It has been established that once in biological media, most nanoparticles (NPs) are covered by the serum proteins which are adsorbed to the NP surface and form the so-called "corona". With 50 nm amine-modified polystyrene (NH_2-PS) NPs, we have demonstrated that the corona and NH_2-PS NPs are trafficked inside the cell together as complexes. Once inside lysosomes, the corona is digested by the lysosomal proteases and the positive NP surface cause damage to the lysosomal membrane and consequential cell death [1]. Moreover we have reported that the cell death induced by NH_2-PS NPs is associated with apoptosis and damage to mitochondria [2,3]. To continue the study, we would like to further investigate how NH_2-PS NPs induce damage to lysosomes and the role of lysosomes in the cell death, which we anticipated to achieve with the expertise of Dr. Patricia Boya in her lab in Centro de Investigaciones Biológicas, Madrid.

To start with, we monitored the morphological changes of lysosomes after treatment of NH_2-PS NPs with transmission electron microscopy (TEM). Figure 1 depicts the evolution of the damage to lysosomes due to NPs. In the untreated control (0h), lysosomes are healthy, membrane bound and electron dense. 3h after exposure to NPs, NPs were found to accumulate inside lysosomes (the ring structures found in lysosomes). 6h after exposure to NPs, lysosomal membrane starts to show signs of perturbation, such as permeabilization (red arrows) and blebbing (green arrows). 8h after incubation with NPs, the lysosomal membrane seems to be no longer visible by TEM (blue arrows), and the NPs seem to be scattered in the cytoplasma instead of being confined inside vesicles (purple arrows), indicating lysosomal membrane permeabilization, which is consistent with our previous results [1,3]. To note that after exposure to NPs for 3h, we found that some lysosomes seem to be engulfed by double membrane structures (black arrow) which could be autophagosomes. Therefore the autophagic process is further investigated below.

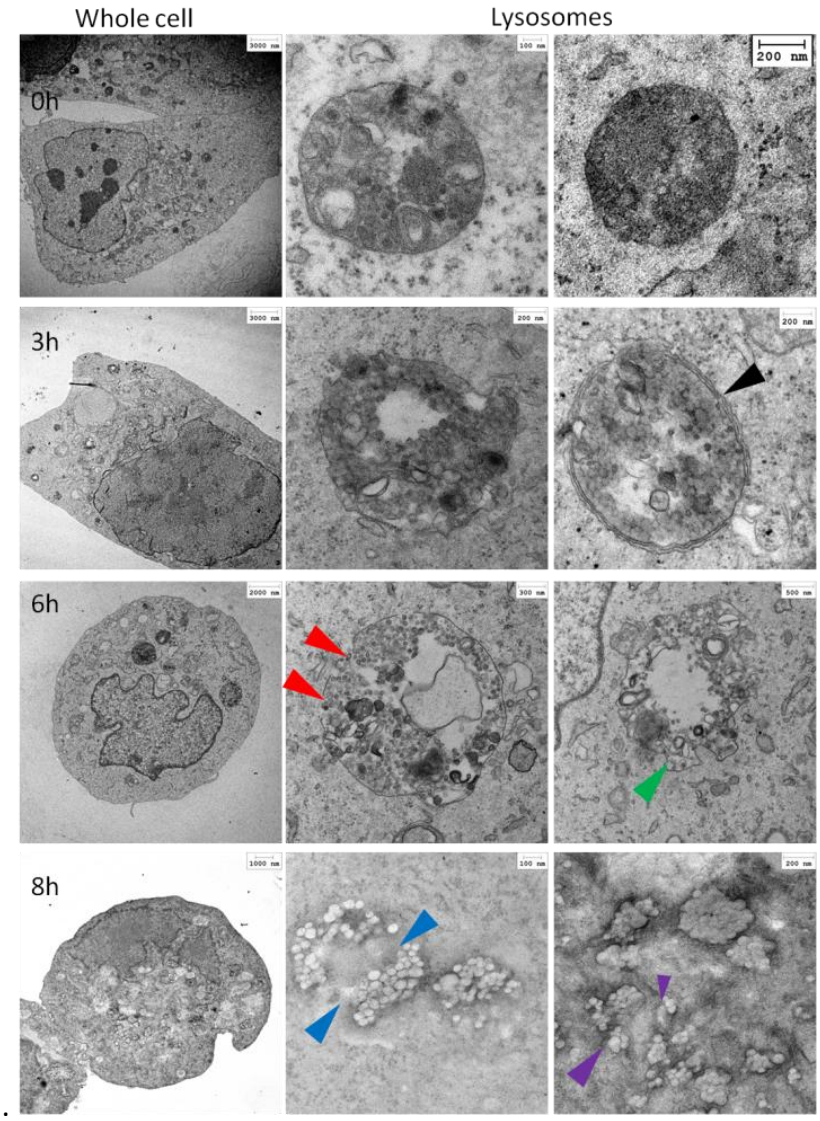

Figure 1 TEM images of cells treated by NH_2-PS NPs for indicated time points. Left panel presents images of the whole cells; middle and right panels present images of lysosomes.

The double membrane structure in the TEM images above indicates autophagy, a constitutive process in which a portion of cytoplasma is engulfed by the double-membrane autophagosomes which later fuse with lysosomes to degrade the engulfed content and regenerate energy. Since NP accumulation into lysosomes cause damage to lysosomes which is essential for autophagy, therefore we speculated that the autophagic process could be consequentially affected. After treatment of NH_2-PS NPs, we carried out kinetic studies with western blot to examine the expression levels of the major proteins involved in this process. This study was done in both 1321N1 cell line (Figure 2A) and mouse embryonic fibroblasts (MEFs) (Figure 2B). Hydrochloroquine (HCQ) was included in this study as an inhibitor for the fusion between lysosomes and autophagosomes. As HCQ blocks the fusion between autophagosomes and lysosomes, therefore if treatment+HCQ increases the LC3 II level than that of treatment alone, it means the treatment induces autophagy; if the treatment+HCQ does not increase the LC3 II level, it means the treatment blocks autophagy.

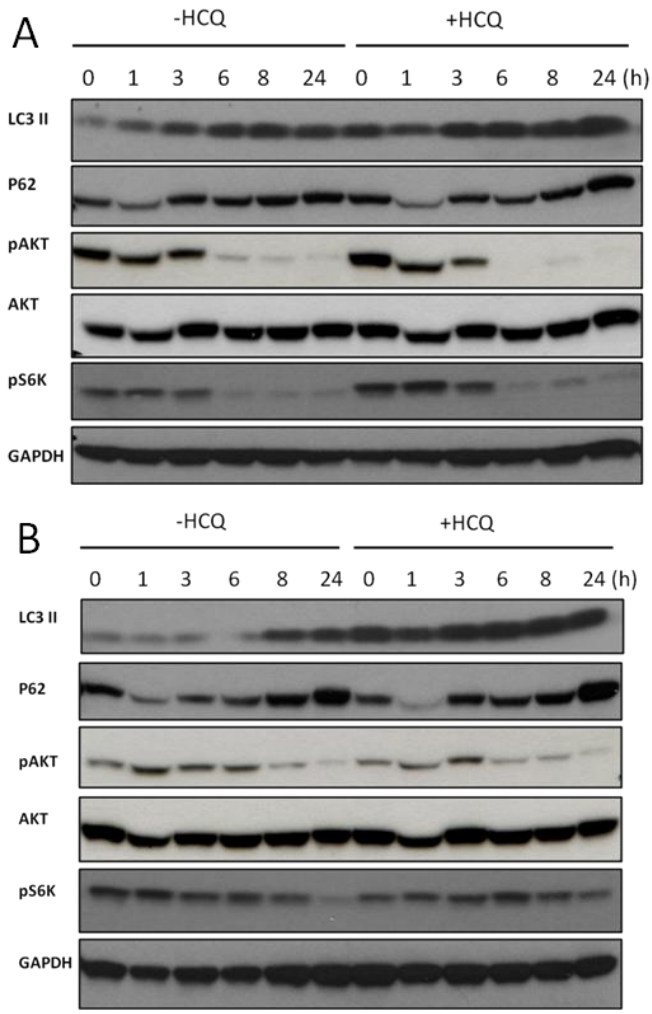

Figure 2: Western blot of 1321N1 cells (A) and MEFs (B) treated by NH_2-PS NPs for indicated time points with or without the presence of the inhibitor HCQ.

LC3-II is a marker for autophagosomes as during autophagy it is recruited to the autophagosomal membrane. The western blot results shows increase of LC3 II amount, indicating that NPs induce accumulation of autophagosomes. This confirms our previous results [3]. p62 is an autophagic substrate, therefore functional autophagy degrades p62; however if autophagy is blocked then p62 will not be degraded. The western blot results indicate there might be functional autophagy at early time points (1h), as there is decrease of p62; however at later time points, there is accumulation of p62, indicating the blockage of autophagic pathway. Both AKT and S6K proteins are involved in mTOR/PI3K/AKT signalling pathway and play an important pro-survival role by inhibiting apoptosis. The phosphorylated forms of both AKT and S6K are the active forms of both proteins. After treated by NPs for 6h both pAKT and pS6K have dramatically decreased, indicating NPs interact strongly with the AKT signalling pathway.

The same experiments were carried out in the mouse embryonic fibroblasts wild type (MEFs wt). From the blot of LC3, autophagosomes seem to start to accumulate after 8h, later than in 1321N1. For p62, there is a decrease at 1h, indicating functional autophagy; then a

gradual increase is followed, indicating blockage of autophagic flux, similar to what has been shown in 1321N1 cells. The changes of pAKT and pS6K protein levels do not seem to be as dramatic as in 1321N1 but it still shows decrease.

Moreover, we examined the cellular localization of autophagosomes and autolysosomes. 1321N1 cells were transfected with tandem RFP-GFP LC3 plasmid. Autophagosomes (not acidic) are labelled by both GFP and RFP (yellow). However when autophagosomes fuse with lysosomes (autolysosomes, acid), the fluorescence of GFP will be quenched in the acidic ambient; therefore autolysosomes show red. HCQ blocks autophagy by inhibiting the fusion between autophagosomes and lysosomes, therefore there are more autophagosomes (yellow) but not autolysosomes (red) can be observed. However another explanation for the quenching of GFP fluorescence could be due the alkalization of lysosomes due to HCQ treatment.

As is illustrated in Figure 3, cells treated by NPs for 3h show both red and yellow, indicating the early autophagic flux is functional; cells treated by NPs for 8h show mostly yellow, indicating the overall blockage of autophagic flux which confirms the western blot results of LC3 and p62 above.

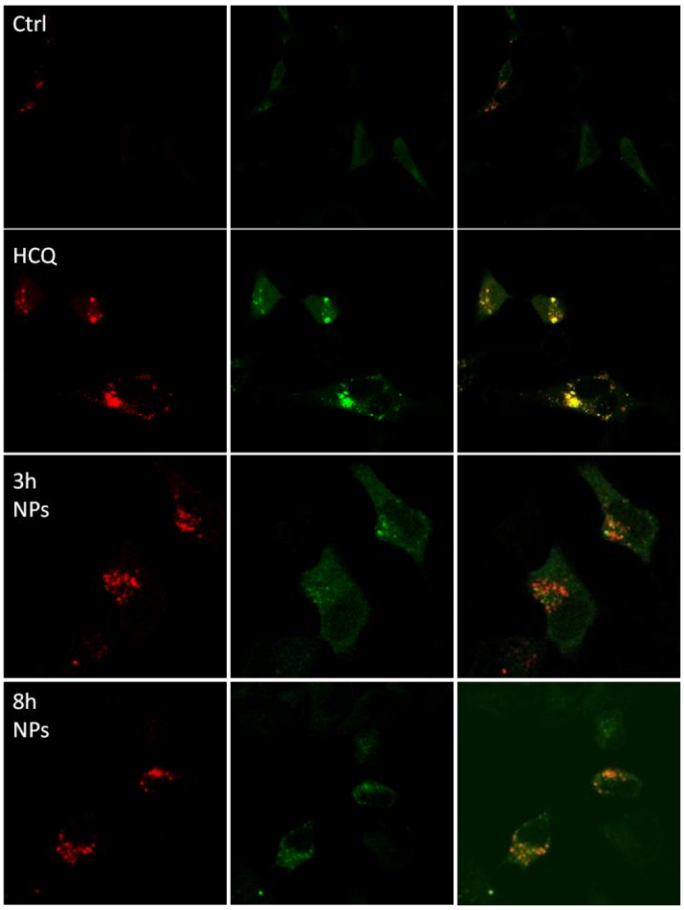

Figure 3: Confocal images of 1321N1 cells treated by NH_2-PS NPs for indicated time points. Cells were transfected by tandem LC3 plasmid. Red (left panel) shows autolysosomes and yellow and green (middle and right panel) show autophagosomes.

We have previously demonstrated that NH_2-PS cause damage to mitochondria. Therefore here we investigate which is the upstream of damage: mitochondria or lysosomes. To investigate this, we compare MEFs wild type and its Bax/Bak double knock out (DKO). Bax/Bak DKO cells cannot carry out mitochondrial membrane permeabilization (MMP) [5], therefore it can protect cells from death induced by the positive control staurosporine (STS). The same result can be observed by the treatment of NPs, indicating that the death induced by NPs is still a MMP dependent process. We have observed that the Bax/Bak DKO cells are protected from cell death induced by NH_2-PS NPs. This indicates that NPs induce MMP and then apoptotic cell death. Moreover we have previously demonstrated that NH_2-PS NPs induce lysosomal membrane permeabilization (LMP) [1,3]. Therefore it is then tempting to speculate that cell death is carried out through NPs-->LMP-->MMP [4].

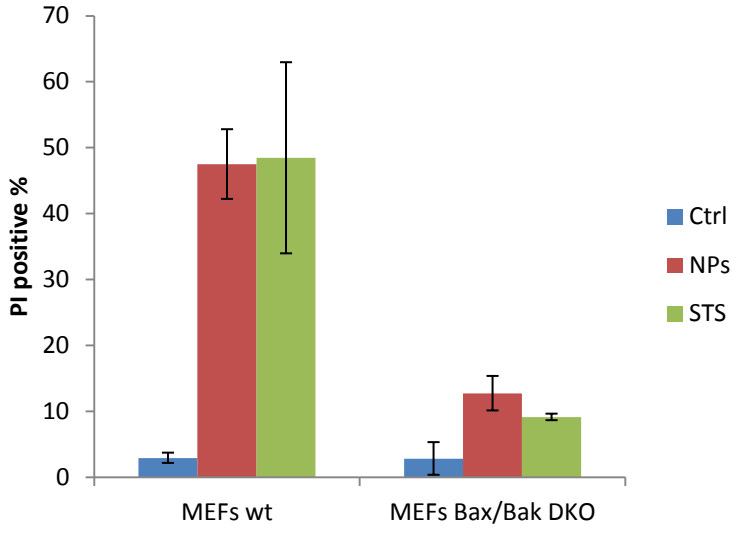

Figure 4: Cell death induced by 50 µg/ml NH_2-PS NPs in MEFs wt and MEFs Bax/Bak DKO after 24h, as indicated by measuring propidium iodide (PI positive).

All the above results indicate that the 50 nm NH_2-PS NPs cause severe damage to lysosomes as indicated by the EM images. Moreover it is very likely that NH_2-PS NPs induce autophagy after a short exposure time to the NPs. However the lysosomes are damaged due to accumulation of NH_2-PS NPs, therefore the autophagosomes generated cannot fuse with lysosomes anymore, leading to an accumulation of autophagy and a blockage of autophagic flux. This observation again suggests that lysosomes play an essential role in the cell death induced by NH_2-PS NPs. Moreover we demonstrated this lysosomal-associated cell death due to NH_2-PS NPs is dependent on mitochondrial membrane permeabilization.

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