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NWs of 5.4 μm in length, as well as for Ni ions. The results show that cytotoxicity markedly increases past 24 hours of incubation. Cellular uptake of NWs takes place through the phagocytosis pathway, with a fraction of the dose of NWs dissolved inside the cells. Cell death results from a combination of apoptosis and necrosis, where the latter is the outcome of the secondary necrosis pathway. The cytotoxicity of Ni ions and Ni NWs dissolution studies suggest a synergistic toxicity between NW aspect ratio and dissolved Ni, with the cytotoxic effects markedly increasing after 24 hours of incubation.

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Abstract

The assessment of cytotoxicity of nanostructures is a fundamental step for their development as biomedical tools. As widely used nanostructures, nickel nanowires (Ni NWs) seem promising candidates for such applications. In this work, Ni NWs were synthesized and then characterized using vibrating sample magnetometry, energy dispersive X-Ray analysis and electron microscopy. After exposure to the NWs, cytotoxicity was evaluated in terms of cell viability, cell membrane damage and induced apoptosis/necrosis on the model human cell line HCT 116. The influence of NW to cell ratio (10:1 to 1000:1) and exposure times up to 72 hours was analyzed for Ni NWs of 5.4 μm in length, as well as for Ni ions. The results show that cytotoxicity markedly increases past 24 hours of incubation. Cellular uptake of NWs takes place through the phagocytosis pathway, with a fraction of the dose of NWs dissolved inside the cells. Cell death results from a combination of apoptosis and necrosis, where the latter is the outcome of the secondary necrosis pathway. The cytotoxicity of Ni ions and Ni NWs dissolution studies suggest a synergistic toxicity between NW aspect ratio and dissolved Ni, with the cytotoxic effects markedly increasing after 24 hours of incubation.

Introduction

Over the past few years, thanks to the progress in nanofabrication and imaging, the field of nanomagnetism has seen a steady growth, and as such, nanoparticles that can perform multiple functions are becoming more relevant (Varadan et al. 2008). Whereas magnetic nanobeads are already widely used in biomedical research and applications (Gooneratne et al. 2013; Wilhelm et al. 2008; Tanaka et al. 2008; Gaihre et al. 2009; Gonzales-Weimuller et al. 2009), magnetic nanowires (NWs) became attractive only recently. The magnetic properties of NWs can be tailored in a wide range, mainly because their diameter and length can be independently modulated (Song et al. 2012; Sun et al. 2005). It has also been shown that magnetic NWs may have a larger magnetic moment per unit of volume compared to beads (Hultgren et al. 2005). Further, due to their shape, magnetic NWs can be used to exert translational forces as well as torques (Hultgren et al. 2004).

Different kinds of NWs are being investigated for biomedical applications. Interactions between normal or stem cells and silicon NWs have been the focus of recent studies (Zhang et al. 2012; Julien et al. 2010), and there has been success in regulating the osteogenic differentiation of stem cells when grown on Si NWs matrices of different lengths (Kuo et al. 2012). In the context of magnetic materials, the biocompatibility of iron NWs has also been validated for the application of cell manipulation (Song et al. 2012). Reich and colleagues have carried out extensive research on cell manipulation and separation using Ni NWs, increasing the separation yield 4-fold when compared to magnetic nanobeads (Hultgren et al. 2003; Hultgren et al. 2004; Hultgren et al. 2005). The effects of antibody coating and gold functionalization of Ni NWs have also been

utilized for this purpose (Gao et al. 2010; Reich et al. 2003). Besides cell manipulation, targeted drug delivery is a potential application that is being extensively studied: it has been reported Ni NWs are capable of transporting colloidal cargo when under a uniform rotating magnetic field (Zhang et al. 2010). Further, studies on destruction of living cells have paved the way for possible cancer treatment therapies. For instance, it has been shown that cells with internalized Ni NWs can be heated and killed under a radio frequency electromagnetic field (Choi et al. 2008). Similarly, an alternating current magnetic field has been proven to increase the inflammation of cells with internalized Ni NWs due to an increase in the pro-inflammatory cytokine interleukin-6 (Choi et al. 2012). More recently, it has been shown that Ni NWs can be used to kill cancer cells with extremely low magnetic fields and frequencies (Contreras et al. 2015).

Internalization of NWs by cells has been documented over the past years. While soluble Ni^{2+} can enter the cell via calcium channels, the divalent cation receptor or by diffusion, insoluble Ni compounds do so by phagocytosis (Kasprzak et al. 2003). More specifically, it has been reported that NW uptake in NIH-3T3 mouse fibroblasts takes place through the activation of the integrin-mediated phagocytosis pathway (Hultgren et al. 2005). After 24 hours of incubation, most NWs are located in the cytosol, with a small fraction ($14 \pm 5\%$) located in late endosomal/lysosomal compartments. Inside these organelles they are broken up into smaller aggregates, probably due to the decrease in pH (Safi et al. 2011). After internalization, Ni NWs tend to have a preference to accumulate close to the nuclear membrane (Prina-Mello et al. 2006).

The aggregation of nanoparticles in solution is a well-known phenomenon that may play a role in the cytotoxic response. One study has reported specific uptake patterns

depending on the cell line between single and aggregated nanoparticles (Albanese et al. 2011), whereas another one showed that aggregated nanoparticles lead to a higher cytotoxicity (Okuda-Shimazaki et al. 2010). Magnetic nanoparticles such as NWs possess a permanent magnetic moment that makes them more prone to aggregation in solution (Safi et al. 2011), with ultrasonic agitation minimizing this effect temporarily. Therefore, for most cytotoxicity studies, NWs are delivered to the target cells in a combination of single NWs and aggregates of varying sizes. A higher cytotoxicity for aggregated magnetic NWs compared to single ones has also been observed (Gao et al. 2010).

As nanowire-cell interactions such as internalization could be hazardous to cells, cytotoxicity studies play an essential role in developing a thorough understanding of the implications of NWs on biomedical applications. For instance, cells incubated with iron (Fe) NWs maintain >80% cell viability for incubation times of 72 hours and for doses as high as 10 000 wires per cell (Song et al. 2010). Similarly, Ni NWs cytotoxicity has been evaluated through a multiparameter cytotoxicity assay, and the results showed that the NWs are not cytotoxic for low incubation times (10 hours) and for concentrations lower than 100 NWs per cell, but toxic effects significantly increased for 24 hours of incubation (Byrne et al. 2009). Induction of apoptosis by Ni NWs has also been reported for HeLa and human pancreatic adenocarcinoma cells (Ma et al. 2014; Hossain et al. 2011). Further, a significant difference in inflammatory response in a mouse peritoneal model was observed between Ni NWs of 4.3 μm and 24 μm in length (Poland et al. 2012).

Given the potential of magnetic NWs as biomaterials, as well as the need for a better understanding of the possible risks involved with their applications, the toxicity of Ni

NWs was assessed at the cell level in terms of cell viability, integrity of the cell membrane and degree of apoptosis and necrosis induced. Intracellular Ni NWs dissolution studies were also carried out, considering that the degradation of the NWs could lead to the release of Ni^{2+} , a compound that could contribute to the cytotoxic response. Additionally, the possible cytotoxic effect of Ni^{2+} was studied by exposing cells to Ni sulfate. To complete the picture, internalization and endocytosis studies with different inhibitors were also conducted. The HCT 116 carcinoma epithelial cell line was chosen for the cytotoxicity experiments. As the epithelium lines both the outside and inside cavities of many different tissues in the human body, this cell line is a good model to study cytotoxicity of NWs aimed to be injected into the bloodstream (Kong et al. 2011). The lower end of the range of doses used was chosen to correspond to those used in previous applications with Ni NWs (Hultgren et al. 2005), whereas the higher end would give an insight into the cell response for a potential cytotoxic dose.

Materials and methods

In this section the main aspects of the methods are mentioned. Detailed explanations of the methodology can be found in the supplementary material.

Fabrication and characterization of Ni NWs

Ni NWs of 5.4 μm in length and with diameters of 30 – 35 nm were fabricated by electrodeposition into nanoporous alumina templates. Following electrodeposition, the alumina templates were then dissolved in 1 M NaOH solution and Ni NWs suspended in it, with the NaOH solution being replenished once every hour until the NWs were dispersed. The NWs were then collected with a magnet and thoroughly washed with ethanol to sterilize them. Sonication for two minutes in an ultrasonic bath (Branson 2510, 40 kHz) was used to break up NW aggregation every time the NaOH or ethanol solutions were changed. NWs were then resuspended and washed in cell medium, which was changed five times to remove traces of ethanol.

NW morphology and composition after release from the template were analyzed in a Quanta FEG 600 (FEI) scanning electron microscopy (SEM) and in a Technai T12 (FEI) transmission electron microscopy (TEM). Energy dispersive X-Ray analysis (EDX) was done using scanning TEM (STEM). The magnetization measurements of the NWs were carried out with a vibrating sample magnetometer (MicroMag™ 3900) using the alumina template with embedded Ni NWs.

Quantification of NW aggregation in cell medium was performed using a Leica TCS SP5 Inverted Confocal Microscope. Over 1000 NW aggregates were analyzed using the ImageJ software. Additionally, 15 TEM images were used to observe the delivery of single NWs and NW aggregates to HCT 116 cells.

NW dose and probe interaction testing

Given that NWs eventually precipitate on cells when added to cell medium, all the experiments in this work were performed using the NW number per cell as a dose. To estimate the number of NWs, it was first confirmed that all the pores of the alumina template were filled with NWs after the electrodeposition process (Supplementary Figure S1). The pores in the alumina template of the electrodeposited sample were individually counted for a small, defined area using an SEM image of the template. Then, the total number of pores (i.e. NWs) was calculated by rescaling the number of pores to the total area in which electrodeposition took place (defined by the fabrication setup), as shown in Equation 1:

$$\text{No. of NWs} = \frac{(\text{No. of pores in SEM image}) * \text{Total deposition area}}{\text{Total area of SEM image}} \quad (1)$$

Additionally, the concentration was estimated using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) following the same methodology described further in this section. The equivalences in $\mu\text{g/mL}$ for all the number concentrations in this study can be found in the Supplementary Table S2.

Finally, NWs were tested for possible interaction with the probes used in this work. No interaction was found for any case. Data for the controls testing can be found in the Supplementary Figure S3.

Cell culture and treatment

HCT 116 (ATCC® CCL247™) cells were cultured in McCoy's 5A modified medium (Gibco®), supplemented with 10% fetal bovine serum (FBS) (Gibco®) and L-glutamine,

and grown in a 37 °C humidified incubator with 5% CO₂. Upon reaching 80% confluence, cells were detached from the culture flasks with 0.25% trypsin-EDTA and counted using trypan blue staining. Following the stabilization incubation period, the cells were treated with Ni NWs. Dilutions were prepared with McCoy's medium to obtain the desired concentrations for treatment. In the case of treatment with NiSO₄·6H₂O (Ni sulfate) (22.3% Ni), dilutions were also performed with McCoy's medium and the concentrations were calculated so that the amount of Ni in mass was comparable to the mass values of the NWs.

Cytotoxicity and cell death assessment

Cytotoxicity in terms of cell metabolic activity was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) cell proliferation assay (Vibrant®) following the standard vendor's procedure. The optical density of the reduced products was evaluated in a microplate reader (XMark™, Bio-Rad) using a wavelength of 570 nm and a reference wavelength of 630 nm.

The Cytotox-ONE™ homogenous membrane integrity assay (Promega) was chosen as the second method to evaluate cytotoxicity. Following the vendor's protocol, fluorescence was recorded with the GloMax – Multi Detection System (Promega), using an excitation wavelength of 525 nm and an emission wavelength range of 580 – 640 nm

The transepithelial electrical resistance (TEER) was measured to study the effects of Ni NWs on the cellular membrane integrity. The EVOM² (World Precision Instruments) epithelial voltohmmeter coupled with the STX100C96 electrode (World Precision Instruments) was used for this purpose.

Finally, the Dead Cell Apoptosis Kit with Alexa Fluor® 488 Annexin V & Propidium Iodide (PI) for Flow Cytometry (Invitrogen™) was used to study apoptosis/necrosis. Analysis was done on a BD FACSCanto™ II Flow Cytometer (BD Biosciences). Fluorescence emission of Alexa Fluor® 488 and PI allowed for the identification of four cell populations within the samples: healthy and viable cells (Alexa Fluor® 488 negative and PI negative), early apoptotic cells (Alexa Fluor® 488 positive and PI negative), late apoptotic or necrotic cells (Alexa Fluor® 488 positive and PI positive) and necrotic cells (Alexa Fluor® 488 negative and PI positive). Data analysis was performed using FlowJo software 7.6.1 (TreeStar Inc., Ashland, USA).

Internalization of Ni NWs

The internalization of NWs was studied with three different methods: a 24-hour time-lapse study, a Z-stack analysis and TEM studies. For analyses with the first two methods, Ni NWs were coated with (3-Aminopropyl) triethoxysilane (APTES) (Sigma-Aldrich) and then labeled with pHrodo™ Red, succinimidyl ester (P36600, Molecular Probes™), a pH-sensitive dye used for detection of endocytosis. The Nikon Biostation IM-Q CELL-S2-P was used for the 24 hours time-lapse microscopy study, whereas the Z-Stack analysis was carried out using a Zeiss LSM 710 upright confocal microscope.

Endocytosis inhibition

Three different endocytosis inhibitors were tested to evaluate the internalization pathway of NWs: nystatin (N6261, Sigma-Aldrich), which blocks caveolae-mediated endocytosis; chlorpromazine (C8138, Sigma-Aldrich), for clathrin-mediated endocytosis

and latrunculin A (L12370, Molecular Probes™), which prevents phagocytosis by inhibition of actin polymerization.

Ni NWs dissolution

ICP-MS (Elan DRC II, Perkinelmer) was used to measure the dissolution of Ni NWs inside the cells and in the surrounding medium. The same experiment was additionally carried out using Ni sulfate, with the concentrations tested being equivalent in Ni to those of 5.4 μm Ni NWs. For dissolution in simulated body fluid (SBF), samples were analyzed using an iCAP 6000 Series ICP Emission Spectrometer.

Statistical analysis

The data are expressed as means $\pm$ standard deviation (SD) or mean $\pm$ range. The statistical comparisons of means were performed by one-way analysis of variance using MATLAB software. $n = 3$ for all experiments performed in this work, except for the apoptosis/necrosis assay, where $n = 2$. Statistical significance was considered for values of $p < 0.01$ vs. the specific control.

Results

Characterization of Ni NWs

The morphology of a single Ni NW is shown in Figure 1A. Following the shape of the template's nanopores, the NWs possess a high aspect ratio and a diameter ranging between 30-35 nm. The conducting channels (dendrites) formed before the electrodeposition step can be observed at one end of the NW. A thin layer of a few nanometers can be observed along and around the core of the NW (Figure 1B). Elemental analysis by STEM-EDX confirmed the Ni composition of the core (Figure 1C). The carbon peak can be attributed to the ethanol used in the washing steps. EDX analysis of a single point in the thin layer around the NW core showed an increase of oxygen (Figure 1D), with lower amounts of Ni when compared to the core. The thin layer is possibly oxidized Ni grown due to the NWs exposition to air and to NaOH during the release process.

The magnetization curve of Ni NWs is shown in Figure 1E. The NWs coercivity (H_c) was 922.8 Oe and the saturation magnetization (M_s) was 50.3 emu/g, a smaller value than the one reported for bulk Ni (55.4 emu/g). This decrease in magnetization could be explained by the oxide layer present in the NWs.

The nanoporous alumina templates and released NWs were observed under SEM to study the pore size and NW length distribution, as well as to look at the NW morphology. Figure 2A shows these properties for the fabricated NW samples. The NW diameter was found to be 33 ± 3 nm, whereas the NW length was 5.4 ± 1.6 μ m. Though small, the difference in pore diameters between the samples can be explained by small differences in the aluminum substrate or fluctuations during the fabrication process,

specifically changes in temperature during the second anodization. Similarly, the rather wide length distribution is explained by inhomogeneous wire growth rate in the electrodeposition step. Ultrasonic agitation during the release and washing process seemed to have little effect on the length of the NWs, as samples that were not sonicated during the release process had roughly the same length reported here (Supplementary Figure S4).

Figure 2B (left panel) shows the distribution of sizes of NW aggregates in McCoy's medium for the highest NW dose used in this study (1000:1 NW to cell ratio). As observed, more than half of the NW aggregates cover an effective area of below $5 \mu\text{m}^2$. However, this analysis is limited by the resolution of the confocal microscope (200 nm), so any single NW or small NW bundle could not be considered and therefore is not included in the distribution presented here. To complement this information and to show that NWs are also delivered to cells as single particles, TEM images of cells incubated with Ni NWs were analyzed. All instances of the presence of single NWs and NW bundles or aggregates were quantified, and the results are shown in the right panel of Figure 2B. Single NWs were observed for most cases, though bigger aggregates were also confirmed to be present.

Effects of Ni NWs on cell viability

The MTT salt is mostly reduced by the reductase enzymes in the mitochondria and plasma membrane of living and metabolically active cells, which under defined cell growth conditions can reflect cell viability. Thus, it allows a quantitative estimation of the

volume of viable cells and because of this it was chosen as the first way to assess NW cytotoxicity.

The MTT assay was carried out with regards to the influence of exposure times and different concentrations of NWs. As observed in Figure 3A, cell viability decreases with both NW concentration and exposure time. The findings are supported by optical microscope images of the HCT 116 cells (Supplementary Figure S5), which show an increase in detached (unhealthy) cells from the well surface related to NW concentration and exposure time.

A cell viability of 80% was observed for a concentration of up to 200 NWs per cell and 24 hours of NW incubation, but the increment of incubation time to 48 hours at concentrations of 50 NWs per cell and higher decreased the cell viability below the 80% value. These results are in agreement with those observed at the 24 hours time point by Volkov and colleagues (Byrne et al. 2009).

An MTT assay was also performed using soluble Ni ions in the form of Ni sulfate, one of the original precursors of the electrodeposition solution used for the fabrication of NWs. The concentrations tested were equivalent to the NW to cell ratios used for the Ni NWs. As observed in the results in Figure 3B, HCT 116 cells show >80% viability when exposed to this form of Ni, except at the highest concentrations and incubation time points.

Effects of Ni NWs on cell membrane permeability

The homogenous membrane integrity assay measures the amount of released lactate dehydrogenase (LDH) enzyme from the cytoplasm of cells with compromised

membrane integrity. This method was chosen due to the possible scenario of NWs causing direct damage to the cellular membrane. The results of the experiment are shown in Figure 4A. No membrane rupture and LDH leakage were present after 24 hours of incubation when compared to the non-treated negative control cells. After 48 hours of incubation, LDH leakage was significant for most NW concentrations when compared to the negative control cells, although the increase was not considerably higher. At the longest exposure time (72 hours), LDH leakage increased abruptly for concentrations of 50 NWs per cell and higher.

In addition to the LDH assay, TEER measurements were performed to assess the cell monolayer integrity and permeability after incubation Ni NWs. A confluent, healthy cell population has a markedly high transepithelial resistance, whereas one with compromised cell membrane permeability or decreased healthy cell number normally shows a reduced resistance value. Figure 4B shows the results of the TEER experiment for three concentrations of Ni NWs at 24, 48 and 72 hours of incubation. As expected, the TEER value for the control cells increases over time, indicating a stable, growing cell population. At 24 hours of incubation, only the highest concentration of NWs shows a significant decrease in TEER. At 48 and 72 hours, in contrast, the decrease of resistance value is significant for all three concentrations.

Internalization of Ni NWs

A 24 hours time-lapse microscopy study was conducted to look at the internalization of NWs (200:1 NW to cell ratio). The series of micrographs shown in Figure 5A denote the full duration of the experiment, from the moment the NWs were added to the culture

until the conclusion of the study. The red signal corresponds to the pHrodo™ Red-labeled Ni NWs, which is activated by the decrease in pH as the dye is internalized. The blue signal corresponds to the Hoechst 33342-labeled cell nuclei. As observed in the images, there is an increase in the number of pHrodo™ red fluorescence spots as time progresses, indicating an increase in NW internalization by the cells.

An interesting event regarding one NW aggregate is marked with a white arrow in Figure 5A. As time goes on, the cells around it seem to break the aggregate apart, internalizing NWs from it in the process and then emitting the red signal.

To further confirm the internalization of the NWs, a Z-stack study was performed using confocal microscopy. Figure 5B shows this analysis for one section that has two different cases: one where a NW is internalized and emits the red fluorescence, and another where a NW is probably not internalized and lacks the red fluorescence.

Figure 5C and 5D show images of internalized Ni NWs for 24 and 72 hours, respectively, as observed by TEM. For both incubation times NWs were observed to localize close to the cell membrane, either outside of the cell or inside of it, as well as inside endosomes. In Figure 5C, a NW aggregate can be observed inside an endosome (right panel), while a single NW, also inside an endosome, lies very close to a lysosome (Figure 5D, right panel).

Finally, the internalization pathway of NWs in the HCT 116 cell line was studied. The results of inhibiting three different endocytosis pathways (caveolae-mediated endocytosis, clathrin-mediated endocytosis and phagocytosis) are shown in Figure 5E. As can be observed, the concentrations used for the three inhibitors had no effect on cell viability.

Caveolae-mediated endocytosis and clathrin-mediated endocytosis seem to play no part in the internalization of Ni NWs, as there is a significant decrease in cell viability when compared to the negative control. Similarly, there is no statistical significance when compared to the NW-only control. This indicates that the cytotoxic effects due to NW internalization are still present when blocking these two pathways.

On the other hand, the inhibition of phagocytosis resulted in a significant difference when compared to the NW-only control, with no statistical difference with the negative control. This means that the blocking of phagocytosis prevented the internalization of NWs and thus its effects on cell viability were prevented. Additionally, the blocking of energy-dependent processes by incubating HCT 116 cells with Ni NWs at 4°C showed no significant decrease in cell viability, suggesting an active mechanism of internalization (Supplementary Figure S6).

Apoptosis/necrosis induction by Ni NWs

Alexa Fluor 488® Annexin V-PI apoptosis/necrosis assays were performed by flow cytometry in order to characterize the activated cell death mechanisms by Ni NWs. The annexin V protein binds to phosphatidylserine (PS), a phospholipid that is normally located in the cytoplasmic surface of the cell membrane. In apoptotic cells, PS is flipped to the outer cell membrane to act as a marker for phagocytosis by macrophages. PI, on the other hand, stains the nucleic acids of dead cells and is impermeant to live and (early) apoptotic cells. Thus, the dye combination allows distinguishing apoptotic cells from necrotic ones.

Figure 6 shows the gated cell populations over time (rows) for increasing concentrations of Ni NWs (columns). After 24 hours of exposure (Figure 6B-D) a small

increase in the apoptotic population due to Ni NW concentration is observed without it being significant. At 48 hours of incubation, the apoptotic population markedly increases, whereas the healthy population is considerably diminished when compared to the negative control. For 72 hours of incubation, cell populations are mostly in the apoptotic and late apoptotic/necrotic stages, while the healthy cell population considerably decreases by increasing NW concentration.

Ni NWs dissolution

To determine the possible causes of the cytotoxic cell response, the total Ni^{2+} content derived from the Ni NWs was measured in both the surrounding medium and inside the cultured cell population. Results from this study are shown in Figure 7A. Ni NWs showed a time-dependent dissolution rate for both conditions tested, though the overall percentage of dissolved dose of Ni NWs is very small ($<2.2\%$).

In order to properly compare the effects of Ni NWs to those of Ni in ionic form, an additional ICP-MS study was performed to look at the amount of internalized Ni^{2+} after incubation with Ni sulfate. Figure 7B shows the percentage of Ni internalized for Ni sulfate at the same dose as that of Figure 7A, as well as an additional one equal to 1000:1 NWs per cell. For both concentrations tested, the percentage of internalized Ni was similar to that observed for NWs. The total amount of internalized Ni in mole atoms for all the conditions tested is shown in Figure 7C. In the case of the 200:1 dose, HCT 116 cells internalize similar amounts of Ni either from NWs or Ni sulfate.

It is also interesting to look at the dissolution rate of Ni NWs in the context of *in vivo* applications. Therefore, an analysis of Ni NWs dissolution in SBF solution was

performed. The SBF solution is considered to be physiologically relevant because its ion content is similar to the human blood plasma (Kokubo 1991). Figure 7D shows the release of Ni^{2+} from Ni NWs in SBF (pH 7.4 and 4.8) as a function of time. Results show a very slow dissolution rate for the standard SBF solution with a pH of 7.4, with similar values to those found in cell medium. However, the dissolution rate was increased when the pH was lowered to a value of 4.8 (to emulate the acidic lysosomal pH).

Discussion

The data presented here shows that the endocytosis of Ni NWs takes place through the actin-mediated phagocytic pathway, with the caveolae-mediated and clathrin-mediated pathways playing no apparent role. Internalized NWs reside mostly in endosomes, as well as close to the cell membrane. HCT 116 cells can internalize both single NWs and NW aggregates, while also being capable of breaking up larger aggregates before the uptake process.

Once internalized, Ni NWs at low doses are initially tolerated (<24 hours), but their cytotoxic effects increase for longer incubation times and at lower concentrations. Such behavior could be explained by the onset of internalization: as was found with the time-lapse study, the cells take up the NWs in a non-synchronized manner, with only a few cells internalizing NWs during the first few hours, whereas after 24 hours some of them still have not initiated the process. This could be explained by the random dispersion and orientation of the NWs upon contact with the cell, as well as the number of NWs in

the aggregate being internalized, as reported elsewhere (Albanese et al. 2011). Overall, the delay in the onset of internalization translates into different cell death times.

The cell viability assays with Ni sulfate show that HCT 116 cells are tolerant to low concentrations of Ni in its ionic form, but higher concentrations ($>10.5 \mu\text{g/mL}$) are toxic. Given that the NWs show a higher cytotoxicity than equivalent amounts of Ni sulfate, it can be assumed that the NW shape plays a role in the cytotoxic effect on HCT 116 cells. On the other hand, the increase of internalized mole atoms of Ni for the 1000:1 dose could explain the sudden drop in cell viability for the Ni sulfate, which indicates that Ni in ionic form also contributes to Ni NWs cytotoxicity.

The combined results of the Alexa Fluor 488® Annexin V-PI apoptosis/necrosis assay and LDH leakage show that cell death resulting from interactions with Ni NWs occurs initially through apoptosis. Then, for higher concentrations and incubation times, cells shift to a late apoptotic/necrotic state, with the effects being dependent on NW concentration and incubation time. A similar response was observed with Annexin V-PI staining in HeLa cells for higher concentrations of Ni NWs (Ma et al. 2014).

Normally a marker of necrosis, LDH leakage in the apoptotic process can be explained by the secondary necrosis pathway, in which late apoptotic cell bodies incur cellular membrane rupture (Majno et al. 1995). Due to the absence of phagocytes, secondary necrosis is the usual outcome of apoptosis in *in vitro* experiments (Silva 2010). Thus, the sudden increase of LDH leakage at the 72 hours time point corresponds with the secondary necrosis pathway in the late stages of apoptosis, as secondary necrosis leakage is detected at a much later stage compared to that of (primary) necrosis (Zhan et al. 1997). The MTT assay results also support this, as cell

viability was significantly reduced for most of the Ni NW concentrations at 24 hours of incubation, indicating a reduction in metabolic activity, yet no significant LDH leakage was detected under the same conditions. Further, the decrease in TEER values correlates with the LDH leakage results, adding to the assumption that cell death occurs through apoptosis and then progresses to secondary necrosis.

A fraction of the dose of NWs was found to be dissolved inside the cells (<1%). As the dissolution in low pH SBF solution shown here suggests, intracellular dissolution is most probably due to the acidic pH of the lysosomal compartments in the cytoplasm. Similar observations have been reported for zinc oxide NWs (Muller et al. 2010). Since the percentage of dissolved Ni NWs in bare cell medium and in the medium of grown HCT 116 cells is similar, there is little or no extracellular Ni^{2+} release after internalization of the NWs. The amounts of Ni^{2+} found in the surrounding cell medium are in accordance to those observed by Ma and colleagues when considering the higher dose used in their study (Ma et al. 2014).

As with the dissolution in cell medium, the percentage of the NWs that were dissolved in SBF solution (pH 7.4) was less than 2%. This proves beneficial for therapeutic applications with Ni NWs, as the possible side effects of the soluble Ni can be minimized.

The low dissolution rates in cell medium and SBF reported here are in agreement with a recent study in which the magnetization of Ni NWs was reduced by less than 1% after 48 hours of incubation with different relevant bio-related solutions (Raphael et al. 2010). There, along with the detection of no traces of Ni^{2+} after 48 hours in phosphate

buffered saline using ICP, Raphael and colleagues also concluded that an oxide passivation layer surrounds the released NWs.

Previous cytotoxicity studies of Ni NWs have only looked at effects occurring during <24 hours (Byrne et al. 2009). Here, cytotoxicity up to 72 hours is studied, and it is found to be increased even for the lower concentrations tested. Similarly, it has been hypothesized that the NW passivation oxide layer would translate into a high cell survival rate (Prina-Mello et al. 2006). In this work, it is shown that despite the oxide layer the Ni NWs are cytotoxic and those that are internalized by the cells are still being dissolved to some degree.

Though the specific cell death mechanisms activated by Ni NWs remain unknown, studies have reported the generation of reactive oxygen species and cell cycle arrest (Ma et al. 2014; Hossain et al. 2011). In the case of Ni in its ionic form, reactive oxygen species generation has also been reported, as well as damage to the DNA and inhibition of DNA-repair enzymes (Shi et al. 2004; Kasprzak et al. 2003). The cell death observed in this work for Ni NWs and Ni ions could very well be a result of these mechanisms, with a possible cytotoxic response scenario being as follows: after cellular uptake through phagocytosis, the presence of Ni NWs and the release of Ni²⁺ induces cell oxidative stress, compromising cell viability and inducing apoptosis, which some time later would translate into cellular membrane instability and secondary necrosis.

It should be mentioned that the overall cytotoxicity reported in this work has a limitation in the form of NW aggregation. While sonication may provide a short-term dispersion in solution, aggregates are still present. It is possible that such aggregates may exert a local acute cytotoxicity (Okuda-Shimazaki et al. 2010; Gao et al. 2010), but

at the same time having a reduced area of effect on the cell culture. Moreover, the cells may also be forming aggregates as they internalize the NWs. Due to all these factors, the characterization of cells-aggregates remains a challenging task.

While this study sheds light on certain aspects of magnetic NW toxicity, there is still a need to broaden the understanding of the biological effects NWs have as biomaterials. Assessing the cytotoxicity of shorter NWs would help to better understand their toxicological properties. Fluorescence microscopy techniques could be utilized to track the NWs over time inside the cell and find out their ultimate fate. The influence of specific surface coatings, providing more functionality to the NWs, as well as a better dispersion, should also be addressed. Finally, information on the cytotoxicity of Ni NWs on non-cancerous cell lines such as fibroblasts or stem cells would be of high value.

Conclusions

We evaluated the cytotoxicity of Ni NWs at increasing incubation times and NW to cell ratios on a model cell line. In contrast to previous studies, we examined Ni NWs toxicity over exposure times longer than 24 hours, observing a significant increase in cytotoxicity on these timescales. Internalized through phagocytosis, Ni NWs induce cell death through a combination of apoptosis and necrosis, with the latter possibly being explained through the secondary necrosis pathway as an *in vitro* outcome of apoptosis. Additionally, we found an increased cytotoxicity of NWs when compared to the same amount of soluble Ni, as well as a slow dissolution rate of Ni NWs in the cell medium, inside the cell population and in SBF solution. These results show that the cytotoxic response is due to a synergistic effect of NW aspect ratio and Ni²⁺ release, and high cytotoxicity levels are observed for doses of 200:1 and 1000:1 NWs per cell for long incubation times (>24 hours).

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure captions

Figure 1. Morphology, composition analysis and magnetic properties of Ni NWs. (A) and (B) TEM images of a single NW, with the arrow showing the oxide layer; (C) and (D) EDX spectra of the NW core and surrounding layer, respectively. Insets show corresponding STEM image. (E) Magnetization curve of Ni NWs embedded in the alumina membrane. The applied magnetic field was parallel to the length of the NWs.

Figure 2. SEM characterization and aggregation of Ni NWs. (A) SEM images of the nanoporous alumina template before NW release and of Ni NWs after the release process. The NW diameter and length distribution are also shown. Data represent mean $\pm$ SD of over one hundred individually counted samples. Figure adapted from (Perez et al. 2013); (B) The left panel shows Ni NW aggregates characterization in McCoy's medium, as per confocal microscopy. Size distribution was obtained from over 1000 samples of NWs. The right panel shows the distribution of the number of instances single NWs or NW aggregates were observed using TEM images of HCT 116 cells incubated with Ni NWs.

Figure 3. Ni NWs cytotoxicity on HCT 116 cells. (A) MTT assay of HCT 116 cells incubated with Ni NWs. Figure adapted from (Perez et al. 2013); (B) MTT assay for Ni sulfate, with the concentrations equal in Ni mass to those tested for the NWs. NC = negative control. Data represent mean $\pm$ range, $n = 3$, $*p < 0.01$; $^ap < 0.05$ vs. control.

Figure 4. Effects of Ni NWs on cell membrane permeability. (A) LDH leakage of HCT 116 incubated with Ni NWs; (B) TEER values of HCT 116 cells after incubation with Ni NWs. NC = negative control. Data represent mean $\pm$ range, $n = 3$, $*p < 0.01$ vs. control; $^ap < 0.05$ vs. control.

Figure 5. Internalization of Ni NWs by HCT 116 cells. (A) 24 hours time-lapse microscopy study with pHrodo™ Red-labeled Ni NWs (200:1 NW to cell ratio). Cell nuclei are stained with Hoechst 33342. A single NW aggregate is pointed at by white arrows as it is broken apart by cells into smaller aggregates. (Scale bars: 100 µm); (B) Confocal microscopy Z-stack performed under the same staining conditions. The black arrow shows a non-fluorescent NW, while the white one denotes an internalized, fluorescent NW. (Scale bars: 10 µm); (C) and (D) TEM images of the localization of internalized Ni NWs after incubation for 24 and 72 hours, respectively. Black arrows mark NWs or NW aggregates; (E) MTT assay of Ni NWs with different endocytosis inhibitors. NC = negative control; Ny = nystatin; Ch = chlorpromazine; LatA = latrunculin A. Data represent mean ± range, $n = 3$, $*p < 0.01$.

Figure 6. Apoptosis and necrosis induced by Ni NWs on HCT116 cells. Plots show the Alexa Fluor® 488 and PI fluorescence intensities of healthy (lower left quadrant), early apoptotic (lower right quadrant), late apoptotic/necrotic (upper right quadrant) and necrotic (upper left quadrant) cell populations under three different NW to cell ratios (100:1, 200:1 and 1000:1) for incubation times of 24, 48 and 72 hours. NC = negative control cells. Numbers in quadrants indicate percentage of total cells and data represent mean $\pm$ SD, $n = 2$, * $p < 0.01$; ^a $p < 0.05$.

Figure 7. ICP analysis of Ni NWs dissolution. (A) Ni NWs at 200:1 NW to cell ratio. 1, 2 and 3 denote different conditions tested with and without NWs. Group 1: bare cell medium; group 2: cell medium from grown HCT 116 cells; group 3: lysed HCT 116 cell population in cell medium for quantification of Ni inside the cells; (B) Ni inside HCT 116 cells at different incubation times with Ni sulfate (equivalences to Ni NWs doses shown in brackets); (C) Mole atoms of Ni inside the HCT 116 cell population, as observed in (A) and (B); (D) Dissolution of Ni NWs in SBF solution. The graph shows the percentage of the total mass of Ni NWs that was dissolved in SBF pH 7.4 and pH 4.8, as well as a control SBF solution without NWs. Data represent mean $\pm$ range, $n = 3$, $*p < 0.01$.