Fig. 7) than the control particles due to this mechanism of entry. The functionalization of the CNT help the particles contact the cell and enter later via receptor mediated endocytosis (Maruyama, *et al.,* 2015). As it is shown in the figure 5, after the same time of exposure, more VMP are found inside cells. The control particles, however do not have this mechanism and it is thought that they enter via phagocytosis, helped by the lamellipodium, which are actin projections involved in exocytosis and clathrin-mediated endocytic cycle.

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Carbon nanotubes trigger lysosomal escape to the cytosol

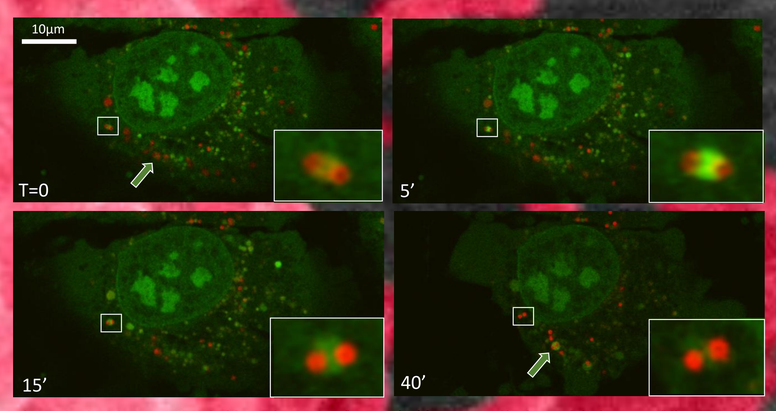


Figure 8. **Time-lapse confocal video microsopy of VMPs inside a HeLa. Single Z- plain images of live HeLa cells stained with Acridin Orange (1:2000) 48h after VMP exposure. Two VMPs escape from a lysosome.**

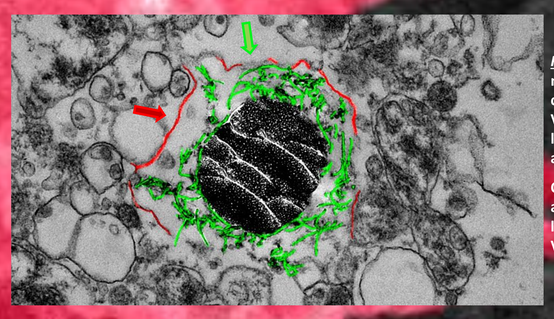


Figure 11. **Tranmission Electron microscopy section of a HeLa cell cytoplasm dysplaying a VMP (black) escaping from the lysosomal membranes (red arrow).**

Carbon nanotubes (green arrow) tear appart the lysosomal vesicle favouring VMP escape to the cytosol.

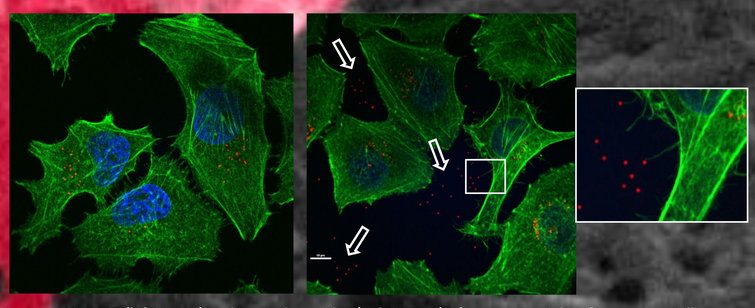


Figure 12. **VMP (left image) and control particles (right image) after an overnight of exposure. Cells were stained with Hoestch (1:4000) and Phaloidine 647 (1:1000) after been fixed with PF 4%. The white arrows point out control particles outside cells.**

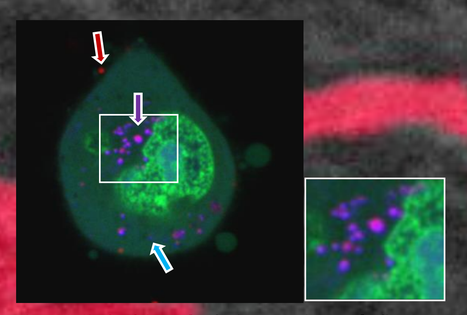


Figure 9. **Single plain confocal microscopy image of a live HeLa cell 2 h after the administration of VMP (red arrow). The cell was stained with Acridine Orange (green), and the lysosomes with Lysotracker (blue arrow). The lysosomes where VMP are, are pointed out with the purple arrow.**

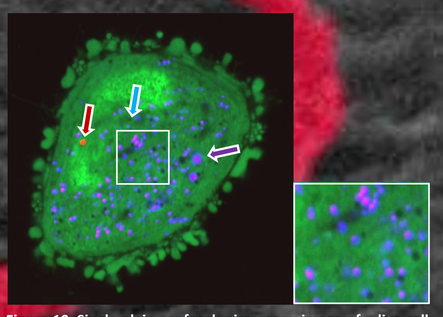


Figure 10. **Single plain confocal microscopy image of a live cell 2 h after the administration control uncoated particles (red arrw). The cell was stained with Acridine Orange (green), and the lysosomes with Lysotracker (blue arrow). The lysosomes where VMP are, are pointed out with the purple arrow.**

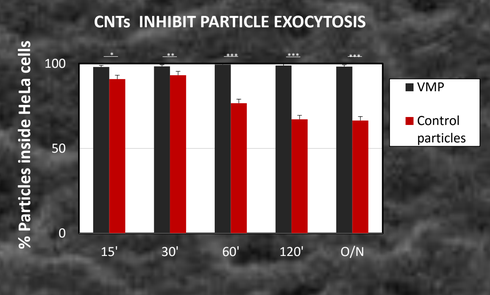
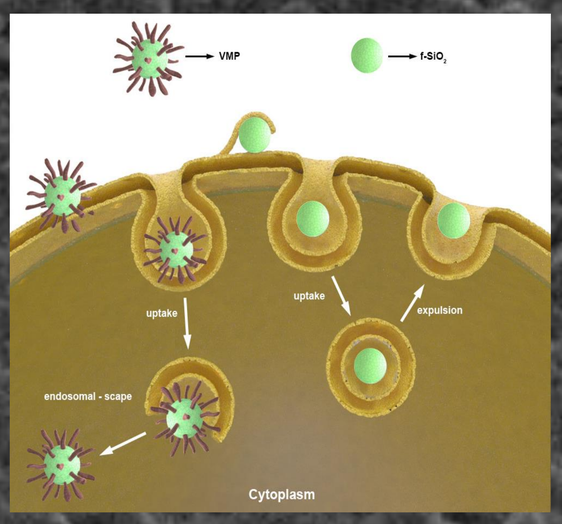


Figure 13. **Percentage of particles within HeLa cells is shown after 15, 30, 60, 120 min and an overnight of exposure, compared to the total particles (inside and outside cells) in each image.**

Particle receptor-mediated uptake is follow by the endosomal-lysosomal route (Fig. 9). The functionalised CNTs of the VMP lose their protein BIOcorona inside the lysosomes triggering lysosomal escape (Fig. 11). Carbon nanotubes themselves are able to escape lysosomes as Al-Jamal, *et al*., 2011 described. The hypothesis is that CNT (which are apolar) of the VMP interact with the lysosomal membrane tearing it apart, and escaping into the cytosol (Fig. 8). On the contrary, uncoated particles are captures and, after trafficking inside the cellular endosomal-lysosomal route (Fig. 10) are finally exocytosed (Fig. 12 and Fig. 13). (Nano)Particle exocytosis is well documented in the literature (Oh, *et al*., 2014).

Characterized model



Representative scheme of the internalization mechanism proposed. The VMP interact with the cell in a virus like mechanism and after internalization are able to escape from the endosome (left). The control silica particles which do not present a nanotube coating are taken up under a typical phagocytosis mechanism and are later exocytosed.

Future perspectives

1) Use empty or porous spheres instead of the solid ones,
2) Bind specific proteins or ligands to the CNT surface for targeted delivery
3) Inject VMP in animal models to test in vivo toxicity and try targeted delivery.

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