Cytotoxic Effects of Nickel Nanowires in Human Fibroblasts

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ABSTRACT

Cytotoxic Effects of Nickel Nanowires on Human Fibroblasts

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There is an increasing interest for the use of nanostructures as potential tools in areas that include biology and medicine, for applications spanning from cell separation to treatments of diseases. Magnetic nanoparticles (MNPs) have been the most widely studied and utilized nanostructures in biomedical applications. Despite their popularity, the regular shape of MNPs limits their potential for certain applications. Studies have shown that magnetic nanowires (MNWs), due to their high-aspect ratio and specific magnetic properties, might provide improved performance for some biomedical applications. As a consequence, MNWs have received increasing attention from researchers in the last years. However, as with any other nanostructure intended for biomedical applications, rigorous studies must be carried out to determine their potential toxicity and adverse effects before they can be successfully incorporated in clinical applications. This work attempts to elucidate the cytotoxic effects of nickel NWs (Ni NWs) in human fibroblasts by measuring cell viability under different parameters.

Ni NWs of three different lengths (0.86 ± 0.02 μm, 1.1 ± 0.1 μm and 6.1 ± 0.6 μm) were fabricated by electrodeposition using porous aluminum oxide (PAO) membranes as templates. Energy dispersive X-Ray analysis (EDAX) and X-Ray diffraction (XRD) were used for the chemical characterization of the Ni NWs. Their physical characterization
was done using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) imaging. MTT assays were performed to assess cell viability of human fibroblasts in the presence of Ni NWs.

NW length, NW/cell ratio and exposure time were changed throughout the experiments to elucidate their effects on cell viability. The results showed that NWs length has a strong effect on internalization and cytotoxicity. Smaller NWs showed higher toxicity levels at earlier times while longer NWs had stronger effects on cell viability at later times. NW/cell ratio did not seem to have a very strong effect at low concentrations. However, at high concentration (1000 NW/cell) significant loss of cell viability was observed, with the effects becoming stronger at later times. Other factors such as cell surface area, presence of oxide layer on NWs, and the cytotoxicity of Ni salts, were also studied and found to affect cell viability.

For our knowledge, this is the first systematic study done in human fibroblasts wi-38 using ferromagnetic NWs; where the toxic effects of equivalent amounts of Ni in its salt and in its NW form are compared. It is also the first study to provide insights of the interaction between wi-38 cells and Ni NWs. The results of this study complement and enrich previous cytotoxicity studies of Ni NWs. This work aims at providing a more comprehensive understanding of the interaction between NWs and biological systems. Despite the advancements, further studies will be required to fully understand the factors affecting NW cytotoxicity. Only when we understand the underlying mechanisms, will we be able to design suitable nanostructures for biomedical applications.
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LIST OF ABBREVIATIONS

Al  Aluminum
AC  Alternate current
Au  Gold
Cu  Copper
DAPI 4',6-diamidino-2-phenylindole
DC  Direct current
DI  Deionized
DMSO  Dimethyl sulfoxide
DMEM  Dulbecco’s Modified Eagle Medium
EDAX  Energy dispersive X-ray analysis
EMEM  Eagle’s Minimum Essential Medium
FBS  Fetal bovine serum
MEM NEAA  Non-essential aminoacids solution
MNPs  Magnetic nanoparticles
MNWs  Magnetic nanowires
MRI  Magnetic resonance imaging
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
NPs  Nanoparticles
Ni NWs  Nickel Nanowires
NWs  Nanowires
OD  Optical density
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>PAO</td>
<td>Porous aluminum oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<tr>
<td>Pt</td>
<td>Platinum</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>Si</td>
<td>Silicon</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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CHAPTER 1

Introduction

1.1 Biomedical applications of magnetic nanostructures

Nanostructures include any structure with at least one dimension in the range of 1 to 100 nm [1, 2]. Most of the nanostructures used nowadays are spherical, made from a magnetic material and with a coating that allows functionalization [3]. However, other geometries have been explored, such as nanotubes, nanowires (NWs), dots and capsules [4] (Fig. 1).

At the nano scale, materials show unique physical, chemical and biological properties that are size dependent [5]. Advancements in the fabrication and characterization techniques as well as the development of new functionalization methods have increased the interest in nanostructures for different areas.

Over the last decade, the field of nanoparticles (NPs) for biomedical applications has drawn increasing attention [6], especially because their small size allows them to penetrate and interact with the whole cell and its structures such as receptors, proteins, biomolecules, and cell membrane. For this reason, such structures could be employed as tools to tackle challenges in biology and medicine. Nanostructures have the potential to provide treatment for certain diseases that were difficult to target due to size restrictions, to improve our understanding of molecular biology and as tools for a wide range of medical applications [7].
While their effects in humans are still being studied, there are examples of nanostructures being successfully used in a wide range of biomedical applications. Depending on particle size, composition, structure and physico-chemical properties, NPs could lead to the development of new tools for fields that include magnetic resonance imaging (MRI), hyperthermia therapies, drug-delivery [5], cancer and gene therapies [8-10], tumor detection [8], cell guidance [11], magnetic separation [12-14] and biosensing [3, 15, 16]. Their advantages, together with the advances and availability of the required technology to fabricate them have facilitated the exploration of the use of nanostructures in biomedical applications.

Iron oxide nanostructures have captured the attention of researchers due to their magnetic properties, their ability to be functionalized and their apparent biocompatibility and non-toxicity, making them good candidates for in vivo experiments [5].
Figure 1. TEM images of (a) spherical, (b) cubic, (c) octopod-cubic, (d) star, (e) rod and (f) bilobar Fe–Pt NPs [7].

1.1.1 Magnetic Nanoparticles (MNPs)

NPs have been the most widely used and studied nanostructures for potential biomedical applications to date. Depending upon the fabrication method, NPs can be engineered with distinctive composition, size, shape, and surface chemistry, characteristics that are desirable for a wide range of biological applications [17] (Fig. 2). Noteworthy types of NPs include quantum dots as well as metallic and polymeric/liposomal NPs, all of which have found their application in drug delivery [18] or as contrast agents in medical imaging [17]. While in many cases the use of NPs is still at the research phase, there are some examples where they have been successfully incorporated for clinical applications [18].
Figure 2. Comparison of sizes of various nanoparticles [4]. Chemical composition and size range are defining features for nanoparticles. Both characteristics are important determinants of nanoparticle in vivo behavior and clearance properties [4].

NPs have many promising attributes for targeted imaging. First, NPs can deliver a large number of imaging agents at a time due to their surface area, allowing for improvement in sensitivity. Second, NPs may passively target tissues in vivo via the enhanced permeability and retention effect or be targeted to accumulate at sites where the molecular target is expressed, increasing the local concentration of contrast agents [19]. The high capacity for NP modification enables their use as amplifiers for in vivo imaging [20]. Finally, they can deliver several different types of imaging agents to perform
multimodality imaging.

For biomedical applications, the implementation of magnetic properties in NPs is of interest as it may lead to the development of new therapies and diagnosis tools [5]. For this purpose, it is desirable to fabricate superparamagnetic NPs with high saturation magnetization and with surfaces that can be functionalized. This will translate into NPs that can be remotely controlled using magnets and that selectively interact with other structures.

Superparamagnetic NPs exhibit magnetic properties only when subjected to external magnetic fields [4], this allows their manipulation using external magnetic field gradients and prevents agglomeration caused by magnetic forces [21]. MNPs have been studied as potential markers for magnetic resonance imaging. As an example, due to their specific uptake by the monocyte-macrophage system, they have been considered as potential tools to diagnose disorders related to high macrophage activity using MRI [4].

The surface of NPs used in biomedical applications is usually functionalized for reasons that include: preservation of physico-chemical properties, to provide specificity (ligands can be attached to the surface) or to carry certain chemicals, such as drugs, to a specific region [5]. Different kinds of surface functionalization have been developed using coatings and biomolecules, including polymers, viruses, antibodies and aptamers [22].

Iron oxide NPs have been the most widely studied and utilized NPs for biomedical
applications [13, 23]. Their magnetic properties and biocompatibility make them good candidates for hyperthermia treatments. Magnetic hyperthermia is a cancer treatment in which MNPs exposed to an external alternating magnetic field transform electromagnetic energy into heat. The transferred heat increases the temperature of the area where the MNPs and the cancer cells are, effectively destroying them [5]. The technique might sound simple but in reality the MNPs must fulfill a list of requirements that include: superparamagnetic behavior (zero magnetization when no external field is applied), to prevent agglomeration, and high saturation magnetization, to ensure efficient heating of the MNPs. The latter is related to the particle size, the bigger the particle, the higher the saturation magnetization. However, if the particles are too big, they become ferromagnetic which is undesired due to agglomeration concerns. A balance should be found between size and magnetic properties. If the MNPs are too small, their hyperthermia effects are negligible, while too big might prevent them from crossing physical barriers such as the endothelial barrier through the continuous capillaries [5]. The success of MNPs in biomedical applications will depend on the ability to direct them to the area of interest.

While previous studies show that some NPs are stable in vivo [5], several NPs clearance mechanisms are known to exist in the human immune system. As an example, neutrophils, monocytes and macrophages form extracellular traps that act as physical barriers for NPs [17, 24].

The factors affecting NPs clearance include material, size, shape, surface charge and
chemistry [1, 17]. To date the most efficient way to prevent clearance of NPs is by coating their surface with uncharged hydrophilic polymers such as polyethylene glycol (PEG) [17, 25, 26].

The materials suitable to fabricate NPs for biomedical applications have to fulfill a long list of requirements that include: magnetic properties (superparamagnetic behavior and high saturation magnetization are desired), biocompatibility, non-toxicity [5]. The most used magnetic materials include iron, cobalt, nickel and silver [27]. There have been several studies, especially of gold and silver NPs, regarding their cell surface distribution and cytotoxicity, but their effects inside the cell are still unclear [28, 29].

An effort has been made to develop coatings that reduce toxicity while preserving physical properties, such as magnetization [4]. Despite the promising achievements a lot of work still needs to be done to fully understand the cell-NPs interaction mechanisms and the associated risks. Only then will it be possible to incorporate NPs into clinical practice [4].

1.1.2 Magnetic Nanowires (MNWs)

Despite all the advantages that NPs represent for biomedical applications, there are some limitations due to their regular shape and size, especially when multiple functional groups are required [22]. This limits the use of MNPs as tools for medicines or biomolecules. NWs are cylindrical, high-aspect ratio nanostructures that might provide improved performance for some biomedical applications. Similarly to NPs, their size,
composition and surface chemistry can be tuned and controlled. Their radius can be tuned in the range of 1.5 nm to 500 nm with lengths reaching up to 100 µm [30, 31]. By adjusting the radius and the length, high aspect ratios are achievable. The composition along the wire can be precisely modulated, allowing for multiple domains with different properties [32]. Single or multiple functionalizations are possible using ligands that bind to the surface of the NWs and allow control over their interactions. In addition, magnetic NWs have higher magnetic moment per unit of volume than MNPs, allowing them to exert large forces and torques [22, 33]. Their large and tunable magnetic moments allow the manipulation of bound cells and molecules.

Two main approaches have been used for NWs fabrication: top-down and bottom-up. The top-down approach includes high-resolution or alternative lithographic techniques that can be used to fabricate simple nanostructures in large quantities; while the bottom-up approach includes self-assembly techniques, vapor-liquid-solid synthesis, and template-assisted methods [34]. Due to its cost-effectiveness, simplicity and the possibility to produce high quality NWs with a precisely controllable aspect ratio, the template-assisted method is the most widely used and it will be further discussed in Chapter 2.

When used for biomedical applications, the diameter of the MNWs is commonly below 100 nm, with lengths ranging from 1 to 10 µm [35]. MNWs can be fabricated using different materials such as semiconductors, superconductors, semimetals and metals [8]. Similarly to other nanostructures intended for biomedical applications, the materials
used for fabrication should fulfill a list of requirements related to biocompatibility and toxicity.

It has been demonstrated that NWs provide many advantages over NPs in terms of separation speed in continuous flow, applying force to living cells, controlling the spatial organization of cells, transporting cells and inducing hyperthermia in cells [12]. Two comprehensive studies compared the performance of nickel nanowires (Ni NWs) and ferromagnetic/superparamagnetic beads for cell separation; these results showed improved performance of Ni NWs over MNPs due to their higher magnetic moment [12, 36]. In addition, their saturated magnetization is over an order of magnitude higher than that of magnetic beads [12, 13].

Recent studies show that silicon (Si) NWs and polycaprolactone NW surfaces support adhesion and proliferation of the cells that have elongated morphologies [37, 38], making them suitable for applications such as tissue engineering and bone regeneration. Further studies have demonstrated that Si NWs can bind large proteins and be internalized by mammalian cells, showing toxic effects only at concentrations above 100 μg/ml [39]. In addition, some studies have shown that silica NWs are potentially a better option for drug and biomolecule delivery when compared to MNPs [39].

Besides drug and biomolecule delivery, NWs could be used for magnetic biosensing. J. Felton and his colleagues [40] demonstrated that the presence and the orientation of a
single ferromagnetic Ni NW can be detectable in the absence of a large external magnetic field, in contrast with superparamagnetic beads.

In recent studies, Reich and his team showed that Ni NWs can be internalized by immortalized fibroblasts [22]. Moreover, Prina-Mello et al. proved that Ni NWs can be internalized by rat marrow stroma cells, MC3T3-E1 osteoblast cells and UMR-106 osteosarcoma cells [41]. Cell viability was reported as more than 95% after 5 days of internalization [41]. Other studies also confirm that cells can internalize NWs of different lengths and materials [22, 42, 43]. These results have opened the possibility of using Ni NWs for biomedical applications.

The available cytotoxicity studies of NWs have shown that depending upon factors like material and size, NWs might produce very low toxicity and good biocompatibility in some cell lines [9, 11, 22, 44], while being highly toxic for other cell lines [45].

Ni NWs of 4.4 μm in length have been proven to be successfully internalized by mouse fibroblasts and induce cell death when a weak external magnetic field is applied, while keeping high cell viability in the absence of the field [44]. In addition, Ni NWs have shown their effectiveness as inducers of cell death in carcinogenic cells, making them promising tools for cancer therapies. Other studies with fibroblasts include cell guidance using 40 μm long Ni NWs [11], cytotoxicity effects of three different Ni NWs lengths (1.5 μm, 3.8 μm and 6.7 μm) [9] and cell death by magnetic field using 20 μm long Ni NWs [46].
A promising application is in early diagnosis of tumors by nanodevices based on NWs that can detect cancer-related molecules [8]. NWs coated with a probe such as an antibody or oligonucleotide could be laid down across a microfluidic channel that allows cells or particles flow through; proteins produced by cancer cells would bind to the antibody changing the NW’s electrical conductance measured by the device and thus detecting the presence of cancer cells [8].

1.2 Importance of cytotoxicity studies: their contribution to the understanding of NW internalization and toxicity

As mentioned before, NWs are structures that have proved their potential for a wide range of biomedical applications; however, there is poor understanding of how they interact with the cells and what kind of adverse effects they could cause. Before any nanostructure can be incorporated into clinical applications, its suitability must be supported by studies of their potential toxicity [39]. For these reasons, studies of cytotoxicity in different cell lines have gained importance lately.

Studies in vitro have shown that factors related to the nanostructure such as chemical composition, shape, surface charge, solubility and functional groups affect the way nanostructures interact with cells and tissues [39], interactions that can yield toxic effects on cells [47, 48]. The goal of cytotoxicity studies is to define the relationship between the physicochemical properties (e.g. size, shape, surface modification, concentration, and aggregation) of nanostructures and their toxic effects on cells [47]...
that might include oxidative stress and inflammatory responses leading to cell death [39]. Information about the adverse interactions of nanostructures with cells and tissues would enable one to design suitable nanostructures for biomedical applications.

Cytotoxicity studies provide vital information about the cellular mechanisms involved in nanostructure internalization and toxicity. Nanostructures interact with the cell membrane, triggering their internalization mainly through endocytosis by enclosing them in membrane vesicles (e.g., late endosomes or lysosomes) to be degraded, recycled back to the extracellular environment, transported across cells, or reach other organelles such as nuclei and mitochondria [47, 49]. Nanostructures can also enter the cells by applying physical stress on the plasma membrane and disrupting it, leading to intracellular leakage and cell death [47].

NWs are internalized by endocytosis, more specifically by receptor-mediated endocytosis, and after 24 hours of incubation most of them are located in the cytosol, with few (14 ± 5%) located in the late endosomal/lysosomal compartments [50]. Nevertheless, some of the studies have shown that the surface charge and the length of the nanostructures have a strong influence on the endocytosis effectiveness by the activation of membrane receptors specific for a cellular uptake pathway [28, 51]. One of these studies suggest macropinocytosis and clathrin-/caveolae mediated endocytosis as the main mechanism for the uptake of positively charged gold NPs, and a clathrin- and/or caveolae-mediated endocytosis for the uptake of negatively charged PEG-coated gold NPs [52]. As well, surface charge seems to influence the amount of nanostructures
uptake by cells. Nonphagocytic cells have shown a preference for cationic NPs while phagocytic cells take up more efficiently anionic NPs [25, 26]. Both works demonstrate the uptake of negatively charged NPs despite their unfavorable interaction with the negatively charged cell membrane. Ted K. et al. [53] compare various research work that demonstrated the influence of polystyrene NPs (24 nm-15 μm) size in the efficiency and mechanism of uptake; it was concluded that generally, smaller NPs are internalized more efficiently than longer ones with similar surface characteristics.

As mentioned before, nanostructure toxicity inside the cells can be driven by their physicochemical properties, such as retention time inside the cell, surface properties, and toxic metabolites. The adverse effects include morphological and structural changes, genotoxicity, and biochemical alterations that trigger different cellular responses such as cell-cycle and proliferation irregularities, diminution in mitochondrial function, activation of cell signaling pathways and cell death [47].

Once inside the cell, the NWs are probably broken up into smaller aggregates due to the internal pH of the late endosomal/lysosomal compartments (6.0–4.8 and approximately 4.5, respectively) to be further degraded [47]. One study [54] revealed that cells were able to split Fe NWs in smaller aggregates that were later degraded; the NWs and the remainders of the degradation were found either in vesicular compartments or directly dispersed in the cytosol. The resulting ionic forms due to NW degradation are able to interact with and alter the intracellular environment [44].
Other studies suggest that factors such as the oxide layer on NWs formed during their release from the template, their surface charge and the release of ions from the NWs have an impact on their toxicity. Fung O. and his colleagues [44] grew 3T3 fibroblasts in the presence of approximately 4 μm long Ni NWs; cell viability was measured after 12 hours of incubation. The results showed low cytotoxicity and low inflammatory response of the cells exposed. This was explained by the interaction of serum proteins with an oxide layer formed on the surface of the NWs. The layer seems to further buffer the Ni from proinflammatory interactions with the intracellular environment [44].

A recent contribution is the research work done by Persson et. al [35], where low motility and low proliferation rate of fibroblasts cultured on gallium phosphide NW substrate was reported. While, others have been reported that the cytotoxicity of NWs is mainly driven by the DNA damage due to reactive oxygen species (ROS) formation [9].

Another study analyzed the cytotoxicity of Tellurium NWs in BALB/3T3 fibroblast cells [55]. The results showed high cytotoxicity of the NWs in a dose-dependent manner and a significant amount of ROS in the cells; necrosis was the basic pattern of cell death. Additionally, it was found that the components of the Dulbecco’s Modified Eagle Medium (DMEM) significantly affect the chemical and structural stability of the NWs, resulting in agglomeration.

All the above mentioned studies highlight the importance of cytotoxicity studies of NWs and the understanding of their toxicity mechanisms before focusing on the study of
possible biomedical applications. Despite the research work done so far, there is a lot of further work that has to be done before completely understanding the NW-cell interaction.

Studies have shown that the toxicity of a material in a certain form (e.g. NW) cannot be inferred from the toxicity of the same material in a different shape (e.g. NP) [22]. A good example is asbestos, a “benign” silicate that is highly toxic in its fibrous form [22]. Therefore, cytotoxicity studies must be carried out for specific materials in specific geometries and for specific cell lines. This work aims at investigating the cytotoxicity effects of Ni NWs in human fibroblast.

1.3 Thesis motivation

Even when the use of Ni NWs has been explored for a wide range of biomedical applications, there is a lack of rigorous studies about their potential toxicity. In addition, previous studies have used cancer cells as models, while not many have tested the effects in non-cancer cells, such as human fibroblasts. This work aims at contributing to the understanding of the factors that influence loss of cell viability when cultured human fibroblasts are exposed to Ni NWs.

Previous cytotoxicity studies of Ni NWs provide limited information because they have focused on short exposure times, 24 hours or less, and have used limited range of NW/cell ratios. Therefore, there is a need for studies that evaluate Ni NWs adverse
effects in different cell lines, at times longer than 24 hours, using a broader range of NW/cell ratios and using NWs with different lengths.

For the reasons above mentioned, this work attempts to study the adverse effects and changes on human fibroblasts cell viability when grown in the presence of short and long Ni NWs, at different NW/cell concentrations and at incubation times that include time points longer than 24 hours.

To do so, cell viability is determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay, a well-known method that assesses metabolic activity of the cells at the mitochondrial level. This, coupled with the different parameters tested such as NW length and concentration, provide insights about the possible mechanisms controlling cytotoxicity of Ni NWs.

My work aims to complement and enrich previous work done by Jose Perez, one of my colleagues at the laboratory, where cell viability of Ni NWs on colon cancer cells was studied using the same parameters [50]. His research work was the first one to show the cytotoxic effects of Ni NWs on colon cancer cells (HCT116) using different aspect ratios, at different NW / cell concentrations and at incubation times longer than 24 hours. My work aims at providing similar information using human fibroblasts (wi-38) cells. Being able to compare the effects on different cell types will provide additional understanding of the mechanisms that control the Ni NW-cell interaction and their toxicity.

As previously mentioned, it is necessary to evaluate the effects on specific materials
with specific geometries interacting with specific cells and tissues to fully understand their adverse effects. Only then will we be able to design suitable nanostructures for biomedical applications.
CHAPTER 2

Cytotoxicity studies

2.1 Basis of cytotoxicity studies

Cytotoxicity refers to the adverse effects that result from the interaction of nanostructures with the structures and/or processes that are crucial for cell maintenance (i.e. proliferation) [47]. There are two approaches to evaluate cytotoxicity: \textit{in vivo} and \textit{in vitro}. \textit{In vivo} experiments are better to observe the overall effects of a nanostructure on living organisms, however they are harder to execute and interpret, and legal approval is required before they can be carried out [50].

On the other hand, \textit{in vitro} studies are easier to perform and commonly used to reveal general mechanisms of toxicity and to predict \textit{in vivo} toxicity [47, 56]. In addition, they offer the possibility to produce results in a rapid and inexpensive manner; hence, this approach was chosen for this work.

Choosing the right cell culture model is very important for \textit{in vitro} studies. The tissue origins and the cell type (i.e. cancer or healthy) must be chosen according to the biological system one is interested in (i.e. respiratory, digestive), and the possible biomedical application of the nanostructure (i.e. cancer therapy, tissue engineering).

The majority of the cytotoxicity studies with NWs have been done on cancer cells, however, cytotoxicity studies on healthy cells are very important considering that NWs
are intended to be inside the human body where both cell types are present. In addition, there are some applications, such as tissue engineering ones, where NWs would interact primarily with healthy cells. For these reasons, a healthy cell line model was chosen for the present work; the next section discusses the cell line in detail.

Once the cell line model has been chosen, cell-doubling time and initial seeding density are two important factors that must be considered when designing the cytotoxicity studies. Cell doubling time refers to the time it takes to the cells to double their number, a parameter that varies across cell lines. Initial seeding density is the number of cells placed in a determined area at time zero of an experiment or when culturing cells. Based on the cells doubling time, the initial seeding density is determined to assure optimal confluence of the cells and their proliferation during the whole experiment. The importance of these two variables is described in more detail in the next sections.

There are currently many different assays that can be used to determine cytotoxicity. The main difference between them is the cellular process they tackle for this purpose. This should be taken into account to choose the method that suits better our experimental needs. The MTT assay was the first homogeneous cell viability assay developed for a 96-well plate format that was suitable for high throughput screening [57]. It has been widely adopted and as of today it remains the most popular method within the research community to observe alterations in specific metabolic pathways linked to cell survival or cell proliferation [1, 2, 58].
Some other variables that need to be defined are related to the characteristics of the nanostructures of interest, in this case Ni NWs, such as morphology, aspect ratio and element composition. Finally, in terms of experimental design, one should define NWs concentration and exposure times to be evaluated. The importance of these parameters is further discussed in the following sections.

2.2 Cell culture and number estimation

The lack of information about the effects of NWs on healthy cells has been previously discussed. It was also discussed how important it is to choose the right cell model. Human fibroblasts cell line wi-38 (ATCC® CCL-75™) (Fig. 3) come from lung tissue and were chosen as cell line model because lungs are commonly exposed to nanomaterials [56]. Mouse and human fibroblasts have been used in previous NWs cytotoxicity studies [11, 33, 44, 59], human fibroblasts being the least studied. The results showed that NWs can be successfully internalized and interact with the cell membrane.

Following the vendor’s recommendations, the fibroblasts were cultured in Eagle's minimal essential medium (EMEM Quality Biological Inc) supplemented with L-Glutamine (Quality Biological Inc), Sodium Pyruvate (Gibco®), Non-essential aminoacids solution (MEM NEAA Gibco®), 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were incubated at 37 °C in a humidified incubator with 5% carbon dioxide (CO₂).
Figure 3. Human fibroblasts. Lung human fibroblasts (wi-38) attached to the bottom of a cell culture flask.

2.2.1 Cell culture and subculture

Cells were cultured in 75 cm² and 25 cm² flasks with EMEM medium and subcultured each two days, with a subculture ratio of 1:2. Cells were observed under the microscope and when the flask area was between 70-80% covered by fibroblasts, the medium was discarded and cells were washed twice with either 5 ml (75 cm² flask) or 2 ml (25 cm² flask) of Dulbecco’s phosphate-buffered saline (DPBS) calcium and magnesium free. The DPBS removes dead cells, debris and traces of serum that could interfere with the action of trypsin on the detachment of the cells from the bottom of the flask. It is important to avoid over confluence of wi-38 cells because the reduced space between the cells can easily induce cell growth inhibition.

Next, either 3ml (75 cm² flask) or 1 ml (25 cm² flask) of 0.05% trypsin was added to the flask and placed in the incubator for 3 minutes. The flask was gently tapped in order to fully detach the cells, followed by the addition of either 3ml (75 cm² flask) or 1 ml (25 cm² flask) of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.
cm² flask) of EMEM medium to the flask to inactivate the trypsin and avoid cell damage. The entire volume (6 ml or 2 ml) was collected in a Falcon tube; if required, a small sample (100 µl) was taken for cell counting. The falcon tube was centrifuged at 1200 rpm for 5 minutes.

After centrifugation, the supernatant was carefully discarded and replaced with either 6mL (75 cm² flask) or 2 ml (25 cm² flask) of DPBS. The pellet was aspirated and dissolved in the DPBS by pipetting; this process was repeated one more time.

Finally, DPBS was discarded and fresh medium (EMEM) was added to the Falcon tube to obtain the desired dilutions: 1.5x10⁶ cells in a 75 cm² flask with a final volume of 21 ml and 0.5x10⁶ cells in a 25 cm² with a final volume of 7 ml.

2.2.2 Cell counting

The hemocytometer (Hausser Scientific) is a counting chamber with a very specific depth (0.1 mm) that consists of a big square divided into nine smaller squares, each one with a surface area of 1 mm² (Fig. 4 A) and was used to determine cell density. On the other hand, trypan blue stain was used to identify dead cells when counting. This technique is based on the fact that live cells are not colored because the dye cannot pass through their selective membrane; in contrast, the dye can traverse the membrane of a dead cell and stain it. As a result, dead cells look blue under the microscope. Both are well-established techniques that have been successfully used in previous studies [47].
To perform cell counting, a 100 μl sample was taken from the Falcon tube containing the cell suspension and placed in an Eppendorf tube. From the Eppendorf tube, 10 μl were taken and mixed thoroughly with 10 μl of trypan blue. Once mixed, the suspension was placed in the hemocytometer chamber using a pipette. The number of cells in each of the nine squares was counted (Fig. 4 A). As each of these squares has a volume of 0.1 mm³, which is equivalent to 0.0001 ml, the cell concentration per ml was determined as follows:

\[ \text{No. of cells} = \left( \frac{\text{Viable cells counted}}{\text{Squares counted}} \right) \left( \frac{1}{0.0001 \text{ml}} \right) (\text{Dilution factor})(V_T) \]  

(1)

The number of cells in each square was counted and averaged. This average
represented the number of cells per 0.0001 ml. After that, it was multiplied by a dilution factor of two and by the total volume ($V_T$) of cell suspension in the Falcon tube from which the 100 μl sample came.

### 2.2.3 Growth curve of wi-38 cell line

The fibroblasts growth curve was calculated to determine the doubling time, saturation density and lag time. Typically, three different phases can be distinguished in a growth curve (Fig. 5):

- **Lag phase**: cells spread and grow slowly because the cell density is very low compared to the area of the flask.
- **Log phase**: cell number increases exponentially.
- **Plateau phase**: cells become overconfluent; contact inhibition slows or stops cell growth.

The doubling time is important for cytotoxicity studies because it allows to estimate the number of cells at the moment of the treatment with Ni NWs. A small number of cells may not be detectable by the cytotoxicity assays and too many cells might quickly lead to overconfluence and cell contact inhibition, resulting in signals that are out of the measuring range. The doubling time for wi-38 was calculated from the growth curve (Fig. 5) and the process to calculate it is explained below.

The cells in the 75 cm$^2$ flask were counted and split in five 25 cm$^2$ flasks, each one with an initial density of $0.46 \times 10^6$ cells, and were later placed in the incubator. Every 24 hours
the cells in one of the flasks were detached and counted using the methods previously described. These values were used to construct a growth curve (Fig. 5).

![Cell growth curve](image)

**Figure 5.** Cell growth curve of wi-38 fibroblasts. In the lag phase the cells are slowly growing. Later the cells start to grow in an exponential manner, log phase. Finally the cells enter the stationary phase where they stop growing (cell inhibition).

The data points that correspond to the exponential (log) phase were used to calculate the doubling time using the Doubling Time software [60]. The growth rate in this phase is directly proportional to the number of cells present at that time point. Therefore, the doubling time can be calculated using the following equation:

\[ N(t) = N_0 e^{rt}, \quad r = 0.032 \]  

(2)

In Equation (2) \( N \) is the number of cells at time \( t \), \( N_0 \) is the initial cell seeding density, \( r \) is the growth rate and \( t \) is the time. In order to calculate the doubling time, the equation
above must be solved for \(\frac{N}{N_0} = 2\)

\[
\ln \left( \frac{N}{N_0} \right) = \ln(e^{rt}) \rightarrow \ln(2) = \ln(e^{rt}) \rightarrow \ln(2) = rt \rightarrow t = 22 \text{ h},
\]

By solving the equation, it was determined that the doubling time was 22 hours.

### 2.3 Optimization of the MTT assay for wi-38 cells: standard curve and incubation time

The MTT assay (Vybrant®) is one of the most used methods for cytotoxicity studies because it is fast, convenient, reliable and provides quantitative results [24, 28, 59, 61, 62]. For those reasons, it was chosen as the method to determine and evaluate NWs toxicity on wi-38 cells.

The MTT assay is a colorimetric assay based on the principle that metabolically active cells are able to reduce MTT tetrazolium salt, a yellow water-soluble salt, to formazan, an aqueous purple insoluble product. The reduction process is carried out mainly at the mitochondrial level [63] by mitochondrial succinate dehydrogenase. The formazan is solubilized, and then the concentration is determined by optical density (OD); value that is used to measure the cell viability.

The cellular response to the MTT dye varies for each cell type and for different cell concentrations [50]. Therefore an MTT standard curve for the cell line is required to ensure that the parameters used in the experiment are within the range where small changes in cell density produce big changes in absorbance, assuring accurate viable cell
number counts (Fig. 6). To build the MTT standard curve for wi-38 cells, different cell concentrations were seeded in a 96 well-plate in 100 μl of EMEM medium, in triplicates. After 24 hours, when the cells attached to the bottom, the MTT assay was performed following the protocol described in section 2.4.4.

To generate the MTT standard curve, absorbance was measured (Section 2.4.4), using the absorbance reader xMark™ microplate absorbance spectrophotometer (BIO-RAD), at different time points. From the curve (Fig. 6) it is clear that the optimal cell seeding density was between $0.5 \times 10^4$ and $1.5 \times 10^4$ cells that correspond to the part of the curve where small changes in density translate into relatively big changes in absorbance, so that cell counting is accurate.

![Absorbance vs Cell Seed Density](image)

**Figure 6.** MTT standard curve for wi-38 cells. Each data represents the mean value of samples in triplicate.

The incubation time for an MTT assay varies depending on the cell line. Depending on their metabolic activity, different cell types reduce MTT at different rates. Therefore,
the optimal incubation time for wi-38 had to be determined before performing any experiment. Wi-38 cells where incubated and absorbance was measured at different incubation times (2, 3 and 4 hours) as an indicator proportional to the number of cells in the sample. The data shows (Fig. 7) that three hours is the optimal incubation period, resulting in values of absorbance two times higher compared to two hours of incubation and slightly higher than four hours incubation.

![Graph showing absorbance vs time of incubation]

**Figure 7.** Optimization of the incubation time for wi-38 cells. The experiment was seeded in triplicates. For the 2 hours incubation, the absorbance value was 0.32; as for next two incubation times was 0.67 and 0.6, respectively.

### 2.4 Methodologies

#### 2.4.1 Fabrication of Ni NWs

The fabrication of Ni NWs was done using a top-down approach where a highly ordered porous aluminum oxide (PAO) membrane was prepared by the two-step anodization
technique and used as a template; the pores were then filled with Ni using DC electrodeposition, resulting in NWs with a perpendicular orientation with respect to the membrane’s surface. The next sections explain the different steps of the fabrication process. For more details about the composition of the solutions and the protocols followed for the Ni NWs preparation please refer to Appendix A.

2.4.1.1. Surface preparation

In this work, high purity Aluminum (Al) disks (99.999% purchased from Good Fellow) were used as templates for NWs fabrication. The surface preparation of the Al disk is very important to ensure high quality electrodeposition, and to remove contaminants from the substrate surface, reducing its roughness. For the surface preparation process, the Al disk surface was washed with acetone, isopropanol and deionized (DI) water. After cleaning, the Al disk was subjected to electropolishing [64].

2.4.1.2 Porous alumina membrane by two-step anodization process

Anodization is the process in which a metal surface is converted into its oxidized form by applying current in an acidic electrolytic solution that provides oxygen ions that react with metal ions to form the oxide [65].

In this work a two-step anodization technique was used to obtain a highly ordered PAO template. This method was selected due to its simplicity and inexpensiveness [66-68]. Among the factors that can be controlled using this technique are pore, pore density,
and thickness of the PAO membrane by changing the anodization conditions such as anodization voltage, temperature and electrolytes [50, 67].

**First anodization**

The electropolished Al disk was anodized using a 0.3M oxalic acid solution as electrolyte, which is known to produce pores in the range of 35 - 50 nm in diameter [50]. The setup consisted of a perforated anodization cell where the Al disk was placed. The system was sealed with an O-ring and secured by a copper (Cu) plate, which acted as cathode. On the top of the anodization cell, a Pt mesh, which acted as anode, was placed together with a cell cap that has a Teflon propeller moved by a DC motor (Fig. 8). The propeller provides the mechanical stirring (~200 rpm) required to maintain homogeneity in the solution. The whole setup was placed in a cooled plate and the temperature was maintained within 2-5 °C.

![Figure 8. Schematic of the first anodization setup. The Al disk is placed in a perforated anodization cell and sealed with an O-ring and a copper (Cu) plate. The Cu plate acted as anode, while Pt mesh acted as cathode. A Teflon propeller moved the oxalic acid solution.](image-url)
The first anodization process was carried out under a constant voltage (40V) applied by a sourcemeter (Keithley 2400-C) for 24 hours [50]. The result was an oxide (alumina) layer on the Al surface with disordered pores (Fig. 9); the pore size and the thickness of the pore walls were not uniform and sub-holes were formed under the main holes, as reported in previous studies [24, 61, 69]. However, on the Al substrate, ordered domains were formed. These domains are very important to obtain an ordered nanoporous layer during the second anodization process. Finally, the oxalic acid solution was removed and the anodization cell was washed with DI water until no trace of the solution was left.

Figure 9. A) After first anodization, an Al₂O₃ layer is formed in the surface of the Al disk with random nanopores structure and well-ordered domains in the substrate (dark gray). B) SEM image [50] of the Al surface after first anodization.
Removal of the alumina layer

Before the second anodization, the alumina layer was chemically removed, resulting in highly ordered domains on the Al surface (Fig. 10). A chrome solution was added to the anodization cell, which was then placed on a hot plate (30°C) and covered with parafilm to prevent evaporation. After 12 hours the chrome solution was removed and the cell was thoroughly washed with DI water.

![Figure 10](image)

**Figure 10.** A) Removal of Al₂O₃ layer using chrome solution for 12 hours in order to reveal the ordered domains in the Al substrate, which will be the template to grow ordered pores during the second anodization step. B) SEM image [50] of the Al disk surface after removal of the Al₂O₃ layer.

Second anodization

The second anodization was carried out using the same setup and under the same conditions of the first anodization process. The anodization time was 20 hours, enough to grow an approximately 50μm thick alumina layer (Fig. 11). The layer was made
several times thicker than the desired length of the NWs to reduce the fragility of the sample, making the handling in further steps easier.

After 20 hours the oxalic acid was removed and both sides of the samples were thoroughly washed with DI water. The result was a membrane with highly ordered nanopores arrays and a narrow diameter distribution.

![Figure 11.](image)

**Figure 11.** A) During second anodization the Al₂O₃ layer grows following the ordered domains in the Al substrate resulting in highly ordered pores. B) SEM image [50] of the Al disk surface after second anodization.

### 2.4.1.3 Removal of the aluminum and pore opening

To prepare the sample for the electrodeposition process, the aluminum on the backside of the Al disk has to be removed, and the pores have to be opened. These two steps are crucial to establish good contact between the pore and the base of the substrate [70]. For removal of the alumina membrane a Cu solution was used. While a 5% phosphoric
acid solution was used to open the pores.

2.4.1.4 **Gold sputtering and direct current electrodeposition**

Before the electrodeposition of Ni on the sample, a gold (Au) layer (~200 nm) was sputter deposited on the backside of the membrane. The Au layer works as a conductive substrate that allows the pore filling with the metal material (Ni in this case).

Electrodeposition, also known as electroplating, is a process where a current is applied between the anode and the cathode, both immersed in electrolytic solution; resulting in the filling of the pores in the surface of the membrane with a different material (Ni in this case).

A similar cell arrangement to the one used for the two-steps anodization was implemented for DC electrodeposition (Fig. 12 A). As the ionic potential of the electrolyte (Ni solution) cannot be measured directly, the electrode of reference is providing a reference signal. For this purpose, a reference electrode with a voltage of -1 V, was submerged in Ni solution. The Ni solution consisted of 2.4% Ni (II) chloride hexahydrate (NiCl₂·6H₂O), 2.77% boric acid (H₃BO₄), 14.5% Ni (II) sulfate hexahydrate (NiSO₄·6H₂O) and 80.3% DI water.

For the electrodeposition process, -2.2 V were applied by a sourcemeter (Keithley 2400-C) between the Cu plate (cathode) and the Pt mesh (anode). The growth ratio of the NWs was ~5.5 nm/s. The time was changed to obtain the desired length.
Figure 12. A) Setup for electrodeposition of Ni NWs with a reference electrode emerged in the solution, very similar to the one used for anodization. The Al disk is placed above the O-ring. B) The sample after electrodeposition.

2.4.1.5 Release of the NWs

Before their use for in vitro studies, the NWs had to be released from the substrate and suspended in solution (Fig. 13). After DC electrodeposition, the gold layer on the backside of the sample was etched using Argon milling. Next, the deposition area of the Al disk was broken into several pieces with thin tweezers for further use.

Figure 13. A) Sample after electrodeposition with a conductive gold layer on the backside of the template. B) Etching of Au layer by ion beam milling process. C) Dissolving of Al₂O₃ by NaOH and chrome solution.
Before continuing the process, an image of this piece together with a scale bar was taken to later estimate the number of NWs in that area; such estimation is further discussed in 2.4.3. The Al disk piece was then put in an Eppendorf tube containing 1 ml of 1 M sodium hydroxide (NaOH) solution and left for 24 hours to selectively dissolve the alumina template. The NaOH solution was replaced with 1 ml of chrome solution consisting of 0.66% chromium (VI) oxide (CrO₂), 3.77% phosphoric acid (H₃PO₄) and 95.55% DI water; and left for 24 hours in a Thermomixer® comfort (Eppendorf) at 40°C and 300 rpm.

Next, the Eppendorf tube was put in a magnetic holder (Dynamag™-2) to collect the NWs, and then the chrome solution was discarded and replaced with ethanol. The NWs were resuspended and shaken for cleaning and disposal of traces of NaOH and aluminum membrane. This step was repeated three more times. In the case of short NWs the tube was sonicated, using an ultrasonic water bath for 30 seconds, every time the ethanol was replaced with fresh one. Long NWs were hand shaken to avoid breaking them into pieces by sonication [50]. Finally, the ethanol was changed one more time to remove traces of contaminants.

### 2.4.2 Characterization of Ni NWs

Samples for SEM, TEM and EDAX (Energy dispersive X-ray analysis) were prepared by taking 1.5 μL of the NWs suspended in ethanol and placing them on a small piece of silicon substrate / Cu- carbon mesh. The samples were allowed to dry before imaging. SEM imaging of the alumina template with deposited NWs was done using a small piece...
of the Al disk. To improve image quality, a thin layer of gold (~0.8 nm thick) was deposited on the front side of the piece using a sputtering machine. Cross-section SEM imaging was done using a small piece of the sample with gold sputtering in the backside, the gold side was attached to a piece of tape while the other side was in contact with the holder.

EDAX was used to verify the chemical composition of the Ni NWs. SEM imaging was used to visualize the filled pores, the alumina template and Ni NWs. TEM imaging was used to take closer look at the NWs to better characterize their morphology and to study the oxide layer formed on their surface. The images are discussed in detail in the results section.

The surface charge of each sample was determined by dynamic light scattering using a Zeta Nanosizer (Malvern). A concentrated suspension of NWs in EMEM medium was added to a disposable capillary cell to perform the measurement. The NWs concentration was adjusted to guarantee accuracy and high quality measurements.

2.4.3 Estimation of the number of nanowires

Determining the NWs concentration accurately was important for this study because the cytotoxicity of Ni NWs at different concentrations was evaluated. A previous study using the same fabrication process for Ni NWs confirmed that every pore had one NW deposited inside [50]. The number of pores corresponding to each sample was calculated for a defined area of the alumina template from SEM images using ImageJ. A picture of the sample was taken before release of the NWs and was used to calculate
the area of deposited NWs. The following Equation (3) was used to calculate the number of NWs [50]:

\[
\text{No. of NWs (Total area surface)} = \frac{\text{No. of pores (At template image)} \cdot \text{Total area surface}}{\text{Area (At template image)}}
\]  

(3)

2.4.4 MTT assay protocol

The following is a general description of the MTT protocol (Fig. 14) used in the experiments:

1. Cells were counted and diluted to obtain the desired seeding density in 100 \( \mu l \) of EMEM medium. Each condition and a negative control (cells without NWs) were seeded in triplicates in a clear bottom flat 96-well plate (Fisher Scientific) with 100 \( \mu l \) of EMEM medium. 100 \( \mu l \) of PBS were added towards the end to the wells surrounding the working wells to avoid medium evaporation. All plates were placed in the incubator for 24 hours to achieve proper attachment and cells density.

2. Next, the cells were treated with the desired NWs concentration in 20 \( \mu l \) of EMEM medium; 20 \( \mu l \) of pure medium were added to the negative control wells. All plates were placed again in the incubator for 24, 48 and 72 hours.

3. After incubation, the 120 \( \mu l \) of media was carefully discarded and the cells were washed twice with 100 \( \mu l \) of PBS. Later, 100 \( \mu l \) of 10% MTT solution (5 mg/ml in PBS) in medium was added.
4. After 3 hours of incubation the insoluble formazan was visible in the bottom of the wells. The 100 $\mu$l of MTT solution was carefully discarded and replaced by 100 $\mu$l of 80% dimethyl sulfoxide (DMSO) / 20% sodium dodecyl sulfate solution (SDS), which acted as a cell lysis buffer, breaking the cells and allowing the purple crystals to be released in the solution.

5. The 96-well plates were then shaken in a Multi-Microplate Genie (Scientific Industries) until a homogeneous color was observed, meaning that the crystals were completely dissolved.

6. The concentration of purple crystals was determined by OD measurements, using a xMark$^\text{TM}$ microplate absorbance spectrophotometer (BIO-RAD). A 570 nm wavelength and a reference wavelength of 630 nm were used. Each OD value was subtracted from the reference wavelength to eliminate the background signal.

7. Cell viability at each condition was determined according to Equation (4). The OD of each treated well was divided by the OD of the negative control (untreated cells). The data points were presented as a percentage of control cells and each one is an average of the respective triplicates.

$$Cell\ viability\ (%) = \frac{OD_{570-630}[\text{Condition}]}{OD_{570-630}[\text{Negative\ control}]} \cdot 100\% \quad (4)$$
Figure 14. Steps of MTT protocol. Each condition was seeded in triplicates.

2.4.5 Fixed cell staining protocol

To prepare the samples for fluorescence imaging, cells had to be stained and fixed. The nucleus of the fibroblasts was stained with 4',6-diamidino-2-phenylindole (DAPI) stain. The staining protocol followed was:

- Cells were washed with 300 μl of PBS.
- 300 μl of 3.7 % formaldehyde diluted in PBS was added and removed after 5 minutes of incubation at room temperature.
- Wells were washed twice with 300 μl of PBS.
- 300 μl from a 300 nM solution of DAPI was added to each well and removed after 8 minutes of incubation at room temperature.
- Finally, cells were washed three times with PBS.
Once the cells were fixed and stained, fluorescence was observed using an excitation and emission wavelength of 360 nm and 460 nm, respectively.

### 2.5 Experimental design of cytotoxicity assessments

In this section parameters such as NW/cell ratios, Ni NWs aspect ratios, exposure times, and initial seeding density are specified and the experimental setup used for the different assays is described.

#### 2.5.1 Nanowire samples

Ni NWs of three different lengths were used in this work. Their characterization is discussed in detail in Chapter 3. Sample A consisted of long NWs while sample B and C consisted of short NWs (Table 1) with the purpose of investigating the influence of NW length on their cytotoxicity. In order to determine the number of NWs that should be added to each well, the number of cells being treated was calculated using Equation (2) from section 2.2.3 for each experiment.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DC Electrodeposition Target charge (C)</th>
<th>Diameter (nm)</th>
<th>Length (μm)</th>
<th>Aspect ratio</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-2.5</td>
<td>29.5 ± 3.7</td>
<td>6 ± 0.6</td>
<td>200</td>
<td>-9.47</td>
</tr>
<tr>
<td>B</td>
<td>-0.5</td>
<td>31.5 ± 3.6</td>
<td>0.86 ± 0.02</td>
<td>27.7</td>
<td>-10.2</td>
</tr>
<tr>
<td>C</td>
<td>-0.5</td>
<td>31 ± 3</td>
<td>1.1 ± 0.1</td>
<td>35.4</td>
<td>-10.1</td>
</tr>
</tbody>
</table>

Table 1. Summary of characterization results and electrodeposition parameters of each sample of Ni NWs used for the different experiments. The length of the NW was controlled by the target charge. The zeta potential was negative for the three samples.
2.5.2 Setup for MTT assays

For all the experiments performed in 96-well plates, the initial seeding density was $0.67 \times 10^4$ cells in 100 μl of medium. This density is optimal for cell proliferation, yielding 60% confluence after 24 hours of incubation. Each experiment consisted of nine 96-well plates; three wells per time point (24, 48 and 72 hours) and each 96-well plate with triplicates for the negative control (untreated cells) and for the different NWs concentrations per cell (10:1, 100:1 and 1000:1) (Fig. 15).

![Setup of MTT experiments](image)

**Figure 15.** Setup of the MTT experiments. Each condition was seeded in triplicates. The negative control (NC) is cells with not NWs added. One representative plate for one incubation time and one NW aspect ratio is shown per assay.

2.5.3 Set up for Confocal Microscopy

The initial seeding density for confocal microscopy was $2 \times 10^4$ cells per well, such that the cells were approximately 60% confluent at treatment time and in a proliferation state during all the time point to reduce natural cell death due to overconfluence.
The cells were seeded in a Lab-Tek™ II Chambered Coverglass (8 wells) (Fig. 16). Each time point was carried out in an independent Lab-Tek with 1000:1 concentration of Ni NWs. Five wells per Lab-Tek were used, cells treated with 1.1 (± 0.1) μm and 6 (± 0.6) μm long NWs were seeded in duplicates, and one well was used for the negative control (untreated cells).

Figure 16. Setup for confocal microscopy. Cell imaging was performed only for the highest concentration (1000:1) with two different lengths of NWs, 1.1 (± 0.1) μm and 6 (± 0.6) μm.

2.5.4 Time points and concentrations for experiments

Most of the previous work in this area consists of cytotoxicity studies tested over short periods of time, ranging from 1 hour to 24 hours, but few studies tested NWs cytotoxicity over longer periods of time. For this work, results were obtained after 24, 48 and 72 hours for three concentrations namely 10:1, 100:1 and 1000:1. These concentrations were chosen to complement and enrich previous studies [50].

Table 2 shows the variables of the cytotoxicity studies and provides a summary of the experimental design. More details of the parameters of the Ni NWs are presented in the next chapter.
<table>
<thead>
<tr>
<th>MTT Experiment with Ni NWs</th>
<th>Exposure time (hours)</th>
<th>Concentration (NW/cell ratio)</th>
<th>Length (µm)</th>
<th>Aspect ratio</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial seeding density: 6,750 cells</td>
<td>24, 48, 72</td>
<td>10:1, 100:1 and 1000:1</td>
<td>6 ± 0.6 0.86 ± 0.02 1.1 ± 0.1</td>
<td>200 27.7 35.4</td>
<td>-9.47 -10.2 -10.1</td>
</tr>
</tbody>
</table>

**Table 2.** Length and zeta potential of samples for both MTT assay and confocal imaging.
CHAPTER 3

Results and discussion

3.1 Structural and morphological characterization of Ni NWs

3.1.1 Elemental composition of Ni NWs

EDAX was used to verify the chemical composition of the Ni NWs. The results show that the NWs are made of pure Ni metal (Fig. 17). The silicon and carbon peaks in the spectrum are the result of using a silicon substrate / Cu- carbon mesh to mount the sample. A small peak of oxygen was due to the Ni NWs being exposed to air and forming a thin oxide layer on the surface.

Figure 17. A) EDAX spectrum of released Ni NWs in solution. B) Selected area for the EDAX analysis.
Figure 18 A shows the morphology of ~6 μm long Ni NWs with a diameter of ~0.3 nm. A closer image (Fig. 18 B) reveals the presence of an oxide layer with a thickness of ~3.1 nm.

![Figure 18](image)

**Figure 18.** TEM images of ~6 μm long Ni NWs. A) Ni NWs with a diameter of ~0.3 nm. Scale bar=1 μm. B) Oxide layer around the Ni NW with a thickness of ~3.1 nm. Scale bar=50 nm.

In order to determine the components of the oxide layer, a second EDAX with higher resolution was performed using TEM. The spectrum showed a small peak of chrome as a result of using the chrome solution during the Ni NWs release (Fig. 19).
3.1.2 Diameter and length distributions of NWs

In order to investigate the influence of the aspect ratio on the cytotoxicity, Ni NWs of three different lengths were fabricated. SEM images were taken to determine the diameter and length distribution by using the ImageJ software (Fig. 20). The lengths of the Ni NWs were 0.86 (± 0.02) μm, 1.1 (± 0.1) μm and 6 (± 0.6) μm; while their aspect ratios are 27.7, 35.4 and 200, respectively.
**A**

- **Pore size**: 29.5 ± 3.7 nm
- **Length**: 6 ± 0.6 μm

**B**

- **Pore size**: 31.5 ± 3.6 nm
- **Length**: 0.86 ± 0.02 μm
Figure 20. Characterization of Ni NWs. A), B) and C) show the SEM images of the filled pores and the alumina template, released NWs, pore sizes and length distributions for each of the samples used during this work.

3.2 Testing cells viability by MTT assays

3.2.1 Treatment with Ni NWs

The first cell viability assay (Fig. 21) was done to evaluate the effects of Ni NWs as a function of NW/cell ratio, incubation time and NWs length. The data was obtained from three biological replicas and expressed relative to the negative control (untreated cells).

One of the challenges of evaluating cytotoxicity after long incubation periods (i.e. 72 hours) is cell overconfluence, which might lead to a decrease of the cell metabolic
activity. Nonetheless, the obtained OD values of untreated cells at different incubation times did not show significant variations. In contrast, OD values showed significant variations when NWs concentration was modified. These results confirmed that confluence at different incubation times did not significantly affect cell metabolic activity, and any loss of cell viability was caused by the presence of Ni NWs.

A  

**MTT 0.86 ± 0.02 μm**
Figure 21. Quantitative MTT assay of cell viability on wi-38 cells. Cell viability after treatment with (A) 0.86 (± 0.02) μm, (B) 1.1 (± 0.1) μm and (C) 6 (± 0.6) μm long Ni NWs. NC= negative control, untreated cells. Error bars show the standard deviation. Percent viability of cells was expressed relative to control cells. Triplicates were seeded for each tested condition.
In the case of the 0.86 (± 0.02) μm long NWs, no significant differences in cell viability (~80%) were observed between the first two concentrations (10:1 and 100:1) after 24, 48 and 72 hours. However, cell viability was notoriously reduced (~70% after 72 hours) when a 1000:1 NW concentration was used, the effects became more apparent at longer incubation times.

The results using 1.1 (± 0.1) μm long NWs showed high cell viability (>80%) when the concentration was 10:1 or 100:1, for any time point. For the 1000:1 concentration this value decreased up to 75% and 72%, after 48 or 72 hours respectively.

For the 6 (± 0.6) μm long NWs, the results showed high cell viability (> 95%) for the 10:1 and 100:1 NW concentrations up to 72 hours. The cell viability decreased to 86%, 74% and 64% after 24, 48 and 72 hours, respectively. Similar results were obtained using shorter NWs (0.86 μm) with the exception of 72 hours incubation where the shorter NWs yielded higher cell viability, more than 70%. Overall, cell viability at mitochondrial level of wi-38 cells seems to be influenced by both, NW concentration and NW length.

Previous studies have shown that nanostructure size influences endocytosis effectiveness by activating different uptake routes [28, 51]. In accordance with these results, the short NWs (0.86 μm and 1.1 μm) exhibited higher cytotoxicity at early exposure times, possibly as a result of faster internalization due to a smaller size. In contrast, longer NWs (6 μm) produced higher cytotoxicity at longer exposure time, probably as a result of slower internalization.
Comparing the results obtained for wi-38 cells in this work to results previously reported using HCTT116 cells [50] it can be concluded that HCT116 cells showed lower viability than wi-38 in the presence of Ni NWs. The lower toxicity of Ni NWs exhibited on wi-38 cells might be explained due to their comparably larger size (fibroblasts ~100 μm and colon ~16 μm length); meaning that even though NW/cell ratios (10:1, 100:1 and 1000:1) are the same for both cell lines, the NWs concentration per volume is less in the case of fibroblasts. The last statement refers to the fact that the number of wi-38 cells was around five times more than the number of HCT116, $1.4 \times 10^4$ and $8 \times 10^4$ cells per well at the time of the treatment with Ni NWs respectively. To confirm this idea, a second MTT assay using wi-38 cells exposed to higher NWs concentrations (5.4 times higher to account for their larger size) was carried out. The experiment was performed using 1.1 (± 0.1) μm long Ni NWs at the following concentrations: 54:1, 540:1 and 5400:1.
Figure 22. Quantitative MTT assay of cell viability on wi-38 cells. Cell viability after treatment with 1.1 (± 0.1) μm long Ni NWs. NC= negative control, untreated cells. Error bars show the standard deviation. Percent viability of cells was expressed relative to control cells. Triplicates were seeded for each tested condition.

Figure 22 shows the results of the 54:1, 540:1 and 5400:1 NW concentrations. For a 54:1 concentration the cells remained more than 84% viable. Increasing the concentration to 540:1 decreased cell viability to 74%. Further increasing the concentration to 5400:1, led to abrupt decreases in cell viability: ~70%, ~56% and ~47% after 24, 48 and 72 hours respectively. The percentages reported by one of my colleagues [50] for HCT116 cells were within the same range of values.

3.2.2 Treatment with Ni salts

The previous results show the cytotoxicity of Ni NWs on wi-38 cells. However the factors responsible for it remain unclear. It was hypothesized that a possible cause for Ni NWs toxicity is the side products of their degradation. To test this hypothesis, a comparative
MTT assays using Ni salts dissolved in medium was performed.

The Ni salt solution was prepared with Ni (II) sulfate hexahydrate (NiSO$_4$·6H$_2$O) and Ni (II) chloride hexahydrate (NiCl$_2$·6H$_2$O) and later diluted in EMEM medium. These were the same salts used to prepare the Ni solution for electrodeposition during NWs fabrication.

For these experiments, the concentration of Ni in μg/ml was calculated to be comparable to each of the NW/cell ratios previously used (10:1, 100:1 and 1000:1) for two lengths of Ni NWs, 1.1 (± 0.1) μm and 6 (± 0.6) μm. To do so, the mass of a single NW was calculated for both lengths using Equation (5), and the equivalent amount of Ni salt was calculated using Equation (6).

\[
\delta = \frac{m}{V} \rightarrow m = \delta \cdot V
\]

(5)

where \(\delta\) is density; and \(m\) is mass and \(V\) is volume of a single NW.

\[
Total \ mass = m \cdot (no. \ of \ NWs) \cdot (no. \ of \ cells) \cdot (no. \ of \ wells)
\]

(6)
Figure 23. MTT assay using different Ni salts concentrations. Results of cell viability for A) concentrations that are comparable with the results of 1.1 (± 0.1) μm and B) 6 (± 0.6) μm long NWs. Error bars show the standard deviation. Three replicates per condition were seeded.

Cell viability for Ni salts concentrations equivalent to those of the 1.1 (± 0.1) μm long NWs experiments, after 24 and 48 hours was above 90% and approximately 88% after
72 hours (Fig. 23 A). Similarly, Ni salts concentrations comparable to that of the 6 (± 0.6) μm long NWs experiments, resulted in cell viability greater than 90% after 24 and 48 hours, and values within ~84-87 % after 72 hours (Fig. 23 B). Surprisingly, cell viability of the last MTT assay (Fig. 23 B) was not significantly lower across the different concentrations, not even when the Ni salts concentration was approximately 6 times higher. The cytotoxicity of Ni salts showed to be concentration and time dependent throughout the experiments. In conclusion, Ni NWs showed to be more toxic than the Ni salts, and this behavior was more pronounced with the 1.1 (± 0.1) μm long NWs.

A review article about Ni carcinogenesis [51] concluded that Ni$^{2+}$ ions released from NPs reached the nucleus in greater amounts than Ni$^{2+}$ ions from water-soluble Ni(II) sulfate, resulting in a higher cytotoxicity. This finding leads to consider a possible degradation of the Ni NWs, releasing an amount of Ni$^{2+}$ ions that is enough to cause higher cytotoxicity than Ni salts. Further studies need to be done to quantify the amount of released Ni$^{2+}$ ions from the NWs, if any. It should also be determined if this release takes place inside the cell and/or before internalization of the NW.

3.3 Other factors that can influence the cytotoxicity of Ni NWs in wi-38 cells

The current knowledge suggests that the uptake of Ni NWs is done trough endocytosis, a process for which its effectiveness has shown to be influenced by the NW's length. In addition, the variations in the concentration of Ni NWs and/or Ni salts caused changes in
cell viability. However, additional factors have been reported previously and are explored below.

3.3.1 Oxide layer

As previously shown in section 3.1.1 (Figure. 18 B), an oxide layer of around 3.089 nm in thickness was formed on the Ni NWs surface. This oxide layer, which is always present, varies in thickness depending on the method used to release the NWs from the alumina template [71-73]. It can be hypothesized that the effects of the Ni NWs on wi-38 cells are buffered by the interaction of the serum proteins with this oxide layer [44].

3.3.1.1 Surface charge

The surface charge is a very important factor and should be taken into account when studying the interaction between nanostructures and biomolecules because it has a strong influence in the adhesion and uptake of nanostructures on the cell membrane. In order to determine the magnitude of the surface charge, the zeta potential was measured. As shown in section 2.5.4 (Table 2), the three values corresponding to each NWs samples were approximately equal to -10 mV.

The magnitude of the zeta potential is related to the colloidal dispersions, where the attraction forces of nanostructures with low zeta potentials (less than ±25 mV) will eventually exceed the repulsion forces, resulting in aggregation. In this case, the three samples of Ni NWs showed a low charged surface [74].
3.4 Cell imaging

Fluorescence and light bright images were taken to identify any change in cell morphology at each incubation time after being cultured in the presence of 1.1 (± 0.1) μm and 6 (± 0.6) μm long Ni NWs. Figure 24 shows some of those images, each of them with their respective negative controls, untreated cells. No changes in cell morphology were observed for any of the samples.

Figure 24. Bright light images. A) wi-38 cells after 24 hours and B) 72 hours of incubation with 6 ± 0.6 μm long NWs, with their controls. Changes in cell morphology were not detected.
In addition, fluorescence images of cells with DAPI stained nucleus were taken using confocal microscopy to obtain a higher resolution image that might show morphology changes in the nucleus. Figure 25 shows images of wi-38 cells after the Ni NWs were added to the wells. Overall, the nucleus morphology seemed to be normal, several cells showed to be under nucleus division at the beginning of the staining process. Something very interesting in these images was that some of the Ni NWs look blurred and lighter in color, which suggests that they are in different focal planes. Based on this, it can be conclude that the blurred and lighter color NWs have been internalized while the rest are interacting with the cell membrane without being internalized yet. This is the first insight of wi-38 cells interacting with Ni NWs that has been reported.

Figure 25. Confocal images of fibroblasts. A) Cells after 24 hours and B) 48 hours of incubation with long NWs (6 ± 0.6 μm) with a 1000:1 concentration. The nuclei (blue) were stained with DAPI. Some of the NWs look blurred and with lighter color (red narrows).
In order to assess if the wi-38 cells took up the Ni NWs, TEM images were taken at different exposure times (Fig. 26). The Ni NWs were taken up even after 24 hours of treatment. The NWs were founded as dark dense clusters inside cellular vesicles (lysosomes). Some of the images showed alteration on the normal size of the endoplasmic reticulum when comparing to the untreated cells, suggesting inflammation as a result of the presence of NWs, the were no visible alteration in the rest of the structures (i.e. mitochondria and nucleus).

![TEM images of wi-38 fibroblasts treated with 1.1 (± 0.1) μm long Ni NWs after 24, 48 and 72 hours with their respective controls. The Ni NWs were founded inside lysosomes. In some of the pictures alteration of the normal size of the endoplasmic reticulum was observed.](image-url)

**Figure 26.** TEM images of wi-38 fibroblasts treated with 1.1 (± 0.1) μm long Ni NWs after 24, 48 and 72 hours with their respective controls. The Ni NWs were founded inside lysosomes. In some of the pictures alteration of the normal size of the endoplasmic reticulum was observed.
Conclusions and future perspective

4.1 Conclusions

This study compared the cytotoxicity of Ni NWs with three different aspect ratios on wi-38 cells (human fibroblasts) by measuring cell viability at different incubation times and NW concentrations. The results showed that cell viability of wi-38 cells in the presence of Ni NWs is especially affected by concentration and NW length. The latter potentially affecting endocytosis effectiveness, favoring the internalization of smaller size NWs.

When comparing HCT116 cells to wi-38 cells at NWs concentrations of 10:1, 100:1 and 1000:1, HCT116 cells showed lower viability. This result might be explained by the comparably larger size of the fibroblasts. When higher concentrations of Ni NWs were tested on wi-38 cells, 54:1, 540:1 and 5400:1, cell viability was similar to those reported for HCT116 cells. For biomedical applications, it is highly recommended to maintain low Ni NW concentration, less than 100:1, and short exposure times, less than 24 hours.

The toxicity of Ni salts in wi-38 cells was also studied. The results showed that Ni salts were less toxic than Ni NWs. The reason for this might be a degradation of the Ni NWs, which in turn leads to the release of Ni^{2+} ions in an amount that is enough to cause higher cytotoxicity than Ni salts. For our knowledge, this is the first time that a systematic study is done in human fibroblasts wi-38 using ferromagnetic NWs; where
the toxic effects of equivalent amounts of Ni in its salt and in its NW form are compared. These comparisons are important to elucidate the effects related to the material and those related to the geometry.

Confocal microscopy images suggested that Ni NWs could either be internalized or just interact with the cell membrane. This research work is the first one to report insights of wi-38 cells interacting with Ni NWs.

The wi-38 (human fibroblasts) cells appeared to be a good in vitro model to evaluate the cytotoxicity of the Ni NWs. Nonetheless, our findings cannot be considered as a general behavior for all the non-cancer cells. More studies using different cell lines and different conditions are required to gain deeper understanding of the effects of NWs and the mechanisms at the base of their cell toxicity. This work represents a useful reference for further studies on the possible applications of Ni NWs in biomedicine.

**4.2 Future perspective**

There is an increasing interest in the evaluation and determination of NWs cytotoxicity and underlying factors that control their effectiveness. This knowledge would allow one to improve and tailor the NWs for their use in biomedical applications.

In addition, there is a clear need to standardize experiments and data related to cytotoxicity studies. This would make the results comparable so that they could be integrated into a general study where a wide range of cell lines, with characteristics such
as doubling time, surface area, metabolic activity, morphology, and biological function can be presented.

Three different lengths of Ni NWs were tested; nonetheless further investigations of wider range of NWs lengths might be useful to determine an optimal size for different biomedical applications that minimizes cell disruption.

The surface charge turned out to be the same for all the samples, so a deeper study regarding this parameter was not possible. Further work needs to be done where NWs with different surface charge interact with the cells. While some of the factors controlling NWs cytotoxicity were evaluated, there are many other factors that have been demonstrated to play an important role in the toxicity and need to be addressed[25, 75-77].
APPENDIX A

Fabrication of NWs

1.1 Surface preparation

The high purity Aluminium (Al) disks (99.999% purchased from Good Fellow) used for the NWs fabrication has a thickness of 0.50 mm and a 2.5 cm diameter. The electrolytic solution used for the cleaning of the Al disk consists of 75% ethanol (C₂H₅OH) and 25% perchloric acid (HClO₄), stored at 4°C.

The surface preparation process consists of two steps: surface cleaning and electropolishing. The protocol used for surface cleaning was:

1. The Al disk was washed three times in a beaker filled with DI water.
2. It was then washed from both sides in this order: acetone, isopropanol and DI water.
3. Finally, the disk was placed in a beaker with acetone and sonicated for 10 minutes. After that the acetone was replaced with DI water.

After cleaning, the Al disk was subjected to electropolishing, an electrochemical process that removes some of the metal from the surface to reduce imperfections, resulting in a smoother surface [64]. For the purpose of electropolishing, the disk was immersed in electrolytic solution consisting of 75% ethanol (C₂H₅OH) and 25% perchloric acid (HClO₄), stored at 4°C.
During the process, a platinum (Pt) wire mesh (Fig. 27) acted as cathode, while the Al disk submerged in the electrolytic solution and connected to the positive terminal of a direct current (DC) R&S®NGPX Programmable Power Supply acted as the anode (Fig. 28); the solution was continuously stirred during the whole process. A voltage of 20 V was applied for 3 minutes. The electrolytic solution acts as a conductor, allowing the current to pass from the anode to the cathode, resulting in the oxidation of the Al metal ions that are then dissolved in the electrolyte.

As a final step, the sample was washed with DI water to remove any residue of solution. The result was a clean, bright and smooth Al disk.

*Figure 27.* Pt wire mesh used for electropolishing. The image shows the front side (left) and backside (right) of the mesh. Dimension: 5x8 cm.
Figure 28. Electropolishing step. A) Schematic of the electropolishing setup. A voltage (20 V) was applied between the Al disk (anode) and the Pt mesh (cathode). B) SEM images [50] of the Al disk surface before (top) and after (bottom) electropolishing. Scale bar= 10 μm.

1.2 Components of the setup for the two-step anodization process

The Figure 29 shows the Pt mesh used for the first and second anodization. The two-step anodization setup and the different O-rings are showed on the Figure 30 A and Figure 30 B, respectively.
Figure 29. Pt mesh used in the setup for the two-step anodization process. It has a diameter (red line) of 7.5 cm and the separation (blue line) of the Pt wires is ~0.6 cm, vertically and horizontally.

Figure 30. Setup for first and second anodization and O-rings. A) Assembled setup and B) the two sizes of the O-rings, 1 cm (top) and 1.6 cm (bottom).
1.3 Removal of the alumina layer

A chrome solution consisting of 0.66% chromium (VI) oxide (CrO₃), 3.77% phosphoric acid (H₃PO₄) and 95.55% DI water was added to the anodization cell.

1.4 Removal of the aluminum and pore opening

To prepare the sample for the electrodeposition process, the aluminum barrier between the bottom of the pore and the Al surface has to be removed, and the pores have to be opened. These two steps are crucial to establish good contact between the pore and the base of the substrate [70]. The protocol we used for removing the aluminum was as follows:

- The sample was placed in a small-hole cell with its backside facing Cu solution, consisting of 1.67% of CuCl₂ · 2H₂O, 49.16% of HCl and 49.16% of DI water. While on the other side a piece of paper was placed to be used as an indicator that the aluminum is completely removed. The whole system was sealed with a Cu plate (Fig.31 A).
- The cell was filled with Cu solution and stirred at 300rpm for 20 minutes. Each 5 minutes the solution was replaced with fresh one.
- The solution was then removed and the cell was washed with DI water. To ensure that the aluminum was completely removed, a small amount of Cu solution was dropped on the Al disk. If a reaction was observed only on the
borders of the sample one could conclude that there is no remaining aluminum in the area of interest (Fig.31 B).

- The setup was disassembled and the sample was dried with N₂.
- The sample was then placed inside a plastic case with the anodized side facing a pH indicator paper, while the backside was filled with 5% phosphoric acid solution. The pores were opened after 2 hours; red spots on the pH indicator confirmed this (Fig.31 C).
- Once the pores were opened, the sample was rinsed with DI water and dried with N₂.

**Figure 31.** Aluminum removal process. B) Top view of the setup showing the perforated anodization cell with the Al disk after removal of aluminum. C) Pore opening. The red spots in the pH indicator paper are result of the solution that went through the membrane indicating that the pores are open.

### 1.5 Gold backside sputtering

Before the electrodeposition of Ni on the sample, the Al disk’s backside should be coated with a noble metal or alloy, this can be accomplished using electron beam
evaporation or sputtering [78]. For the samples used in this work, a gold (Au) layer of approximately 200 nm was deposited on the backside of the membrane using a Q300TD sputtering system (Quorum Technologies) (Fig. 32).

**Figure 32.** Gold backside sputtering. A) Backside of the sample coated with a 200 nm thick layer of gold deposited by sputtering. B) Cross section SEM image of gold layer on one side of the membrane, which is filled with Ni NWs.

### 1.6 Direct current electrodeposition

The Ni solution consisted of 2.4% Ni (II) chloride hexahydrate (NiCl$_2$·6H$_2$O), 2.77% boric acid (H$_3$BO$_4$), 14.5% Ni (II) sulfate hexahydrate (NiSO$_4$·6H$_2$O) and 80.3% DI water.


34. Nanowires, in Nanoscience, D.P.H.P. Dr. Claire Dupas PhD, Dr. Marcel Lahmani PhD, Editor. 2007, Springer Berlin Heidelberg. p. 325-347.


