Sara Antunes de Almeida Teixeira
Licenciada em Biologia

Biological effects of cellulose nanofibrils in a lung epithelial cell line

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Doutora Maria João Silva, Departamento de Genética Humana do Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P.

Co-orientador: Doutora Maria Henriqueta Louro, Departamento de Genética Humana do Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P.

Júri:

Presidente: Prof. Doutora Margarida Casal Ribeiro Castro Caldas Braga
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Abstract

Cellulose nanofibrils (CNFs) are plant-derived nanomaterials, showing advantageous physicochemical properties, renewable nature and low cost. CNFs have great potential in several industries, including forest, food, pharmaceutical and biomedicine, as drug-delivery systems or for tissue repair and regeneration. The increased production and application of CNFs leads to greater exposure of both workers and consumers, being extremely important to evaluate possible negative outcomes for human health. Due to their similarities with other fibers, such as carbon nanotubes, they may reveal adverse effects, such as carcinogenesis. The objective of this work was to evaluate the biosafety of CNFs produced by different pre-treatments - TEMPO-mediated oxidation and enzymatic hydrolysis - from a bleached industrial *Eucalyptus globulus* kraft pulp. For this purpose, the internalization, cytotoxicity and genotoxicity of these CNFs was analysed through different parameters: intracellular localization, cell viability and chromosome damage. Human lung epithelial cells (A549) were used, since the most frequent route of exposure is inhalation of CNFs, especially in occupational context. Preliminary results suggested that CNFs may not be internalized. Both CNFs did not show cytotoxicity and genotoxicity effects, regardless of dose and exposure time. However, an increase in cell proliferative capacity was observed when exposed to both types of CNFs. In conclusion, although the studied CNFs have different properties, *in vitro* safety assessment in A549 cells confirmed that both are biocompatible and showed a stimulating action on cell proliferation that needs to be explored through other parameters or *in vivo* assays. Understanding the physicochemical properties that possibly influence biological effects will contribute to a safe-by-design approach, enabling a more responsible and sustainable development of CNFs.

Keywords: cellulose nanofibrils; A549 cells; biological effects; *in vitro* toxicity; safety assessment; nanotechnology

Resumo

As nanofibras de celulose (CNFs) são nanomateriais derivados de plantas, que exibem propriedades físico-químicas vantajosas, natureza renovável e baixo custo. As CNFs apresentam um grande potencial em diversas indústrias, incluindo a florestal, alimentar, farmacêutica e biomedicina, como sistemas de distribuição de fármacos ou para reparação e regeneração de tecidos. O aumento da produção e de aplicações das CNFs, leva consequentemente a uma maior exposição, tanto de trabalhadores como de consumidores, sendo de extrema importância avaliar possíveis efeitos negativos para a saúde humana. Devido às suas semelhanças com outras fibras, como os nanotubos de carbono, podem revelar efeitos adversos, como carcinogénese. O objetivo deste trabalho foi avaliar a biossegurança de CNFs produzidas por diferentes pré-tratamentos – oxidação mediada por TEMPO e hidrólise enzimática – provenientes de uma pasta de *Eucalyptus globulus.* Para este propósito, foi analisada a internalização, a citotoxicidade e genotoxicidade destas CNFs através de diferentes parâmetros: localização intracelular, viabilidade celular e danos na estrutura dos cromossomas. Foram utilizadas células epiteliais do pulmão (A549), dado que a via de exposição mais frequente ocorre pela inalação das CNFs, principalmente em contexto ocupacional. Resultados preliminares sugeriram que as CNFs podem não ser internalizadas. Ambas as CNFs não demonstraram efeitos de citotoxicidade e genotoxicidade, independentemente da concentração e tempo de exposição. No entanto, foi observado um aumento da capacidade proliferativa das células quando expostas aos dois tipos de CNFs. Em conclusão, apesar das CNFs estudadas apresentarem propriedades distintas, a avaliação de segurança *in vitro* em células A549 confirmou que ambas são biocompatíveis e mostrou uma ação estimulante na proliferação celular que necessita de ser explorada, através de outros parâmetros ou ensaios *in vivo*. O conhecimento das propriedades físico-químicas que possivelmente influenciam os efeitos biológicos das CNFs irá contribuir para uma abordagem "*safe-by-design*", permitindo um desenvolvimento mais responsável e sustentável das mesmas.

Palavras chave: nanofibras de celulose; células A549; efeitos biológicos; toxicidade *in vitro*; avaliação de segurança; nanotecnologia

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List of Acronyms and Abbreviations

A549 - Human Epithelial Lung Adenocarcinoma Cell Line
ATCC - American Type Culture Collection
ATM – Atomic Force Microscopy
BEKP – Bleached *Eucalyptus* Kraft Pulp
BC – Bacterial cellulose
BNC(s) – Binucleated Cell(s)
CBMN – Cytokinesis-Block Micronucleus
CBPI – Cytokinesis-Blocked Proliferation Index
CNC(s) – Cellulose Nanocrystal(s)
CNF(s) – Cellulose Nanofibril(s)
CNT(s) – Carbon Nanotube(s)
Cyt-B – Cytochalasin-B
DLS – Dynamic Light Scattering
DMEM – Dulbecco's Modified Eagle Medium
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribonucleic Acid
DP – Degree of Polymerization
DSB(s) – Double Strand Break(s)
EDTA - Ethylenediaminetetraacetic Acid
EU-OSHA – European Agency for Safety and Health at Work
FBSi – Heat-Inactivated Fetal Bovine Serum
HEPES – 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
ISO – International Organization for Standardization
INSA – Instituto Nacional de Saúde Doutor Ricardo Jorge (National Institute of Health, Portugal)
JRC – Joint Research Centre
LDH – Lactate Dehydrogenase
MMC – Mitomycin C
MN – Micronucleus
MNBNC – Micronucleated Binucleated Cell(s)
MTT – 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NB(s) – Nuclear Bud(s)
NM(s) – Nanomaterial(s)
NP(s) – Nanoparticle(s)
NPB(s) – Nucleoplasmic Bridge(s)
OECD - Organisation for Economic Co-Operation and Development
PBS – Phosphate Buffered Saline
RI – Replication Index
ROS – Reactive Oxygen Species

RPM – Revolutions per Minute
SD – Standard Deviation
SDS – Sodium Dodecyl Sulfate
SEM – Standard Error of Measurement
SSB(s) – Single Strand Break(s)
TB – Trypan Blue
TEM – Transmission Electron Microscopy
TEMPO – 2,2,6,6-tetramethylpiperidine-1-oxyl
WHO – World Health Organization

1. Introduction

1.1. Nanomaterials

Nanomaterials (NMs) are chemical substances or materials used at a very small scale and have the potential to increase the quality of life by improving some already existing products or to create new ones in numerous sectors, including public health, industry, environment, energy and nutrition. However, defining what is considered a nanomaterial is never straightforward. According to the International Organization for Standardization (ISO) the term 'nanoscale' comprises the length range approximately from 1 nm to 100 nm. Since many materials have different properties at nanoscale when compared to the macro or microscale, in nanomedicine, particles up to 1000 nm were also included, regarding materials with potentially new medical applications (Wagner et al. 2006). Other international authorities consider broader ranges of sizes for NMs and have released alternative definitions (SCENIHR, 2010). In 2011, the European Commission published a specific recommendation (2011/696/EU) proposing a definition for nanomaterial as "a natural, incidental or manufactured material containing particles, in an unbound state, as an agglomerate or as an aggregate and where, 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50%." (Rauscher et al. 2019; European Commission, 2011).

Recently, the British Standards Institution has proposed definitions for nano-objects, nanoparticles and nanofibers. A nano-object is a material that has one or more peripheral nanoscale dimensions, whilst nanoparticles (NPs) are considered a nano-object with three external nanoscale dimensions. Moreover, a nanofiber is defined as a nanomaterial with two similar exterior nanoscale dimensions and a third larger dimension (Jeevanandam et al. 2018). Even though these materials are distinguishable, some authors do not differentiate between NPs and NMs definitions. Hence, to facilitate the comprehension, the two terms will be assumed as being the same in this study.

Nanomaterials can have different classifications due to their dimensions, materials or origin. Regarding their origin, NMs can be classified in three main categories: natural nanomaterials, which can be found in nature from volcanic eruptions, forest fires or by biological species; incidental NMs, which are produced incidentally from human activities, as a by-product of industrial processes such as nanoparticles produced from manufacturing, refining and combustion processes and food preparation; or engineered NMs, which are manufactured deliberately by humans to have certain required properties for specific applications (Louro, Borges and Silva 2013). In some cases, incidental NMs are considered as subcategory of natural NMs (Jeevanandam et al. 2018). Compared to incidental NMs, the morphology of engineered NMs can generally be better controlled. Moreover, they can be purposely designed to exploit new features due to their small size.

There are currently specific references to NMs in various technical guidelines that support the implementation of legislation, where often the same materials are used in different contexts. For this reason, an objective of the definition is to create a coherent cross-reference to ensure conformity between different areas. Therefore, the main goal is to ensure that when a nanomaterial is considered in one sector, it is also treated as such when used in another sector. Therefore, the recommendation intends to identify materials for which these considerations make sense by stipulating unambiguous criteria.

1.1.1 Characterization of Physicochemical Properties

Compared to non-nanometric materials of the same chemical composition, NMs are developed to exhibit different characteristics, giving them more advantageous properties such as mechanical, optical, electrical and magnetic properties (Louro, Borges and Silva 2013).

Within its unique properties, the most important to consider is size, since it regulates the possibility of NM internalization, which may cause a response of the body's immune system. Specifically, a reduced size is directly linked to the increased reactivity of NM since the smaller the size the higher the surface area-volume ratio. In fact, by modifying their surface chemistry and reactivity, an interaction with other molecules is promoted (Arora, Rajwade and Paknikar 2012). Previous studies indicate that smaller particles (<100 nm) show a higher ability to penetrate and may cause a greater inflammatory reaction due to their biopersistence, resulting in the disruption of cellular systems of many organisms (Ferreira, Cemlyn-Jones and Cordeiro 2013; Jeevanandam et al. 2018).

Other significant characteristics include shape, charge, dynamic behaviour, corona protein formation, resistance, crystal structure or surface functionalization (Louro, Borges and Silva 2013; Oberdörster 2009; SCENIHR 2009; “Nanomaterials Definition Matters” 2019)

Shape/morphology and surface charge also seem to alter the NM uptake. NMs can come in many shapes, such as fibers, tubes, spheres. This feature directly influences the membrane internalization process. When compared, sphere NMs have already shown easier endocytosis than fiber NMs (Gatoo et al. 2014). Considering the surface charge, it is known that cationic NMs exhibit greater internalization efficacy compared to anionic or neutral NMs but induce higher toxicity when interacting with cellular components (Louro et al. 2015).

When NMs contact with the biological medium, give rise to the corona protein, a layer of various proteins capable of interacting with the surface of materials and whose composition depends on changes in the environment and a continuous association and dissociation of proteins, which may lead to alterations in their properties (Louro et al. 2015). In addition to modifications in material properties, these interactions can also promote changes in cell uptake capacity, different conformations and protein depletion in cell media. It is considered a dynamic process, as the composition of the protein layer may vary depending

on the different compartments where the particles are in the biological system (Kane and Stroock 2007).

Another relevant property is the dynamic behaviour of NMs, i.e., their ability to form aggregates and agglomerates. When dispersed particles come together through weak physical interactions, they form precipitates called agglomerates. In the case of aggregates, the particles are strongly bounded and form a cluster, being an irreversible process (Oberdörster 2009). These phenomena depend on the size, chemical composition, surface charge and dispersant medium under study. The presence of agglomerates/aggregates leads to an increase in particle size, which may influence its uptake and distribution (Gatoo et al. 2014).

It is also important to consider the presence of impurities resulting from air or water contaminants or waste materials that remained during the synthetic process. These may adhere to the exterior of the particle and alter its properties, such as its charge or reactivity (Oberdörster 2009).

1.2 Nanotechnology

Nanotechnology is based on the application of these NMs to the technologic area, which according to the ISO means the “application of scientific knowledge to manipulate and control matter predominantly in the nanoscale to make use of size- and structure-dependent properties and phenomena distinct from those associated with individual atoms or molecules, or extrapolation from larger sizes of the same material” (ISO, 2015).

1.2.1 General Applications

Nanotechnology development has been progressing at a great scale with high levels of innovation, thanks to the need of improvement in the quantity and quality of new technologies. Due to their innovation and specific characteristics, NMs are broadly used in several fields of human life (Louro, Borges and Silva 2013). A short list of some of the main uses of manufactured NMs in consumer products can be found in **table 1.1**.

This development and large-scale production of NMs is driven by the rapid growth of the human population combined with increased product consumption (Louro, Borges and Silva 2013). However, this recent and rapid expansion raises some health and safety concerns since the NMs are used in the production and are present in numerous final products, which are daily consumed (Jeevanandam et al. 2018; Vance et al. 2015).

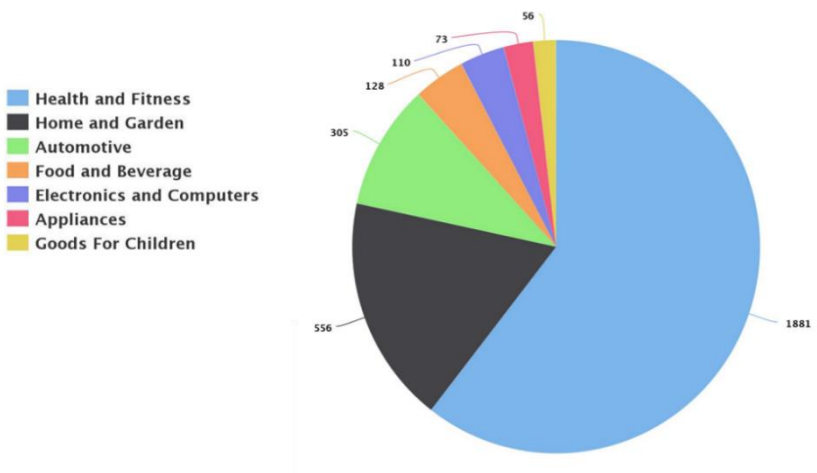

Figure 1.1 - Industries that produce consumer goods containing NMs in their composition. Source: http://nanodb.dk/en/analysis/consumer-products/#chartHashsection. Accessed on August 6, 2019.

Table 1.1 - Some examples of manufactured nanomaterials applications, in everyday consumer products. Adapted from Wijnhoven *et al.*, 2010.

Product category	Examples
Biomedical applications and Health products	Targeted drug-delivery systems; diagnostic systems; artificial skin or bone for regenerative medicine; wound dressing, hearing aids, orthopaedic prostheses
Personal care products and cosmetics	Sunscreens; baby care products; shampoo, gel, hair dyes; shower gel; lotions; deodorants; shaving soap; toothpaste; make-up products and nail care; jewellery
Appliances	Refrigerators, washing machines, air conditioning; air/water sanitizers/ neutralisers, batteries
Electronics and computers	Audio; cameras; hardware; mobile devices; television
Home furnishing and household products	Cleaning products; cooking utensils; pillows; construction materials, paint, coating
Textile	Clothes; sheets
Packages (including those for food)	Food packaging and sensors in food packages
Sporting goods	Rackets, bats, golf clubs, bowling balls, etc.
Motor vehicles	Paint; tires; air purification systems; coating/cleaning
Toys and games	Toys, Games
Cross-cutting (multi-functional)	Coatings (anti-fog, self-cleaning, etc.)

1.2.2 Biomedical Applications

Thanks to the distinct and valuable properties above mentioned, the increasing application of NMs to biomedicine indicate more efficient and less expensive healthcare (Louro, Borges and Silva 2013). In fact, nanomedicine has emerged with the intention of adopting these materials for medical diagnosis or therapeutics to improve health strategies. For instance, the ability of NMs to cross biological barriers can provide applications in drug delivery, imaging and diagnostic, fluorescent labels and biomarkers and drug discovery therapies (Wagner et al. 2006; Oberdörster 2009)

Therefore, nanotechnology is promising in the field of biomedical applications, as some nanoparticles have the ability to cross impermeable membranes, such as the blood-brain barrier, and corona protein may indeed increase the absorption of nanoparticles in cells. Thus, the efficiency of transport into the brain can be drastically increased by binding specifically targeted proteins and drugs to these nanoparticles, possibly enabling the treatment of neurological diseases (Oberdörster 2009).

There are now several laboratory groups and medical industries investigating countless possible applications such as: orthopaedic prostheses; hearing aids; artificial skin or bone for regenerative medicine; wound dressings; tumour targeting, analysis and therapy; molecular therapy or immunotherapy; diagnostic systems and targeted drug-delivery systems. The latter occurs by drug encapsulation, as NMs allow to protect drug molecules and prevent them from being denatured or degraded by the body. Besides that, this area also allows the use of nanomaterials as contrast agents in optical images or as a dye in fluorescence-based bioanalytical techniques (e.g. quantum dots) (Bacakova et al. 2019; Abitbol et al. 2016; Louro, Borges and Silva 2013).

The use of these materials in industrial and consumer products is increasing, leading to direct and indirect exposure of humans (Arora, Rajwade and Paknikar 2012).

1.3 Human Exposure to Nanomaterials

1.3.1 Types and Routes of Human Exposure

From what has been mentioned previously, it is possible to recognize a substantial increase in human exposure to NMs, especially in the occupational context, due to the expansion of nanotechnology, because of the increasing development, production and use of manufactured NMs. Nevertheless, the levels and the health consequences of NMs to which the human population may be subject, whether as consumers, workers or in the environment, remain unclear (Louro, Borges and Silva 2013).

Human exposure may occur throughout all stages of the nanomaterial life cycle (**figure 1.2**), involving occupational exposure, consumer exposure and finally, environmental exposure due to their disposal and consequent accumulation in the environment.

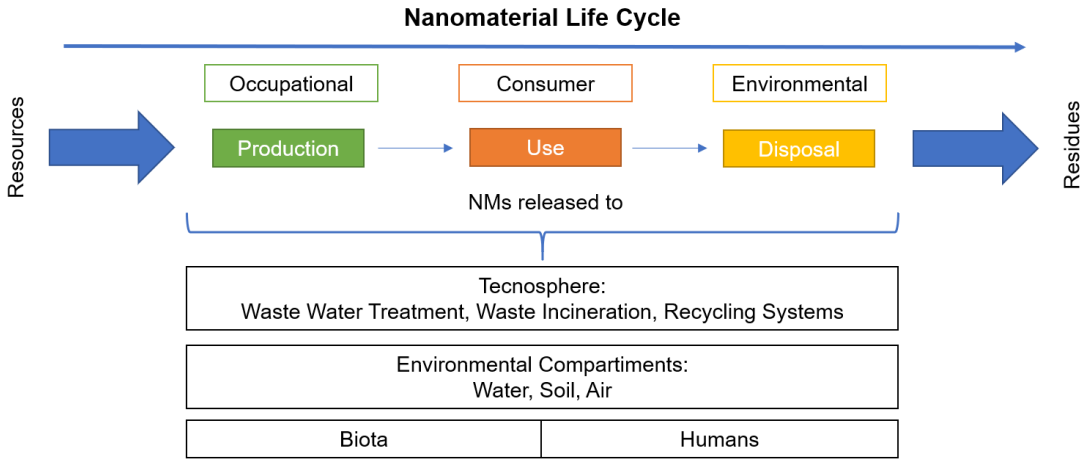

Figure 1.2 - Life cycle, exposure scenarios and possible fates of a nanomaterial. Adapted from Som *et al.*, 2010.

In the occupational context, exposure to NMs may occur in their production phase. However, exposure can also occur in various professional contexts where NMs are handled, treated or applied, meaning that many more workers can be exposed without even knowing they are in contact with NMs. Therefore, there is a risk of inhalation in the case of airborne particles or contact with the skin. As an example, exposure can range from healthcare or laboratory work to maintenance or construction work. (https://osha.europa.eu/pt/themes/nanomaterials, accessed on August 8, 2019).

As regards worker's protection, European legislation applies to NMs but does not explicitly refer to them. This means that it is up to employers to prudently assess and manage the risks of NMs in the workplace. When the production and use of NMs cannot be eliminated or replaced by less hazardous materials and processes, it is important to minimize worker exposure through preventive measures such as source risk control or the use of personal protective equipment (https://osha.europa.eu/pt/themes/nanomaterials, accessed on August 8, 2019).

To assess the toxicity of NMs it is required information about the route of exposure. Of the human exposure routes already evaluated, the most relevant is inhalation, especially in occupational and environmental circumstances, affecting the respiratory system. It is well known that lungs are easily exposed to airborne NMs, which may be caused by pollution, production/manipulation of nanoparticulate powders in industrial processes or by erosion of manufactured materials (Medina et al. 2007).

Oral exposure may occur due to the addition of NMs to food, food supplements or even food packaging, as well as those that may be absorbed through the intestines of mammals from contaminated soil or water, possibly resulting in systemic effects. The transdermal route is also important, both in occupational settings and in the use of cosmetic and personal care products containing NM, despite the knowledge about the possible ability of NM to penetrate the skin remains insufficient (Louro, Borges and Silva 2013).

One of the main issues is the fact that there is no traceability system to properly inform consumers about which products on the market contain NMs, making it difficult to understand and communicate the risk. In most countries, the use of the designation 'nano' on consumer product labels is not legally substantiated, with products incorporating and not referring to NMs and others indicating their presence but not actually containing them (Louro, Borges and Silva 2013).

For this reason, many agencies that regulate the safety of NMs, such as EU-OSHA, ISO or WHO, are increasingly concerned about human exposure to NMs, considering that there is a need for full life-cycle assessment to identify all worker exposure situations as well as the implications on their health (Carriere et al. 2016; Louro, Borges and Silva 2013).

1.4 Cellular Interactions and Nanotoxicology

1.4.1 Nano-bio Interactions

Nano-bio interactions consist of physicochemical interactions, kinetic and thermodynamic exchanges between the nanomaterial surface and biological units such as membranes, proteins, organelles and DNA. These interactions, which are facilitated by considering nanometric materials and biological structures within the same dimensions, can occur at the cellular and molecular levels and may cause severe effects (Nel et al. 2009). Relevant physicochemical properties that have been proved to affect NM behaviour as well as their possible impact on cells are shown in **figure 1.3**.

Since membrane structures can involve and capture NMs, their most likely entry in cells is by endocytosis, leading to new cellular interactions, with inter and intracellular effects (Nel et al. 2009), including direct or indirect reactions with cell genome (Louro, Borges and Silva 2013).

Due to their small size, NMs can be absorbed and once inside the body, these particles are able to translocate to the circulatory and lymphatic system and can reach various organs and tissues. Also, these materials may reach the nucleus, thus being important to consider their interaction with the DNA and consequently the possibility of DNA damage. This may lead to genetic instability and appearance of neoplasms (Carriere et al. 2016; Louro, Borges and Silva 2013).

The consequences of NMs exposure, mainly by inhalation, have been investigated in several *in vivo* experiments regarding the occurrence of lung damage, inflammation and tumour formation (Medina et al. 2007). After being inhaled, NMs reach the alveolar epithelial surface, where they can interact with alveolar macrophages and epithelial cells (Herzog et al. 2007). Their accumulation occurs mainly in the alveolar region. Nanomaterials can translocate through the pulmonary epithelium, entering the blood and lymphatic circulation to reach bone marrow cells, lymph nodes, spleen, heart, among other organs (Arora, Rajwade and Paknikar 2012).

The oral route is also under study, since NMs can be absorbed from the intestine, moving into the internal environment of the organism. From there, the route is similar to the one previously described, as particles are also able to reach diverse organs and tissues (Louro, Borges and Silva 2013).

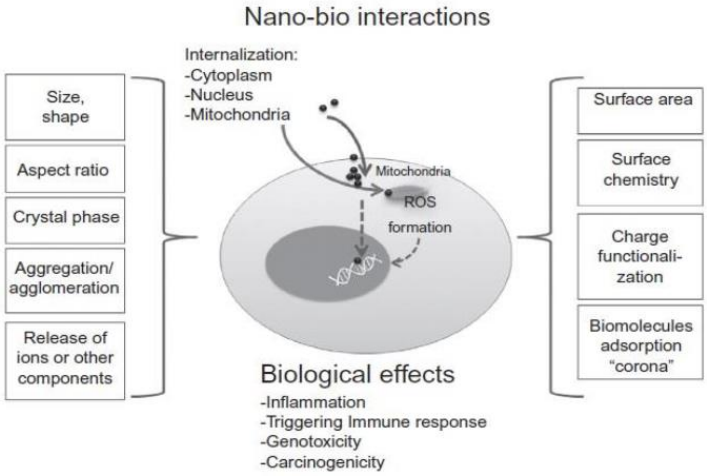

Figure 1.3 - Physicochemical interactions between nanomaterials and biological compartments and their biological effects. Adapted from Louro *et al.*, 2015*.*

1.4.2 Nanotoxicology

Nanotoxicology is a recent area of toxicology that explores the adverse effects of nanoscale materials, either *in vitro*, *in vivo* or in the ecosystems. For the development of a sustainable and safe nanotechnology, the physicochemical properties of NMs are considered (Donaldson et al. 2004; Oberdörster 2009).

Initially the toxicity of NMs was studied in the same way of common chemical compounds or larger sized materials. However, the distinct physicochemical properties that guarantee their innovation, such as size, shape, charge, solubility, surface area and structure are often the same ones that pose risks to the environment and raise safety concerns (Collins et al. 2017).

As a consequence of the increased manufacturing and subsequent release of NMs, triggered by their increased use in consumer products and industrial applications, there is a need to create risk assessment strategies. Despite the high utility in predicting the behaviour and fate of NMs produced, the major challenge of these risk assessment strategies is whether exist enough knowledge about these NMs to predict their overall behaviour or if according to the environment their behaviour is distinct from natural NMs**.** The Scientific Committee on Emerging and Recently Identified Health Risks (SCENIHR), concluded that although NMs are not dangerous per se, in many aspects their safety still leaves significant scientific uncertainty so the safety assessment of materials should be done on a case-by-case basis (SCENIHR, 2009).

According to Carriere *et al.* (2016), the toxicology of NMs involves two paradigms: their inflammatory potential and oxidative stress. The most evident pathway that correlates NMs exposure with tissue damage, already proven *in vivo*, is the increase of the oxidative stress, from the production of reactive oxygen species (ROS), caused by inflammatory response. Nevertheless, it has also been found that even in the absence of local inflammatory cells, NMs can cause oxidative stress through cell constituents such as mitochondria (Louro, Borges and Silva 2013). If this stress persists, it can induce DNA damage, that may cause cell death or mutations. If these lesions are not repaired, chromosomal abnormalities may occur and consequently the potential of cancer development increase (Carriere et al. 2016).

Regarding the inflammatory response of the tissue, it was possible to establish analogies with recognized materials, like silicosis and asbestos, where chronic inflammation can lead to genotoxicity, mutations, cell death and cancer (Louro, Borges and Silva 2013). For example, carbon nanotubes have a fibrous structure like asbestos, which have been causing concern about their inflammatory and toxic potential (Ilves et al. 2018), emphasizing this analogy.

Even though some materials are characterized and approved as biocompatible, in their macro- and microforms, that does not mean their nanoform reveal the same behaviour. As the reactivity of NMs is different, it may have implications on the interactions with biological systems, meaning that even if not observed for the same non-nanometric materials, various types of modifications may be induced (Louro, Borges and Silva 2013). For this reason, cell interactions must also be considered to the safety assessment of a NM, being aggregation or agglomeration important factors (Jeevanandam et al. 2018).

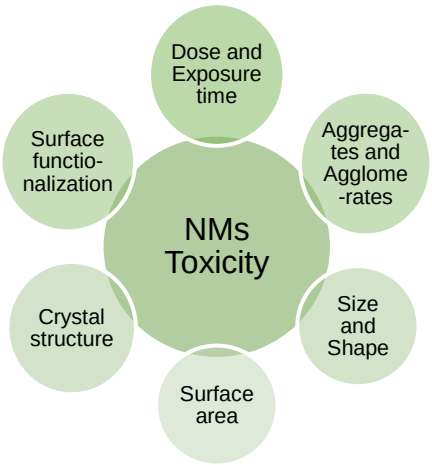

Figure 1.4 - Several factors can influence the toxicity of nanomaterials. Adapted from Jeevanandam *et al.*, 2018.

The number of NMs that penetrate cells depends directly on their molar concentration in the surrounding medium (dose) and the exposure time. Regarding the toxicity of NMs at different concentrations, there are many contradictory studies. However, higher concentrations of NM are known to promote aggregation. Since most NM aggregates are micrometric, it means that a significant amount of NM aggregates can no longer penetrate cells, losing their toxicity (Jeevanandam et al. 2018).

Typically, the toxicological effect of NMs increases with decreasing particle size and increasing surface area. Different shapes (aspect ratios) can lead to different toxicity levels. For example, asbestos fibers 10 μm in length can cause lung cancer, while shorter fibers can cause mesothelioma (5–10 μm) or asbestosis (2 μm) (Jeevanandam et al. 2018). In the case of nanofibers, with the same composition, characteristics such as length, diameter, shape and surface energy can cause distinct adverse effects (Ilves et al. 2018; de Lima et al. 2012).

Despite numerous efforts, consensus on the toxicity assessment of these materials has been difficult to reach and reliable guidelines are still under development. In this perspective, it is difficult to compare existing studies due to the great diversity of protocols used, affecting the scientific robustness and statistical power of the studies (Soares et al. 2018; Collins et al. 2017).

Before their utilization, it is required more studies of NMs in the format in which they will be applied clinically (Louro et al. 2015). So, their full physicochemical characterization should be a priority, since it alters the pharmacodynamics and pharmacokinetics of NMs (Soares et al. 2018).

1.4.2.1 Potential adverse effects of nanomaterials and their possible mechanisms in human health

Some authors have found that nanometric particles (<100 nm) appear to be more toxic when compared to larger particles of identical chemical composition (Ferreira, Cemlyn-Jones and Cordeiro 2013), which raised concerns about their safety profiles. One particular concern is airborne NMs, with occupational or environmental origin, and possible damage to the respiratory tract (Oberdörster 2009). The most worrying effects that occur in the lungs include inflammation, tissue damage, the appearance of fibrosis and tumours, among others (https://osha.europa.eu/pt/themes/nanomaterials, accessed on August 8, 2019).

Nanomaterials can mediate cytotoxic or genotoxic responses by biological constituents through diverse endpoints inherent to NM toxicity. Thus, the NM can directly or indirectly induce cell dysfunctions, affecting essential cellular components such as the membrane, mitochondria and nuclear compartment, which lead to DNA damage. In case of accumulation and biopersistence of the nanomaterial, it may occur chronic inflammation.

Comparing to their larger analogues (>500 nm), nanoparticles have some distinct biological characteristics and effects. Among these, it is possible to highlight the higher surface area to volume ratio, occurrence of agglomeration, protein interaction capacity and uptake by different cellular components (Oberdörster 2009). More data can be seen in **table I.1** in the annexes.

Some *in vitro* studies have demonstrated the influence of agglomeration stability and polydispersity on NMs toxicity. For this reason, it is important to observe and characterize the behaviour of the NM under

study, when in culture medium, together with the seeded cells. As already mentioned in **Section 1.1**, the formation of aggregates can determine the actual size of the particles that will interact with biological systems (Louro, Borges and Silva 2013; Oberdörster 2009). To facilitate the disaggregation of NMs, they are sometimes suspended in serum, cell culture or a surfactant-coating vehicle.

Due to the lack of agreement between nanotoxicology studies, several international organizations, such as the Center for Disease Control (CDC), the Organisation for Economic Cooperation and Development (OECD) and the European Union (EU), have been working to promote and ensure the correct use of NMs (Louro, Borges and Silva 2013). Currently, to ensure that a substance is safe for human health there are international recommendations establishing the adequate battery of assays and other relevant bioassays that assess the toxicity of chemicals. Thus, concerning NMs for biomedical applications, it is important not only to evaluate their toxicity potential, but also understand their biocompatibility. And for that there are few standard operational procedures to allow the standardization of bioassays towards an entire evaluation of the adverse effects of nanomaterials.

In summary, in nanotoxicology studies, it is essential to consider not only the physicochemical properties of the tested NMs, but also the cellular interactions and their behaviour in biological systems.

1.4.2.2 The safe-by-design approach

To ensure the safety of NMs and their application, it is essential to understand and classify their outcomes. Several groups have been investigating nanotechnology in order to recognize its benefits and minimize its potential risks.

With this in mind, the “safe-by-design” approach has gained considerable importance in recent years, by guaranteeing safe and sustainable manufacturing processes for innovative and functional products, with a reduction in their possible adverse effects for the population. By knowing the physicochemical properties of a NM, it is possible to identify which characteristic leads to a possible toxicity, allowing to turn the toxicity into biocompatibility (Louro et al. 2015). Meaning that a material should be designed, in a profitable way, in its least hazardous nanoform. To select the best formulations, predictive tools such as high throughput screening (*in vitro/in vivo*) should be used (Dekkers et al. 2016). It is therefore important to consider the risk of exposure to consumers and the environment and to reduce them as much as possible. Beyond the need for safe infrastructures, industrial safety procedures also include safe conditions for workers to handle, store and transport NMs. Thus, a product must be built based on 3 pillars: safe design of products, safe use of products and safe industrial procedures. This will allow the enhancement of safety in human health and in ecosystems (http://www.nanoreg2.eu/safe-design, accessed on August 12, 2019).

The challenging issue remains associated with the different behaviours between materials with similar chemical composition, but with different dimensions (nanoscale and macroscale). Indeed, the existence

of toxicity appears to be influenced by variation in size, shape or specific surface charge (Dekkers et al. 2016). Thereby, to assess the potential adverse effects on our health and the environment, it is essential a broad characterization of the chemical and physical properties of each NM.

1.4.2.3 The ToxApp4NanoCELFI Project

The increased number of NMs, as well as variations of them, make it difficult to assess their risk as it is impossible to toxicologically characterize them by conventional tests. Alternatively, a predictive toxicological approach can be adopted based on the explanation of the mode of action (MoA) of representative NMs. It is more cost-effective and can be achieved, for example, by identifying key factors and pathways that govern interactions between NMs and biological systems and their consequences (Oberdörster 2009; Arora, Rajwade and Paknikar 2012).

With this purpose, the European Commission approved a project called ToxApp4NanoCELFI, which involves the Department of Chemical Engineering at the University of Coimbra, the National Institute of Health Doctor Ricardo Jorge, I.P (INSA) and a private non-profit Institute (RAIZ). The main objective of ToxApp4NanoCELFI is to investigate the potential respiratory effects of various CNFs, using monocultures and cocultures. Briefly, this project aims to provide relevant data on the possible ability of CNFs with different properties to produce immunotoxic, genotoxic, genomic and epigenomic effects on the respiratory tract and the underlying mechanisms. These results will allow the development of predictive nanotoxicology strategies, ensuring the safety of the studied CNFs or, if toxicity is detected in any CNF, allowing its modification to reduce adverse effects/outcomes, thus promoting the safe-by-design approach.

1.5 Nanocelluloses

1.5.1 Origins, Structure, Production and Modification

Cellulose is the most abundant biopolymer in nature, being the main structural constituent of the plant cell wall. It is found mainly in wood, cotton, hemp, flax and other plant-based materials, but can also be produced by algae, fungi and various bacteria (within the genera Acetobacter, Agrobacterium, Rhizobium and Sarcina) (Halib et al. 2017).

Regarding its chemical and structural composition, cellulose is a linear homopolysaccharide, with high molecular weight. The constituent monomers are β-D-glucopyranose linked together by β-1,4-glycosidic bonds. However, according to **figure 1.5**, in nature cellulose is not present in this form, since molecules are joined in individual nanofibrils, nanofibrils in microfibrils and microfibrils in macrofibrils/bundles (Abdul Khalil et al. 2014).

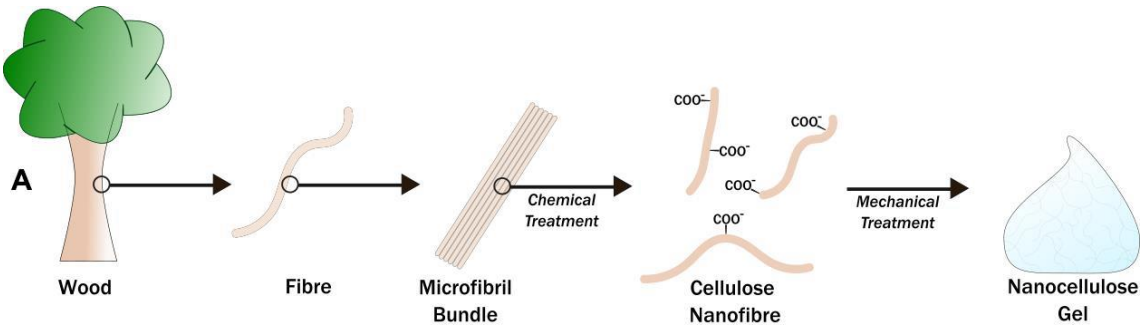

Figure 1.5 - Representative process to produce nanocellulose gels by physical electrostatic repulsion. Cellulose fibers are obtained from wood as bundle of microfibrils. TEMPO-mediated oxidation allows to individualize cellulose nanofibrils. High shear mechanical treatments (e.g. high-pressure homogenization) transforms the nanocellulose into a hydrogel. Image from Curvello, Raghuwanshi and Garnier, 2019.

Over time this material has been used for a variety of purposes and more recently, due to increased demand for renewable and biodegradable products that fit the current and global sustainability trend, there has been a clear growth (Ilves et al. 2018).

Through nanotechnology it was possible to reduce cellulose to the nanoscale, thus improving its physicochemical properties, giving rise to nanocelluloses. Based on its source, processing methods and final characteristics, nanocellulose is divided into three categories (**table 1.2**): cellulose nanocrystals (CNCs or nanocrystalline cellulose), cellulose nanofibrils (CNFs, also known as nano/microfibrillated cellulose) and bacterial cellulose (BC, also called microbial cellulose) (Lin and Dufresne 2014).

Table 1.2 - Comparison of morphology and physicochemical properties between BC, CNF and CNC. Adapted from Klemm et al. 2018.

Nanocellulose type	Length	Cross-section	Degree of polymerization	Crystallinity/crystal structure
Bacterial nanocellulose (BNC)	Different types of nanofiber networks	20–100 nm	4000–10,000	Iα (shell) and Iβ (core) – highest degree of crystallinity
Cellulose nanofibrils (CNF)	0.1–2 µm	5–60 nm	≥500	Primarily Iβ – lowest degree of crystallinity
Cellulose nanocrystals (CNC)	100–250 nm (from plant celluloses); 100 nm to several micrometers (from celluloses of tunicates, algae, bacteria)	5–70 nm	500 – 15,000	Primarily Iβ, sometimes Iα – medium degree of crystallinity

Among nanocelluloses, their chemical composition does not differ; However, the morphology of BC is less consistent compared to the others, which gives it different characteristics. As for its structure, BC forms fibrils that are organized in ribbons, with a cross-linked and more crystalline appearance. It is considered highly hydrated, leading to the formation of selectively permeable, porous, non-toxic hydrophilic structures, suggesting similarities to collagen fibrils (Halib et al. 2017). Compared to other polymers, their production is not economically competitive. Thus, plant cellulose emerges as a good alternative (Nordli et al. 2016).

In biomedical application context, there are compounds present in some types of nanocellulose that must be removed, such as lignin or hemicellulose. These compounds are generally associated with plant cellulose, so BC does not need to undergo chemical treatments to be used as it is considered pure cellulose. In addition, it has better characterization and fewer safety problems compared to other types, so it is more present in the market today (Halib et al. 2017).

Due to the presence of previously mentioned compounds, e.g. lignin and hemicellulose, plant-derived cellulose is not as pure as bacterial, leading to biocompatibility problems. Lignin grants stiffness to fibers, increasing their resistance to possible threats such as wind or gravity. Hemicellulose allows the binding between lignin and cellulose. Lignin is known to be non-biodegradable and has a recognized toxicity potential. If it remains in the body, its effects are unpredictable and therefore nanocellulose should be subjected to chemical processes to eliminate these compounds. However, these processes may increase the toxicity of nanocellulose as they give rise to residual chemicals (Halib et al. 2017). For this reason, in order to ensure biocompatibility when these compounds are present, it is recommended to analyse possible cytotoxic and inflammatory effects or to purify plant nanocelluloses (Nordli et al. 2016).

From cellulose microfibrils two different materials can be obtained: cellulose nanofibrils (CNFs) or cellulose nanocrystals (CNCs) (Halib et al. 2017). CNFs are renewable and cost-effective manufactured materials with advantageous mechanical, optical and rheological properties. Fiber dimensions may vary by source and production method, typically they range between 5-30 nm in width and length in the order of micrometers, with a length to width ratio (aspect ratio) greater than 50 (TAPPI standard proposal WI3021; Chinga-Carrasco *et al.*, 2011; Halib *et al.*, 2017). This nano-object consist of alternating crystalline and amorphous domains, with long, flexible and entangled cellulose nanofibrils that form a bundle of stretched cellulose chain molecules (Abdul Khalil et al. 2014).

Different production methods, such as a combination of chemical and mechanical methods, allow not only chemical modification but also materials with different sizes, surface energies and physical structure from the same raw material (de Lima et al. 2012; Halib et al. 2017). Nanocelluloses may undergo different chemical modifications, such as carboxymethylation, acetylation, esterification, cationization, carboxylation or silylation depending on the purpose. Although the most common are the first two, they all allow slightly altering the properties of nanocelluloses to suit a variety of applications. As these modifications alter the basic structure of nanocellulose, it may become less natural and more expensive, somewhat disadvantageous from a regulatory point of view (Halib et al. 2017).

Concerning CNFs production, generally they are obtained by wood pulp delamination through intensive mechanical treatment, e.g. with a high-pressure homogenizer (Siró and Plackett 2010; Li et al. 2012), and can be associated before and/or after with chemical or enzymatic treatment to reduce energy consumption (Klemm et al. 2011; Abdul Khalil et al. 2014). As for chemical treatments, one of the most effective is an oxidation mediated by 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO), which

introduces carboxylate and aldehyde functional groups into the cellulose fibers, facilitating their deconstruction process (Saito and Isogai 2007; Lourenço et al. 2017).

1.5.2 Physicochemical Properties and Applications

As already mentioned, nanocelluloses have unique properties, which differs thanks to their source and production method. The most explored features include high specific surface area, high aspect ratio and high tensile strength and stiffness, with the addition of being renewable and biodegradable in nature (Ventura et al. 2018).

Nanocelluloses are considered accessible, both economically and in nature, making their use expected to increase. They are of interest not only to industry, including paper, coatings, food and formulations, but also to innovative biomedical purposes, such as drug-delivery carriers, antimicrobial materials and tissue repair and regeneration (Lin and Dufresne 2014).

Considering some industrial examples, gels or emulsions containing nanocelluloses can be used as rheology modifiers, for example. Furthermore, CNFs are great for strength-enhancement of the mechanical properties of other materials, such as paper, carton and packaging materials, as they have remarkable mechanical resistance and low density (Ventura et al. 2018).

1.5.2.1 Biomedical applications

Within the biomedical field, applications of nanocelluloses are numerous and progress has been increasingly visible. Since CNFs are considered biocompatible, several investigations have been made regarding their use in areas such as wound healing, surgical suturing, regenerative medicine, long-lasting drug delivery systems or 3D cell culture scaffolds (Ventura et al. 2018).

In wound healing, CNCs and CNFs have already been used as delivery systems for bioactive substances such as anti-inflammatory drugs, antibiotics and growth factors, especially in case of burns where there is a high loss of fluid that can result in the patient's death or cancer patients with impaired tissue vitality. These materials, besides being biocompatible, allow the incorporation of various molecules as they have a large surface area. Also, due to their ability to form a cellular matrix and maintain their mechanical properties in humid environments, mesenchymal cell-associated CNFs have also been used as sutures (Halib et al. 2017).

As for the regenerative capacity, these materials have also demonstrated their value. In bone regeneration, CNCs have been used as a matrix thanks to their mechanical properties, their ability to fix calcium, which allows a more uniform deposition and the ability to interact with mesenchymal cells, that promotes differentiation into osteoblasts. Since CNFs have a fiber shape that mimics the extracellular matrix, they were also tested, showing good results and allowing cell proliferation beyond the matrix

boundaries. In addition, a 3D matrix made of cellulose acetate was created and showed excellent porosity for the formation of new bone and blood capillaries, with great diffusion of oxygen and nutrients (Halib et al. 2017).

Although cartilage regeneration remains limited and challenging due to the absence of blood vessels, there has been a growing focus on its replacement using 3D bioprinting technology. In association with other materials, such as alginate, CNFs have been used as constituents of a bioink, resulting in cartilage-like viscoelastic structures (Markstedt et al. 2015; Chinga-carrasco 2018). These nanocelluloses are also used in cartilage tissue matrices, with the advantage of being able to add cells or other bioactive substances that favour chondrogenesis.

Currently the most widely used nanocellulose for biomedical purposes is BC. Although plant-based CNFs are increasingly emerging as potential competitors, especially in economic terms, they have major concerns about their biosafety, constitution and production methods (Halib et al. 2017).

1.5.3 Toxicity Assessment of Nanocelluloses

Even though there is already a significant amount of toxicological studies on different cell types, most have focused on nanocellulose types with different properties than CNFs, such as BC (Lin and Dufresne 2014; Saska et al. 2012; Moreira et al. 2009) and CNCs (Catalán et al. 2015; Yanamala et al. 2014; Clift et al. 2011). Some studies on nanocrystalline cellulose indicate possible induction of cytotoxicity and immunotoxicity *in vitro* and *in vivo* (Yanamala et al. 2014; Clift et al. 2011). Generally both nanocelluloses are considered non-toxic and because they show interesting properties, they are exploited in the current market (Ventura et al. 2018).

CNFs have a fiber-like structure, such as carbon nanotubes and asbestos, which may have undesirable genotoxic effects, often associated to carcinogenesis (Ilves et al. 2018). Despite the smaller number of published studies, their cytotoxic, genotoxic and immunotoxic effects have already been evaluated, and most of them did not show relevant toxicity levels (Souza et al. 2018; Rashad et al. 2017; Lopes et al. 2017; Čolić et al. 2015; Pitkänen et al. 2014; Alexandrescu et al. 2013; Nordli et al. 2016). Several authors even report an increase in cell viability after treatment with CNFs (Ventura et al. 2018; Rashad et al. 2017; Lopes et al. 2017; Nordli et al. 2016). As for genotoxicity, there is no uniformity of results. While Catalán *et al.* (2017) did not observe DNA damage, other authors detected genotoxic effects on cells exposed to CNFs (Ventura et al. 2018; de Lima et al. 2012). Others also analyse the inflammatory potential of nanocelluloses, as they can lead to genotoxic effects later.

Inflammation is a parameter for assessing immunotoxicity, by analysing the presence of cytokines and the recruitment of inflammatory cells. There are some general results from broader studies available in the literature indicating that CNF has no proinflammatory effect (Ventura et al. 2018; Nordli et al. 2016; Čolić et al. 2015; Hua et al. 2015; Vartiainen et al. 2011). Even so, in the *in vivo* study by Catalán *et al.*

(2017), an increase in the recruitment of inflammatory cells to the site of CNFs exposure was observed which resulted in an acute inflammatory response. These results were consistent with those obtained by Yanamala *et al.* (2014) for the exposure of mouse to two types of CNCs by pharyngeal aspiration. Given the *in vitro* studies, nanocelluloses did not appear to be efficient stimulants in cytokine production. Vartiainen *et al.* (2011) found no increase in expression of various cytokines and chemokines in mouse macrophages or PBMCs exposed to microfibrillated cellulose. Čolić *et al.* (2015) also observed no increased proinflammatory cytokine secretions in PBMCs exposed to various concentrations of CNFs (31.25 µg/ml–1 mg/ml). A more recent study by Lopes *et al.* (2017), detected an increase of inflammatory cytokines from one of the CNFs studied, which had been considered non-cytotoxic. This may indicate that cytotoxicity and inflammation are not necessarily related. Menas *et al.* (2017) also found that exposure to CNCs, considered low cytotoxic, for 72 hours significantly increased the secretion of various proinflammatory cytokines in A549 cells. While CNFs, despite the higher cytotoxicity detected, did not show significant results. In contrast, Ventura *et al.* (2018) detected no increase in inflammatory cytokines in a co-culture of A549 cells with THP-1 cells exposed to CNFs. Contrary to what occurs in a living organism where there are several mechanisms and interactions occurring simultaneously, in a cell culture this is not true, which excludes several variables. These data indicate the possibility of CNFs being genotoxic through several mechanisms, being necessary to increase the knowledge regarding the inflammatory potential of these materials.

Due to the disagreement between results, it is clearly necessary to further investigate the effects of these biomaterials and their mechanisms. This knowledge will allow the assessment and prevention of human exposure to potentially hazardous CNFs, cost-effectively preventing occupational and consumer illness and contributing to a safe-by-design approach. This approach enables industries to develop innovative and safe materials without risk to human health.

1.6 *In vitro* Cellular Assays

In vitro and *in vivo* studies should be performed for all newly developed and commercially available materials to investigate their biosafety and biocompatibility, to allow their subsequent approval and/or use (Nordli et al. 2016).

At an early stage, to assess the toxicity of NMs in a short period of time, *in vitro* cellular assays can be used. Typically, these assays arise as a first line, since they are less time consuming, cost-effective and are simpler to perform, control and interpret the results. In addition, they reduce the number of animal tests required, avoiding ethical problems (Collins et al. 2017). There are other advantages, such as less variability between experiments and less need for test materials, which reduces the amount of toxic waste generated (Arora, Rajwade and Paknikar 2012).

There are a growing number of materials tested for toxicity through these assays, which are considered suitable for high throughput screening (HTS). The HTS approach, according to Collins et al. (2017), is

defined as “the use of automated tools to rapid execution of a large number and variety of biological assays that may include several substances in each assay”. In other words, it allows testing numerous materials at different concentrations in different cell types, but with a significant reduction in inter-laboratory variation. Several methods are used to mimic *in vivo* assays, as well as possible, saving time and money.

In vitro studies may be useful for studying and establishing effect-concentration associations – which may be excessive for many organisms – and for explaining possible mechanisms that cause toxicity (Arora, Rajwade and Paknikar 2012).

Despite their usefulness, they have some limitations. The use of cell lines does not fully mimic the interaction that occurs between various intracellular molecules, cell types and even organs within a complex biological system. Thus, it is more difficult to assess *in vitro* the organism response to material, as there is no function and dynamics that subsequently gives rise to the coordinated response of tissues in the body. This may influence the outcome of exposure to the agents under study. In order to achieve reproducible results, it is also very important to ensure that the NMs themselves do not interfere with the chosen assay (Magdolenova et al. 2014). If they interfere, there may be formation of agglomerates, changes in the optical properties or pH of the medium (Collins et al. 2017). Another disadvantage is that cell cultures cannot be used to identify potential exposure targets in the human body (Stone, Johnston and Schins 2009).

Given the strong recommendation to replace animal testing, there is currently a high incentive to research innovative cellular systems that better mimic, in culture, the exposure of an entire organism. According to the study’s purpose, different cell lines (transformed or non-transformed) with specific characteristics can be selected. It should be taken into consideration that their behaviour differs when placed in culture medium and may influence susceptibility to the evaluated material, due to changes in the medium or cell density, and consequently studies on its toxicity (Magdolenova et al. 2014). It can also be created more complex *in vitro* systems, including various cell types, e.g. co-culture of epithelial cells and macrophages, to bring the situation closer to *in vivo* (Stone, Johnston and Schins 2009).

Cancer cells are widely used in these assays, since they can proliferate indefinitely and be established in simple culture media, unlike non-transformed cells. Despite the limitations already mentioned, the most standardized systems for bioassays are adherent two-dimensional (2D) cell monolayer (Edmondson et al. 2014).

The choice of the cell line for *in vitro* toxicity assessment is often influenced by the exposure/absorption route and the potential target organ of the tested substance or material (Stone, Johnston and Schins 2009).

1.6.1 Cellular Uptake

As a first step in assessing NMs toxicity, it is important to measure their absorption by the cell, which can be done by labelling some particles with fluorescent compounds. If the goal is to use the NM as a nanocarrier, its absorption must be extremely efficient. The use of fluorescent probes allows the observation and evaluation of some effects at specific sites, which can go down to the nucleus level, thus identifying possible sites of interest. Some of the most commonly used fluorescent probes are fluorescent organic dyes (Forster et al. 2012).

Cellular internalization of NMs can occur by different mechanisms: for smaller particles, by diffusion or by transport channels and for larger particles by endocytic pathways. In the latter case, cell membrane invaginations occur (e.g. through clathrin-mediated systems) or even extensions of the membrane, with processes such as macropinocytosis or phagocytosis (Höcherl et al. 2012).

Although there are several studies on these uptake mechanisms, most of them do not distinguish between adsorbed NMs and those that were actually internalized. The interaction between the adsorbed proteins on the surface of the NMs and the cell membrane gives rise to a global fluorescence signal wrongly associated with the internalization of NMs, which may lead to an inadequate determination of the NM concentration that is actually internalized. As it is not easy to differentiate between these two types of fluorescence, sometimes adaptations in the methodology used may be necessary (Vranic et al. 2013).

1.6.2 Cytotoxicity Assessment

There are different types of toxicity, such as cytotoxicity, genotoxicity, immunotoxicity, among others. The first two have distinct adverse effects, as cytotoxicity is related with damage at the cellular level and genotoxicity at the genetic level (Stone, Johnston and Schins 2009). Nevertheless, both may result from a direct effect or a cellular stress response due to the production of reactive species.

In biological environments, e.g. respiratory system, the biopersistence of inhaled particles determines their toxicity (Herzog et al. 2007). NMs can interact with cellular and mitochondrial membranes or alter mitochondrial function, provoking the production of ROS and inducing DNA oxidation. ROS, due to their high chemical reactivity, can react with DNA, proteins, carbohydrates and lipids causing cell death either by apoptosis or necrosis or even inflammatory responses.

Generally, cytotoxicity assays are used as a first screening to test different experimental conditions, observe cellular response and suggest the most adequate concentration-range to further explore (Stone, Johnston and Schins 2009). There are many methods available to study the viability and capacity of cell proliferation in culture and characterize the cytotoxic potential of a compound.

The optimization of suitable assays to be made in 96-well culture plates enabled rapid and simultaneous analysis of a large number of samples. The use of colorimetric or luminescence-based assays allows the measurement of the samples to be made directly on the plate through a reader specific to the type of plate used (Stepanenko and Dmitrenko 2015). However, these assays are not always appropriate for the study. There are some concerns about adsorption of NMs (e.g. carbon nanotubes) into dyes that may lead to false positives (Herzog et al. 2007).

Cytotoxicity may interfere with the outcome of genotoxicity assessment of a given substance, as cell death increases the amount of fragmented DNA, which can induce false positive results in genotoxic assays. Therefore, it is recommended to first perform cytotoxicity assays to define the non-cytotoxic dose range to be further investigated and to complement the genotoxicity studies (Donaldson, Poland and Schins 2010).

In order to increase the reliability of the cytotoxicity assessment, it is recommended to combine at least two different assays that measure different outcomes, thereby generating complementary results (Stone, Johnston and Schins 2009).

1.6.2.1 The MTT assay

Within cytotoxicity studies, there is a wide variability of assays, usually based on measurement of cell viability. One of the most common is the MTT assay or other equivalent tetrazolium salt-based assays (MTS, XTT or WST-1). The principle of this assay, developed by Mosmann (1983), is to quantify cell viability by determining mitochondrial enzyme activity. Occurs due to the action of mitochondrial dehydrogenases of viable cells, which allow the reduction of a water-soluble yellow tetrazolium dye, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into a water-insoluble purple compound (formazan) by cleavage of the tetrazolium ring (**figure 1.6**). It is considered a colorimetric assay and can be quantified by spectrophotometric readings at a specific wavelength. The absorbance value obtained is representative of the number of cells and their viability. Thereby, they allow the detection of proliferation as well as cell death (Stepanenko and Dmitrenko 2015; Stone, Johnston and Schins 2009).

Before performing an MTT assay, there are several control conditions to be aware of. Overestimation of cell viability may occur if some particles generate an absorbance at the same wavelength as the final coloured product. This interference can be controlled by subtracting the background absorbance of the cells exposed to the particles, but without the assay reagents (Stepanenko and Dmitrenko 2015). In contrast, an underestimation of cell viability may also occur. Some surface properties, such as their large surface area, can lead to high adsorption capacity, allowing the particles to extract the coloured product from the cells. As this phenomenon is more difficult to control, an alternative assay may be considered. Another relevant factor is the oxidative properties of NPs. Through an oxidative reaction, they can produce colour that will trigger increased absorbance. Therefore, it is important to evaluate the

behaviour of the particles in the absence of cells. It is also important to include a negative and a positive control to compare with the particle under study and linked to the hypothesis being tested. An example of a positive control may be a relatively toxic known nanoparticle, such as copper oxide. As a negative control, could be the same tested particle but larger (Stone, Johnston and Schins 2009).

During this test, a suspension is formed which contains cell debris, the dissolved formazan and the particles themselves. In order to reduce possible NM background interference, the sample can be centrifuged, the supernatant transferred to a new 96-well plate and then read the absorbance of the supernatant obtained, free of cell debris and particles, thus obtaining a more faithful result (Stone, Johnston and Schins 2009).

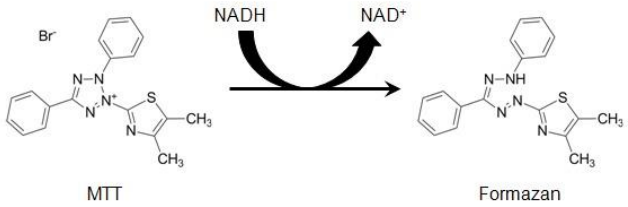

Figure 1.6 - MTT conversion into formazan crystals inside the mitochondria. Adapted from Riss *et al.*, 2013.

1.6.2.2 The clonogenic assay

As referred before, some NMs interact with various colorimetric indicator dyes, which are used for assays that measure fluorescence or absorbance after exposure to NMs, such as MTT. This can lead to false readings and misinterpretation of particle toxicity, making these tests unsuitable for quantitative toxicity assessment.

The clonogenic assay – also called colony formation efficiency (CFE) assay – can be used as an alternative, eliminating the risk of NM-dye interactions and ensuring the assessment of true cytotoxicity as it avoids the use of any colorimetric or fluorescent indicator dye. This assay, first described by Puck and Markus (1956), is based on the ability of a single cell to form a colony, assessing cell survival *in vitro* (Herzog et al. 2007).

Before or after exposure, cells are seeded at appropriate dilutions to form colonies depending on the duration of the assay. Subsequently, colonies arising from individual cells are counted (Franken et al. 2006). For an effective counting it is important that plating density is adequate, if too high the colonies will become indistinguishable, making individualized colony counting impossible (Buch et al. 2012).

It is widely used in oncology to determine cell reproductive death after radiation and to measure the effect of a given drug concentration. However, it has recently been considered appropriate to determine cell survival after exposure to different compounds (Franken et al. 2006).

1.6.3 Genotoxicity Assessment

Genotoxicity assays are considered essential in the safety assessment for several potentially harmful compounds, including NMs. A genotoxic agent interacts with DNA, compromising the integrity of the genetic material. Such assays not only allow to detect the presence of lesions and evaluate their possible consequences, but also detect changes in repair mechanisms (Carriere et al. 2016).

Genotoxicity can be promoted by primary or secondary interactions, where the first is subdivided into direct and indirect interactions. Within primary interactions, direct interactions arise from a direct contact between particle and DNA. That is, it requires internalization in the nuclear compartment, through physical or chemical processes, causing physical injury to the DNA structure. When this occurs, genetic instability may arise, contributing to the development of carcinogenic processes. Among the most frequent lesions are strand breaks and intercalation between NMs and DNA bases. In indirect interactions, damage is induced by other molecules, such as proteins involved in replication, transcription and repair. Thereby, the replication and cell cycle can be affected, by by-products of the reaction between NMs and cellular molecules, such as ROS, which may deplete available antioxidants defences or toxic ions released by soluble nanoparticles, generating oxidative stress (Donaldson, Poland and Schins 2010; Magdolenova et al. 2014).

Secondary interactions are associated with an inflammatory response due to the presence/aggregation/accumulation of NMs. This inflammation can lead to oxidative stress and damage to cells and DNA. If oxidative stress persists, accumulation of DNA damage may eventually lead to carcinogenicity (Donaldson, Poland and Schins 2010). They occur between different and organized cells, as happens in the *in vivo* situation during a body stimulus (Evans et al. 2016). In this case they correspond to a more realistic genotoxic evaluation of NM, since they combine different cellular responses and mechanisms. The latter can only be explored through *in vivo* models, although several *in vitro* tests should be performed first to characterize the toxicity potential of NMs and to support safe-by-design practice.

There are several variables which can influence the results obtained through genotoxicity assays. From the method applied for the preparation of NMs, the use of biologically significant concentrations, the existence of impurities over the analysed samples, to the choice of the cell lines themselves, or even the way the particles are absorbed at a certain cellular level (Magdolenova et al. 2014).

Within the different assays developed to detect DNA damage, the most widely used are those that can assess this damage based on individual cell analysis, requiring only small cell samples, such as the micronucleus assay.

Most studies identify surface area and its reactivity as the two main factors involved in dose-dependent genotoxic events. As previously stated, a protein corona may be formed on the particles surface, which can influence their biological reactivity and imply a variation on their genotoxic potential, while making it hard to identify the source of the effect. It is also difficult to study this phenomenon *in vitro* since, in the biological organism, the particles can transverse different combinations of proteins, impossible to simulate in the cell culture environment (Donaldson, Poland and Schins 2010).

In addition to micronucleus assay, the comet assay is also widely used. Also known as single-cell gel electrophoresis, this assay is a technique for assessing DNA damage and repair in individual cells of different tissue types (Collins 2004). It is based on the detection of DNA strand breaks, either strands (double strand breaks, DSBs) or single strand (single strand breaks, SSBs). In addition, it can be modified to detect oxidative damage to the bases and even DNA repair (Collins et al. 2008). Quantified DNA lesions correspond to primary and reversible lesions that can be repaired or, on the contrary, lead to cell death if highly damaged.

This method represents a simple, inexpensive, fast and sensitive technique as it can be applied to virtually all cell types without the need for cell culture and can detect low levels of DNA damage (Tice et al. 2000). It can be performed *in vitro* using appropriate cultured cell lines or *in vivo* as a standard test with analysis of various experimental animal tissues (OECD 2016b; Arora, Rajwade and Paknikar 2012). This test has multiple applications in detecting genotoxic potential, testing monitoring (ecological/environmental or even human biomonitoring) and clarifying fundamental mechanisms of DNA damage and repair (Collins 2004). Other methods to study genotoxicity include the Chromosomal Aberration assay and HPRT gene mutation assay.

Currently, the great diversity and non-conformity of the results among the various genotoxicity studies performed are due to several characteristics, such as the origin of the NM, the preparation method, the protocols used and the experimental conditions (pH, temperature, presence of impurities or irradiation), the type of cell line or animal model used, the concentration and exposure time. In order to harmonize these methods for *in vitro* toxicity assessment, protocols for the characterization and preparation of dispersion of NMs should be developed as well as a suitable battery of tests should be established (Cohen et al. 2013).

1.6.3.1 The Cytokinesis-Block Micronucleus Assay

In normal situations, cell cycle can be blocked with the intention of allowing DNA repair, and, if this is not possible, programmed cell death (apoptosis) may be triggered. When cellular repair mechanisms

fail to reverse DNA damage, it can result in permanent damage to the cell. If cell division continues, the damage is transmitted to the daughter cells by genetic mutations or chromosomal aberrations, inducing deleterious defects and possibly leading to carcinogenesis (Fenech 2000).

Among the most used methods to assess genetic damage and characterize cytotoxicity and genotoxicity of a specific compound/material is the micronucleus assay, suggested in the HTS approach. The micronucleus assay is one of the most important *in vitro* procedures (Fenech 2000). It is based on the evaluation of chromosomal abnormalities usually caused during anaphase, when the nucleus divides into two separate nuclei.

When cells experience high levels of toxicity, micronucleus (MN) can reflect a pronounced effect of chromosome damage, such as breakage (clastogenic effects) or loss (aneugenic effects). Micronuclei arise either from chromosome fragments and/or whole chromosomes that are incapable of migrating to the poles of the cell at anaphase. Their existence may also be a sign of DNA hypomethylation, an event related to epigenetic processes of regulating DNA expression. A nuclear membrane is formed around the genetic material and this corpuscle remains in the cytoplasm, morphologically identic to main nuclei but smaller, and it can be detected upon staining (Fenech 2000).

Although this assay is commonly recommended for genotoxicity testing, the possible interference of NMs has been discussed (Magdolenova et al. 2012) and therefore slight adaptations have been made in the original assay. According to Guideline 487 (OECD, 2016), the *in vitro* Cytokinesis-Block Micronucleus (CBMN) assay is described as a standard procedure to detect genotoxic events at the chromosome level with international validation.

As MN can only be expressed in “active cells” capable of completing the cell cycle, it is crucial to identifying and distinguish dividing and non-dividing cells. CBMN assay is an improved version of the traditional methodology since by adding a cytokinesis blocker, such as Cytochalasin-B (Cyt-B), allows to distinguish between this type of cells and, therefore, exclude interference from micronuclei originated before the assay. Cyt-B inhibits actin polymerization, which is essential for the formation of the microfilament ring that constricts the cytoplasm and individualizes the two daughter cells during cytokinesis. Thereby, the process of mitosis results in binucleated cells (Fenech 2000, 2007). Conversely, there are also cells that did not divide or made more than once, being called mononucleated or multinucleated, respectively. OECD recommends that chemicals under study should be removed after the addition of cytochalasin B; with NMs it is not possible to do, as they remain adsorbed to cells after washing (Magdolenova et al. 2012).

Within chromosomal abnormalities, others important parameters that represent a common phenotype of chromosomal instability can also be measured by CMBN assay, such as nucleoplasmic bridges (NPBs), nuclear buds (NBs) as well as cytotoxic (necrotic and apoptotic aspect) and cytostatic effects (**figure 1.7**)

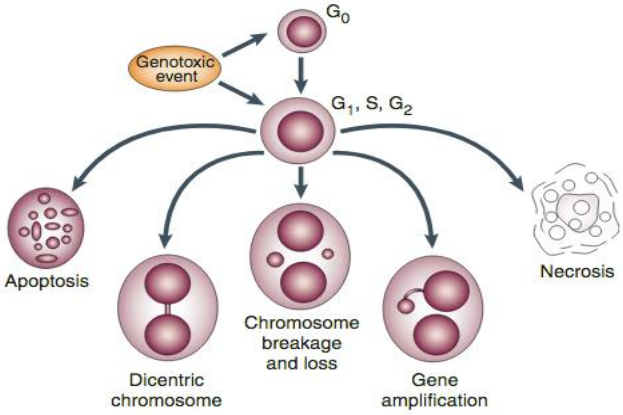

Figure 1.7 - Potential fates of cultured cytokinesis-blocked cells following exposure to cytotoxic/genotoxic agents. Adapted from Fenech, 2007.

NPBs can be formed between nuclei of a binucleated cell, from dicentric chromosomes that are pulled to opposite poles of the cell in anaphase, the existence of these chromosomes may be by telomere union or failures in DNA repair. Both phenomena allow to measure the chromosome rearrangement that cannot be assessed only by the presence of micronuclei, thus NPBs evaluation is an important complement (**figure 1.8**). NBs have a homologous structure to MN, but they are still attached to the main nucleus by a narrow stalk of nucleoplasmic material and is an important biomarker for gene amplification. Cytostasis represented by the ratio between mononucleated, binucleated and multinucleated cells, evaluates the ability of cells to replicate. Cytotoxicity is measured by necrotic and/or apoptotic cellular ratios (Fenech 2000, 2007).

All these endpoints improve the detection of a possible genotoxic response caused by a chemical compound or NM. One sign of a genotoxic effect is the increase in micronucleated cells after exposure to a chemical compared to their basal frequency in unexposed controls. Several studies have linked this higher frequency of micronucleated cells with an increased incidence of cancer (Fenech 2007).

Although this method can identify micronucleus-inducing agents, to differentiate whether micronuclei originate from clastogenic and/or aneugenic events, specific techniques such as immunochemical labelling of kinetochore or fluorescence *in situ* hybridization (FISH) are required (OECD, 2016).

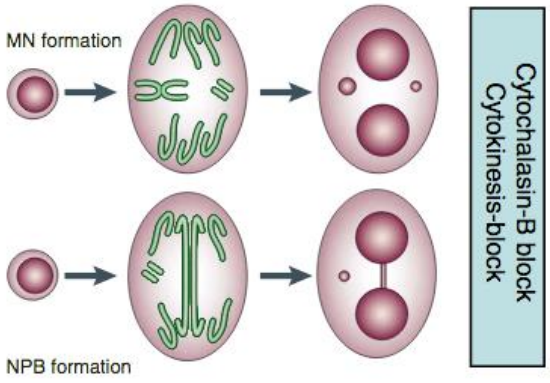

Figure 1.8 - Process of micronucleus formation (MN) and nucleoplasmic bridges (NPBs) during cell division. MNs are formed from chromosome or whole chromosome fragments that are left behind and NPBs from errors in repairing DNA strand breaks or telomere linkage. Adapted from Fenech, 2007.

Each essay is focused on evaluating distinct endpoints, so each one can have its own strengths and weaknesses. One of the possible shortcomings of CBMN assay is that it can be considered a subjective assay as it generally requires manual identification of micronuclei. If not careful, an operator may be influenced by knowing whether the sample was treated or not, which may lead to skipping some micronuclei simply because they do not fit the collected data, resulting in a kind of manipulation of the results (Magdolenova et al. 2014). On the other hand, operator experience also plays a role in the variability of the tests. Generally, to minimize experimental variation, it is desirable that the tests be performed by the same operator or following specific guidelines as described by Fenech (2000).

By knowing that the micronucleus assay is one of the most important *in vitro* procedures for evaluating potential genotoxic effects, for this study, CNFs were evaluated through this assay.

2. Objectives

The potential toxicity inherent to CNFs is one of the least explored topics, so it is important to study their outcomes to promote the development of safe CNFs and thus a more responsible and sustainable industry.

The main objective of this work was to contribute to the safety evaluation of cellulose nanofibrils produced from *Eucalyptus globulus* by a combination of high-pressure homogenization with distinct pre-treatments in human respiratory tract cells.

To achieve this purpose, using *in vitro* methodologies, the specific aims were:

- Explore the internalization of the CNFs with different properties by cells.
- Evaluate their biological effects through cytotoxicity and genotoxicity assays.
- Compare the biological effects caused by each type of CNFs, to try to understand the most relevant properties that may mediate toxicity, if existent.

3. Materials and Methods

3.1. Nanocellulose Production and Characterization

The nanocelluloses used in this study were produced from an industrial bleached *Eucalyptus globulus* kraft pulp (BEKP), in the Chemical Engineering department of the University of Coimbra. Due to worries in previous studies about possible bacterial contamination, the kraft pulp that originated these samples was autoclaved (120ºC, for 15 min).

In order to facilitate fiber disintegration, two different pre-treatments were applied: CNF TEMPO suffered an oxidation mediated by 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), according to a procedure described elsewhere (Lourenço et al. 2017; Saito and Isogai 2007). CNF Enzymatic was hydrolysed with endoglucanases by a similar process described by Tarrés *et al.* (2016), Afterwards, a mechanical treatment was performed on a high-pressure homogenizer. The pre-treated fibers were passed twice in the homogenizer, the first at 500 bar and the second at 400/1000 bar (TEMPO/Enzymatic), in order to reduce the fiber size to nanoscale (Ventura et al. 2018).

As a result, the final consistency of nanocellulose suspensions was 0.77 wt% for CNF TEMPO – exhibiting gel-like behaviour – and 0.85 wt% for CNF Enzymatic, showing a more liquid aspect. After being produced, the samples were kept cold to better preserve their properties.

After their production, nanocelluloses were characterized by their fibrillation yield, number of carboxylic groups (CCOOH) attached to the cellulose chain as well as the degree of polymerization (DP) and the data obtained for the two samples are presented in **table 3.1**. The yield was determined in duplicate by submitting 40 mL of 0.2 wt% dispersions to centrifugation at 9000 rpm for 30 min (Gamelas et al. 2015). The carboxylic content (CCOOH) was determined from a conductivity curve, for this purpose, a conductometric titration of the aqueous suspensions of CNFs was performed according to a methodology reported elsewhere. DP was calculated by intrinsic viscosity measurements using the Mark-Houwink equation, requiring dissolution of the CNF samples in cupriethylenediamine (Lourenço et al. 2017).

Moreover, in order to control possible bacterial contamination, it is recommended that prior to the use of CNF samples, a process of autoclaving should be performed, especially when there are results indicating that their inoculation in certain media revealed contamination. However, the possible consequences of the autoclaving process on the structure and composition of nanocelluloses are not fully known. Therefore, we chose not to autoclave before use and there did not appear to exist any apparent contamination throughout the experiments.

Table 3.1 - Data obtained for the characterization of CNF samples.

CNF Sample	Pre-treatment	Dry Content (%)	Yield (%)	C_{COOH} (µmol/g)	DP	Zeta Potential (mV)
TEMPO	NaClO oxidation mediated by TEMPO	0.77	100	1408	365	-60
Enzymatic	Enzymatic Hidrolysis (endoglucanases)	0.85	ND*	ND*	1591	-20

*ND- value not determined

3.2 Cellular Assays

3.2.1 Cell Culture and Maintenance

The A549 cell line (ATCC® CCL-185™; VA, USA) used in this study represents the human alveolar epithelium and is derived from a 58-year-old Caucasian male lung carcinoma. It has an epithelial-like morphology and is adherent under culture conditions. The doubling time of this cell line is approximately 22 hours.

The growth medium used for the A549 cell cultures (ATCC® CCL-185™) was Dulbecco's Modified Eagle Medium (DMEM; with 1g/L glucose, L-glutamine and pyruvate) supplemented with 10% heat-inactivated fetal bovine serum (FBSi), 1% penicillin/streptomycin mix (Pen/Strep; with 10000 units/mL penicillin and 10000 µg/mL streptomycin), 1% fungizone (amphotericin B; 250 µg/mL) and 2.5% HEPES buffer solution (1M) (Invitrogen, CA, USA). Cells were maintained in culture flasks in an incubator, at 37 °C and 5% CO_2. When the cells reached approximately a confluence of 80%, a subculture was performed: the culture medium was removed, the cells were washed with preheated sterile PBS (1X) and incubated at 37°C for 5 minutes, with preheated trypsin-EDTA (0.05%) (Invitrogen, CA, USA). Then culture medium was added to inactivate the trypsin-EDTA. With detached cells from the flask, viable cells were counted using Trypan Blue dye and the cell density was determined. Cells were seeded at appropriate dilutions in new culture medium and incubated in the same conditions as before (**figure 3.1**). All these reagents were provided by Gibco (Scotland, UK). Cell culture was continuously controlled for mycoplasma, for which no contamination signal was detected.

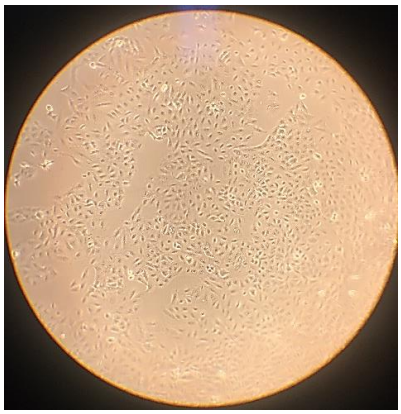

Figure 3.1 - A549 cells in DMEM culture medium. A549 cells at approximately 70% confluence, forming "colonies" of cells interspersed with some empty spaces. Despite the high cell-cell adhesion, it is possible to distinguish the normal morphology of cells, which are elongated and growing in monolayer. (Telaval 31, Zeiss, 630x Magnification)

3.2.2 CNFs Solution

Some NMs show a high tendency to form agglomerates/aggregates. In this case, constant resuspension is required to maintain a homogeneous solution (Magdolenova et al. 2014).

CNFs were dispersed in PBS at stock concentrations of 770 µg/mL for CNF TEMPO and 850 µg/mL for CNF Enzymatic. For cell exposure, CNF suspensions were then diluted in complete culture medium. Careful homogenization was performed by resuspending the sample with the pipette and with the aid of vortex until no aggregation was visually detected. The solutions have always been freshly prepared and applied immediately to avoid deposition of fibers or small aggregates. The concentration range chosen was based on some guidelines and previous work. All concentrations (µg/mL) applied to 6, 24 or 96 well plates were converted to µg CNF per well area (µg/cm^2).

3.2.3 Cell Uptake Assay

For a correct evaluation and better understanding of the mechanisms responsible for NM toxicity, it is important to show experimentally or based on the literature whether cellular internalization of NMs occurs. This knowledge also allows, in the case of negative results, to discard the hypothesis of using the inadequate cellular model due to the lack of interaction of NM with cellular components.

For this assay, previously sterile coverslips were placed in each well of 24 well plates. Cell cultures were trypsinized, counted and a cell suspension was made. A549 cells were seeded on these 24-well plates containing sterile glass coverslips (Greiner, Germany) at a density of 0.8x10^5 cells/well and incubated at 37°C and 5% CO_2 for approximately 24 hours. After this time, cell growth was observed, and subsequently exposed to 6.25, 12.5 and 25 µg/cm^2 of each type of CNFs diluted in complete culture medium for 24 hours, with the same conditions as before. These concentrations were obtained from a stock solution at 770 µg/mL and 850 µg/mL of a 0.77% CNF (CNF TEMPO) and 0.85% (CNF Enzymatic)

diluted in PBS, respectively. For each experiment, negative control was included and refers to culture media-only.

Two different methodologies were carried out regarding the exposure of CNFs: exposure with previously stained CNFs or exposure to unstained CNFs and a posteriori labelling at the time of fixation. The objective of this difference was to try to understand if the dye interferes with cellular internalization or with the structure of the CNFs themselves. The compound used to stain the CNFs was Calcofluor White, a fluorochrome already explored in previous studies, which stains structures containing chitin and has known excitation and absorbance wavelengths. To stain the cell nuclei was used propidium iodide.

After incubation, the coverslips were transferred to a new 24-well plate. The cells were washed with PBS before and after fixing with cold methanol for 20 minutes, protected from the light. Calcofluor White was then added to the CNFs, or if the CNFs were already stained, only propidium iodide was added and quickly removed. The cells were fixed again with cold methanol for 10 minutes and washed with PBS. The coverslips were air dried and slides were mounted in antifade mounting medium (Vectashield; Sigma-Aldrich, UK).

3.2.3.1 Fluorescence and Confocal Microscopy

Fluorescence was observed and recorded on a fluorescence microscope (Axioplan 2 imaging, Zeiss, Germany) equipped with an *Axiocam HRc* camera (Zeiss, Germany). Image acquisition was possible using Isis Fluorescence Imaging System (MetaSystems, Germany). Due to the software used, the images obtained were already merged.

Confocal microscopy images were acquired on a Leica TCS-SPE equipment (Leica Microsystems, Germany) using the 405 nm (Calcofluor White) and 532 nm (Propidium Iodide) lasers at 20% intensity for excitation. The emitted fluorescence was detected with a spectral detector set at ± 50 nm of the respective emission fluorophore peaks. Analysis in the XYZ axis was performed by capturing 30 sequential XY images in the Z axis at 500 nm intervals.

3.2.4 Cytotoxicity Assays

Cell viability assays allow the detection of cell death following exposure to various concentrations of a compound or material. Furthermore, they are quite useful for determining the correct concentration range for use in genotoxicity assays, since necrosis or apoptosis phenomena are not desired. The effect of CNFs on cell viability was first evaluated by two distinct methods – MTT and clonogenic assays – which measure different endpoints.

For all assays performed, A549 cells were trypsinized, the cell suspension obtained was centrifuged at 1200 rpm for 5 minutes and then resuspended in 2-4 mL of culture medium. From the detached cell

suspension, a sample was taken and a dilution factor of 2 (50:50 µL) was applied by adding Trypan Blue (TB) to the sample. The cells were then counted by microscopic analysis (Telaval 31, Zeiss, Germany) with a Neubauer chamber and the number of unviable cells was compared to the total number of cells. The use of this vital dye allows to assess the integrity of the cell membrane, since TB is unable to penetrate intact cell membranes, which means that it only stains unviable cells.

3.2.4.1 MTT Assay

As mentioned above, the MTT assay is a quantitative method that measures cell viability. This assay uses a soluble dye – MTT – which contains a ring that, when broken by living cell mitochondrial dehydrogenases, gives rise to formazan crystals. These crystals are further dissolved with dimethyl sulfoxide (DMSO) to form a purple coloured compound. Therefore, formation of intracellular formazan is directly proportional to the number of viable cells. Viability is then quantified by spectrophotometric readings, which translate the colorimetric intensity into absorbance values.

A549 cells were plated at a density of $0.5x10^5$ cells/well in 96-well plates and incubated at 37°C and 5% CO_2 for 24 hours. Cells were then exposed for 24, 48 or 72 hours to 1.5, 3.125, 6.25, 12.5, 25 and 50 µg/cm^2 of the two types of CNFs diluted in complete culture medium. These concentrations were obtained from a stock solution at 770 µg/mL and 850 µg/mL of CNF TEMPO and CNF Enzymatic diluted in PBS, respectively. It should be noted that CNF solutions, especially CNF TEMPO, are difficult to disperse. Hence, to avoid the formation of agglomerates, dilutions in the culture medium were always made with the solvent at 37°C, followed by vortexing and resuspended by pipetting.

For positive control, SDS (1 µg/mL; Sigma-Aldrich, UK) at 0.1% diluted in culture medium, from a stock solution at 10% diluted in PBS, was used and added 1 hour before incubation time ended. After each time of incubation, cells were washed with PBS and 100 µL of MTT (5 mg/mL; Sigma-Aldrich, UK) was added at a final concentration of 0.5 mg/mL (previously prepared in PBS and culture medium) to each well and incubated for 3 hours in previously defined conditions. After this time, the MTT-containing medium was removed and 100 µL of DMSO (Sigma-Aldrich, UK) was added in each well to dissolve the formazan crystals. Plates stayed for 30 minutes under agitation and light protected. Finally, absorbance was measured at 570 nm (with a reference filter of 690 nm) on a Multiscan Ascent Spectrophotometer (Labsystems, Helsinki, Finland). Throughout the process, a microscopic control was performed regularly.

Previous tests were performed in order to define an appropriated cell density and an effective concentration of SDS.

For both types of CNFs, at least 3 independent assays were measured, and 6 absorbance values were considered for each condition. Assuming that the absorbance of the unexposed culture represents 100% cell survival, absorbance values were converted to relative cell viability (%) as the following equation:

$$\textbf{Cell Viability}\ (\%) = \frac{\bar{x}\ \textbf{Absorbance}\ (\lambda = \textbf{570 nm})\ \textbf{Exposed Cultures}}{\bar{x}\ \textbf{Absorbance}\ (\lambda = \textbf{570 nm})\ \textbf{Unexposed Culture}}\ \textbf{x 100}$$

3.2.4.2 Clonogenic Assay

The clonogenic assay was performed as described in a study coordinated by the JRC (Report EUR 27009:2014). It is based on the ability of cells to form colonies, allowing to evaluate the proliferative potential of these cells (Herzog et al. 2007). A549 cells were plated in a very low density – approximately 200 cells per well – in a 6-well plate and incubated for 24 hours, at 37°C and 5% CO_2. Then, cells were exposed to CNF TEMPO and CNF Enzymatic at the same concentrations used in the MTT assay (1.5, 3.125, 6.25, 12.5, 25 and 50 µg/cm^2). For each experiment, negative (non-exposed cells), positive (Mitomycin C 0.004 µg/mL final concentration, Sigma-Aldrich, UK) and solvent controls (PBS exposure) were included. Cells were then incubated for 8 days, at 37ºC and 5% CO2 to allow colonies formation. During this time, plates were continuously controlled by observation under the microscope. The cells were then washed twice with PBS, fixed in cold absolute methanol (Sigma-Aldrich, UK) for 10 minutes. After a drying period, the colonies were stained with 10% Giemsa (Merck, Darmstadt, Germany) in phosphate buffer, pH 6.8, for 10 minutes, washed twice with Gurr's phosphate buffer (VWR, PA, USA) and allowed to dry. The colonies were observed and counted with a magnifying glass (Wild M7A, Heerbrugg, Switzerland). Several parameters were analysed, such as the plating efficiency (PE), determined using the following equation:

$$\textbf{PE}\ (\%) = \frac{\textbf{No. of colonies formed Unexposed}}{\textbf{No. of cells seeded}}\ \textbf{x 100}$$

The surviving fraction (SF) for each CNF concentration was calculated as follows:

$$\textbf{SF} = \frac{\textbf{No. of colonies formed Exposed}}{\textbf{No. of cells seeded x PE/100}}$$

The cytotoxicity was determined as the decrease in the SF in when compared to negative control, based on the results from three independent experiments.

3.2.5 Genotoxicity Assay

3.2.5.1 Cytokinesis-Block Micronucleus (CBMN) Assay

The assay was performed according to the indications described in the Guideline 487 (OECD, 2016). Cells were plated at a density of 1x10^5 cells/mL in 6-well plates and incubated for 24 hours, at 37ºC and 5% CO_2. Then, the culture medium was removed, and cells were exposed to previously defined

concentrations – 1.5, 3.125, 6.25 and 12.5 µg/cm^2 – of CNF TEMPO and CNF Enzymatic diluted in complete culture medium and incubated for more 22 hours in same conditions. These dilutions were prepared from a stock solution diluted in PBS with a concentration of 770 µg/mL for CNF TEMPO and 850 µg/mL for CNF Enzymatic. For each experiment, negative (non-exposed cells) and positive (Mitomycin C exposure) controls were included. MMC was chosen since it produces chromosome breaks and is generally used as genotoxic agent for tumour cells.

A first test based on a well-established protocol for A549 cells was performed, however, the procedure was adapted to compare with results already obtained for these CNFs in a previous study with different cells. Thus, after 21 hours MMC (1 µg/mL) was added at a final concentration of 0.1 µg/mL (diluted in PBS and culture medium) and incubated for 1 hour before Cyt-B addition. Following this, MMC was removed and the respective wells were washed twice with PBS, followed by the addition of fresh culture medium. Cyt-B (2 mg/mL; Sigma-Aldrich, UK) with a final concentration of 6 µg/mL was added to each well and the plates were incubated, under the same conditions as above, for more 42 hours.
After this time, cells were washed twice with PBS, detach by trypsinization and inactivate with fresh medium. Then, contents of each replicate (2 wells) were transferred to a 15 mL tube and centrifuged at 1000 rpm for 5 minutes, the supernatant was discarded and the pellet resuspended. Thereafter, cells were submitted to a hypotonic treatment by the addition of a previously prepared and heated solution – DMEM:dH2O:FBSi (37.5:12.5:1) – drop-by-drop, while vortexing. The samples were immediately centrifuged under the same conditions as before and the supernatant discarded. The cells were resuspended and spread on the slides. The cell density was verified and adjusted for each slide. Four slides were made for each replica and allowed to dry at room temperature for 4-6 hours. After this time, slides were fixed in a cold methanol:acetic acid (3:1) solution for 20 minutes. After air-drying, slides were stained with a 4% Giemsa solution diluted in Gurr's phosphate buffer for 15-20 minutes and then washed twice with Gurr's phosphate buffer. Slides were mounted with 2-3 drops of Entellan (Merck, Darmstadt, Germany) and a coverslip.

Several parameters resulting from this assay were considered, using the criteria described by Fenech (2007). Nonetheless, some issues are important to refer: cells should have a well-limited cytoplasm and normal nucleus morphology; MN should only be considered if had until 1/3rd diameter of the main nuclei; NBs must be linked to main nucleus and NPBs have to present a width of 1/4th of the diameter of the nuclei. All these biomarkers should have the same staining intensity as the main nuclei.

Slides were scored under an optical microscope (Axioskop 2 Plus, Zeiss, Germany) with 400x magnification to assess the amount of mono-, bi- and multinucleated cells and 1000x magnification to confirm MN, BNPs and NBs. Apoptotic and necrotic cells were not considered in this work. At least 2000 binucleated cells from two independent cultures were scored per treatment condition.

The Cytokinesis-Block Proliferation Index (CBPI) and Replication Index (RI) were calculated as presented in the equations below (OECD, 2016):

$$\boldsymbol{CBPI} = \frac{(\boldsymbol{No.\ Mononucleate\ cells}) + (\boldsymbol{2\ x\ No.Binucleate\ cells}) + (\boldsymbol{3\ x\ No.Multinucleate\ cells})}{\boldsymbol{Total\ number\ of\ cells}}$$

$$\boldsymbol{RI} = \frac{\left(\frac{\mathbf{(No.Binucleate\ cells) + 2\ x\ No.Multinucleate\ cells}}{\boldsymbol{Total\ number\ of\ cells}}\right)\mathbf{Treated}}{\left(\frac{\mathbf{(No.Binucleate\ cells) + 2\ x\ No.Multinucleate\ cells}}{\boldsymbol{Total\ number\ of\ cells}}\right)\mathbf{Untreated}}\ \boldsymbol{x}\ \mathbf{100}$$

3.2.6 Statistical Analysis

Data obtained from independent experiments were expressed as mean and standard error of mean (mean ± SEM) for cytotoxicity assays and as mean and standard deviation (mean ± SD) for genotoxicity assays.

In the cytotoxicity assays - MTT and clonogenic - after confirming the data normality, statistical comparisons between exposed cells and control were performed using One-way ANOVA analysis, followed by Tukey's multiple comparison test. The Student's t-test served to analyse significant differences between controls and between different exposure times. In the micronucleus assay, the two-sided Fisher's exact test was applied to compare the frequency of micronucleated cells (MNBNC) obtained in cultures exposed to the different concentrations with the negative control. The Student's t-test served to analyse significant differences between the positive and negative control. The same test was also used to compare CBPI and RI results between exposed cells and control. A p-value<0.05 was accepted as statistically significant. All statistical analysis of the results was performed in IBM SPSS statistics v.20.

4. Results

4.1 Nanocelluloses Production and Characterization

As aforementioned, CNF TEMPO was subjected to TEMPO-mediated oxidation while CNF Enzymatic suffered enzymatic hydrolysis. To evaluate which properties could be more significant for cytotoxicity and genotoxicity tests, both CNFs were characterized (**table 3.1**).

The yield corresponds to the percentage of material that was effectively transformed to nanoform. The CNF TEMPO obtained a maximum yield of 100%, which suggests that the entire sample subjected to treatment is in the nanoscale. Regarding enzyme pre-treatment (CNF Enzymatic) a large heterogeneity in fibril thickness was obtained which led to a large deviation. Thus, although the value has not been determined, based on previous experience, we know that its yield range is around 10-20%. Thus, the yield obtained for CNF Enzymatic is low compared to that of CNF TEMPO, which means that only a portion of it is in nanometric format, being mostly micrometric. For this reason, this CNF should be more properly named cellulose microfibrils (CMF). However, the denomination CNF Enzymatic will be kept for coherence along the text.

Measurement of carboxylic content only applied to CNF TEMPO, since in the case of CNF Enzymatic no functional groups were introduced. Therefore, its value should be similar to the source kraft pulp (BEKP), being between 130-150 µmol/g. Regarding the DP, the CNF Enzymatic has a higher value when compared to the CNF TEMPO, justified by the larger dimensions of the obtained fibers.

4.2 Cellular Assays

A valuable part of this work is reproduced by *in vitro* cellular assays. Both types of CNFs, CNF TEMPO and CNF Enzymatic, were evaluated for their biosafety in a pulmonary epithelial cell line (A549), described above.

4.2.1 Uptake Assay

Distinguishing between internalized and non-internalized NMs is extremely important to understand the results obtained in cytotoxicity and genotoxicity assays, so the uptake assay should be considered the first step for the toxicological evaluation of nanocelluloses. In this context, the particles were stained with a fluorescent dye, Calcofluor white, with known wavelengths of excitation and absorbance in which the amount of fluorescence was observed by microscopy techniques.

4.2.1.1 Fluorescence Microscopy

Using fluorescence microscopy techniques, an optical analysis of the two Calcofluor white-labelled nanocelluloses was performed. As described in the materials and methods, a two-staining process was used, which allowed to distinguish the nuclear compartment in the observed cells. **Figures 4.1 and 4.2** show the acquired images, which allowed the visualization of the behaviour of both types of CNFs, at concentrations of 6.25 and 25 µg/cm^2 in cell culture. Other concentrations were tested but found to be of little significance for the purpose. Based on these images, it was possible to verify that these nano-objects do not appear to be located in the nuclear compartment and that the interaction between them and the cells seems to be superficial, through the formation of a cellulose matrix on the cell surface.

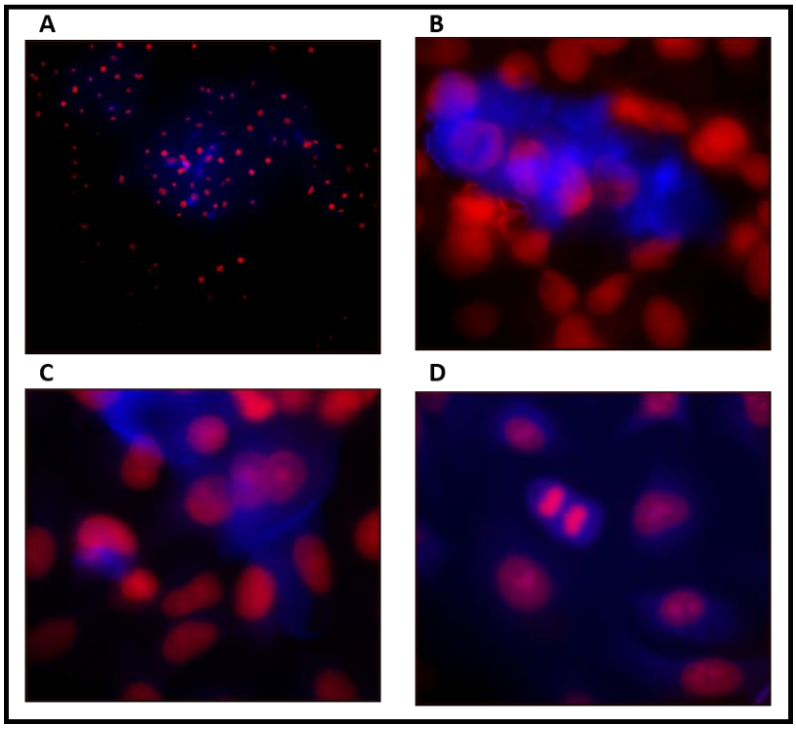

Figure 4.1 - Fluorescence microscopy images showing CNF TEMPO (blue) and A549 cells (nucleus, red) after 24h incubation. (A) and (B) with 25 µg/cm^2, at 100x and 630x magnification, respectively; and (C) and (D) with 6.25 µg/cm^2, 630x magnification (Axioplan 2 imaging, Zeiss).

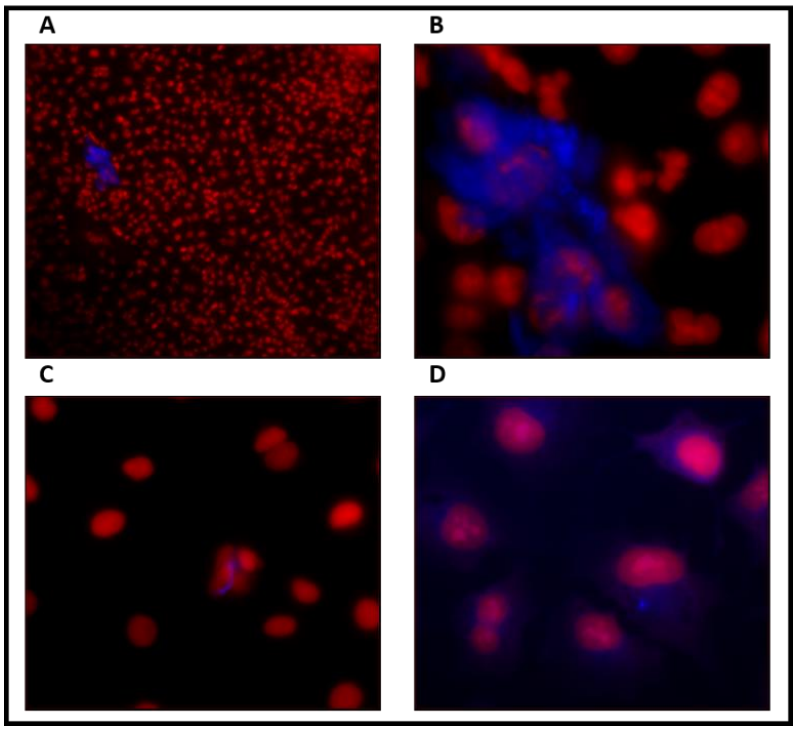

Figure 4.2 - Fluorescence microscopy images showing CNF Enzymatic (blue) and A549 cells (nucleus, red) after 24h incubation. (A) to (C) with 25 µg/cm^2, 100x and 630x magnification and (D) with 6.25 µg/cm^2, 630x magnification (Axioplan 2 imaging, Zeiss).

Moreover, it was not possible to notice in the images present in **figures 4.1 and 4.2** an effect dependent of the internalization of both CNFs related to the tested concentrations, that is, a direct relation between the amount of CNFs available and the cellular uptake was not observed.

4.2.1.2 Confocal Microscopy

In order to obtain more information about the uptake of these nanofibrils, the samples were observed by confocal microscopy. This technique allows a more in-depth analysis, making it possible to have a tridimensional image (3D images). **Figures 4.3 and 4.4** show both Calcofluor-white-labelled CNFs (blue sign). Given the greater complexity of this type of analysis, only one concentration (25 µg/cm^2) was selected for each nanocellulose.

Although intense labelling of the two nanocelluloses are seen, CNF Enzymatic seems to be less present around cells compared to CNF TEMPO.

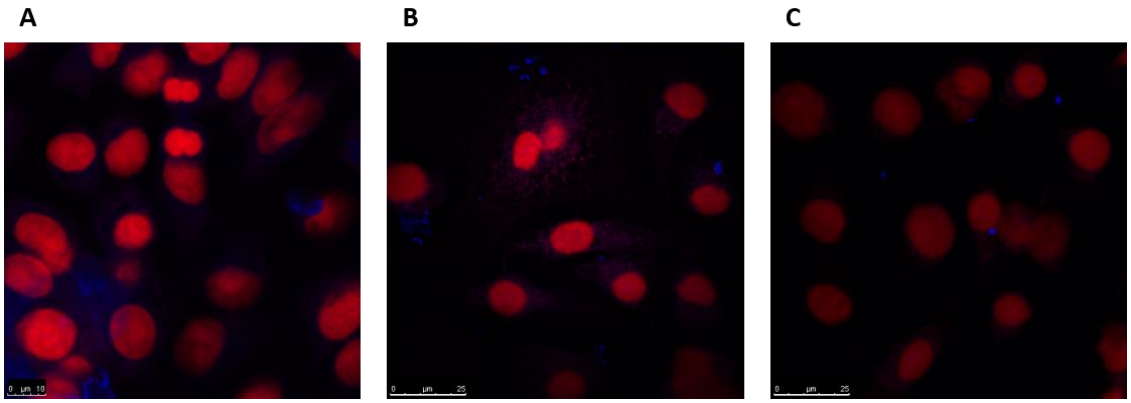

Figure 4.3 - Confocal microscopy images showing CNF TEMPO (blue) and A549 cells (nucleus, red) after 24h incubation (Leica TCS-SPE, Leica Microsystems). (A) is on a scale of 10 µm, whereas (B) and (C) are on a scale of 25 µm.

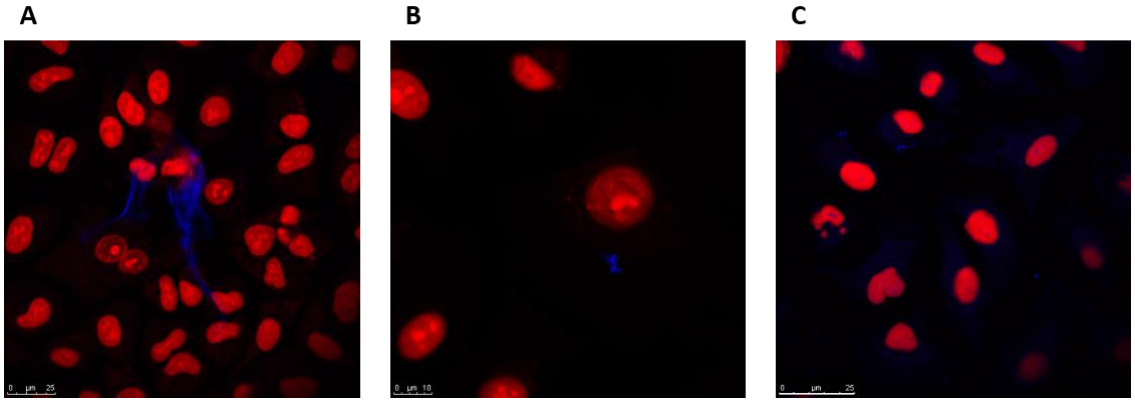

Figure 4.4 - Confocal microscopy images showing CNF Enzymatic (blue) and A549 cells (nucleus, red) after 24h incubation (Leica TCS-SPE, Leica Microsystems). (B) is on a scale of 10 µm, whereas (A) and (C) are on a scale of 25 µm.

4.2.2 Cytotoxicity Assessment

Cytotoxicity was assessed by two assays, covering different endpoints: alterations in cell metabolic activity (MTT assay) and the ability of cells to proliferate in the presence of nanofibrils (clonogenic assay).

The MTT results are plotted in **figures 4.5 to 4.7**, as the mean (Mean ± SEM) percentage of viable cells, relative to the negative control (assumed as 100% viability). For both CNFs, four independent experiments with 24 hours exposure and three independent experiments for 48 and 72 hours exposure were considered. SDS was used as a positive control and was strongly lethal to A549 cells in all assays.

The shorter exposure time explored showed no significant decrease in cell viability ($p > 0.05$). On the contrary, there was a slight increase in most of the tested concentrations of the two nanocelluloses (**figure 4.5**).

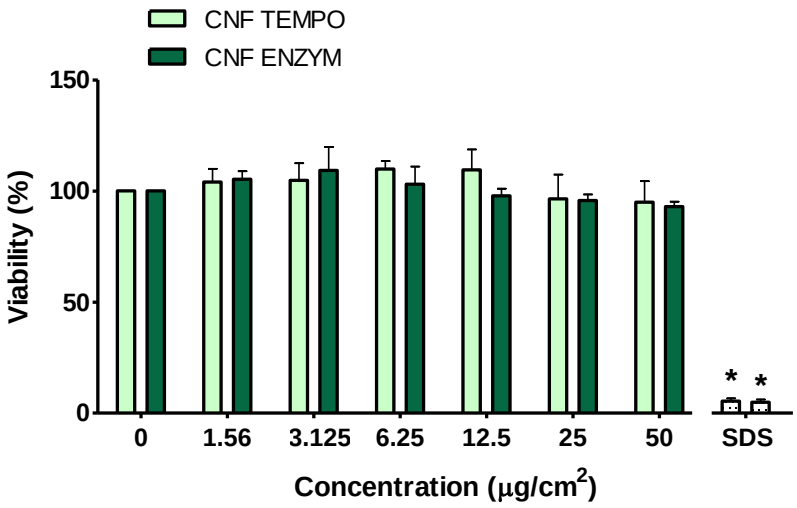

*Figure 4.5 - Results of the MTT assay on A549 cells after 24 hours of exposure to CNF TEMPO and CNF Enzymatic. SDS is the positive control. * significantly different from control (p<0.05).*

After 48 hours of exposure (**figure 4.6**), cells exposed to CNF TEMPO were generally more viable, reaching a maximum value of 113.30% at a concentration of 6.25 μg/cm^2 and a minimum of 96.85% at a concentration of 25 μg/cm^2 (p>0.05). While the percentage of viable cells exposed to CNF Enzymatic decreased slightly, reaching 80.24% at the highest concentration – 50 μg/cm^2 (p>0.05).

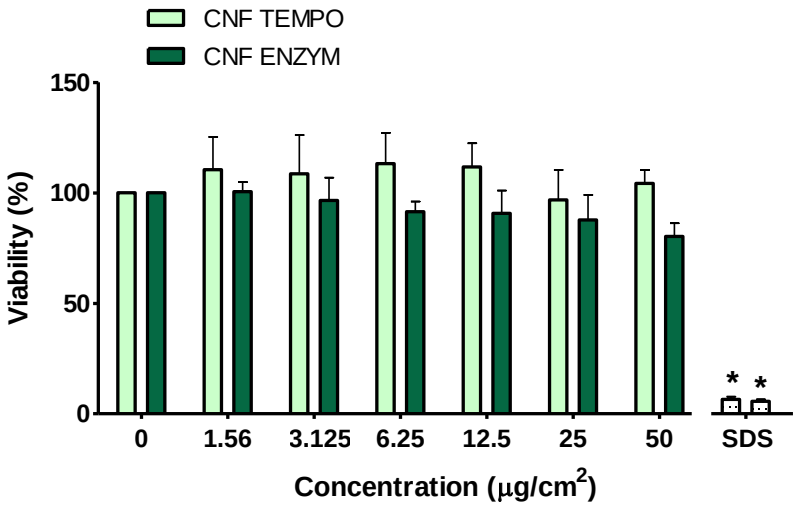

*Figure 4.6 - Results of the MTT assay on A549 cells after 48 hours of exposure to CNF TEMPO and CNF Enzymatic. SDS is the positive control. * significantly different from control (p<0.05).*

At 72 hours of exposure (**figure 4.7**), the behaviour of cells incubated with CNF TEMPO or CNF Enzymatic was similar to that mentioned for the other timepoints, and any significant variation in cell viability was detected (p>0.05)

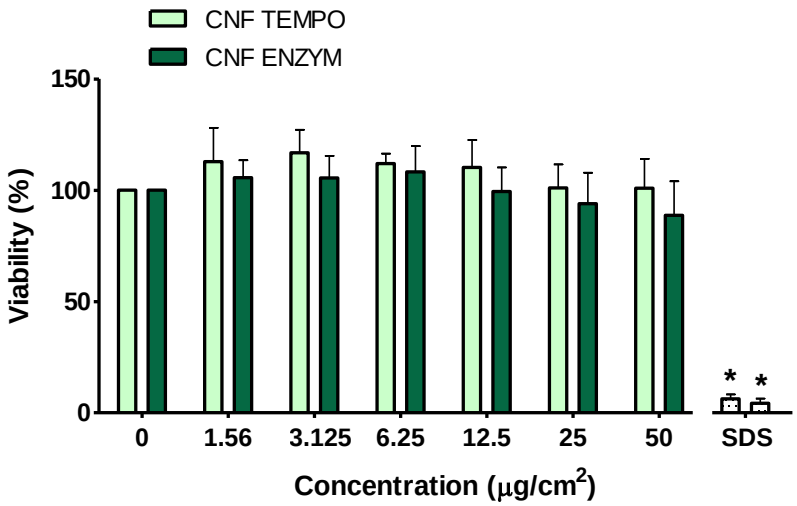

*Figure 4.7 - Results of the MTT assay in A549 cells after 72 hours of exposure to CNF TEMPO and CNF Enzymatic. SDS is the positive control. * significantly different from control (p<0.05).*

The clonogenic assay has a longer exposure time and allows to assess the ability of a single cell to form a colony while exposed to an NM (**figure 4.8**). The results obtained in A549 cells exposed to each of the CNFs studied are shown in **figure 4.9**. For each nanocellulose three independent experiments were considered.

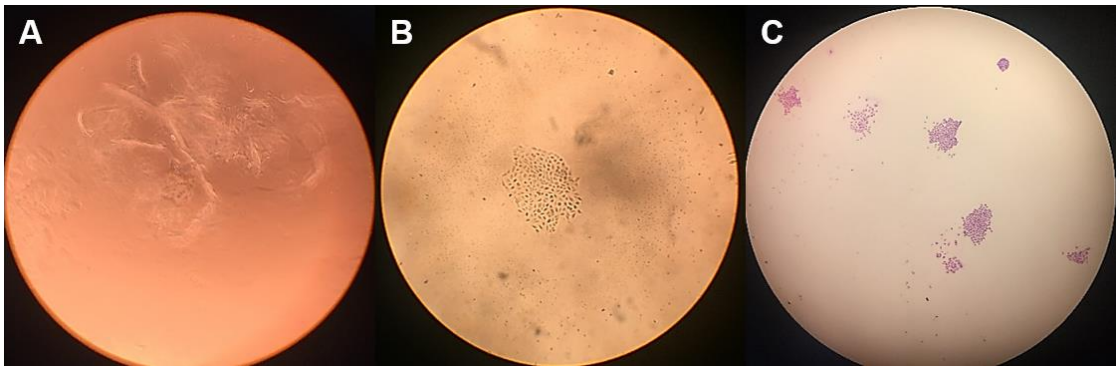

Figure 4.8 - Representative images of the different stages of the clonogenic assay in A549 cells exposed to CNF Enzymatic. (A) Cultured cells incubated with CNF Enzymatic, which is visible above the cells, (B) Colony formed from a single cell after 8 days of exposure (Telaval 31, Zeiss. 630x Magnification) and (C) Fixed and stained colonies for further evaluation of CNF toxicity (Wild M7A, Heerbrugg).

In exposure to both CNF TEMPO and CNF Enzymatic, no statistically significant decreases in cell viability, and therefore proliferative capacity, were observed at all concentrations tested ($p>0.05$). In fact, an increase in the number of formed A549 colonies was detected when exposed to CNF TEMPO, with a maximum value of 143.66% at the intermediate concentration of 12.5 μg/cm^2. Cells exposed to CNF Enzymatic maintained viability similar to the control except at the highest concentration – 50 μg/cm^2 – which reached 129.61%. MMC was used as a positive control and was significantly toxic to A549 cells as compared to the negative control ($p<0.001$).

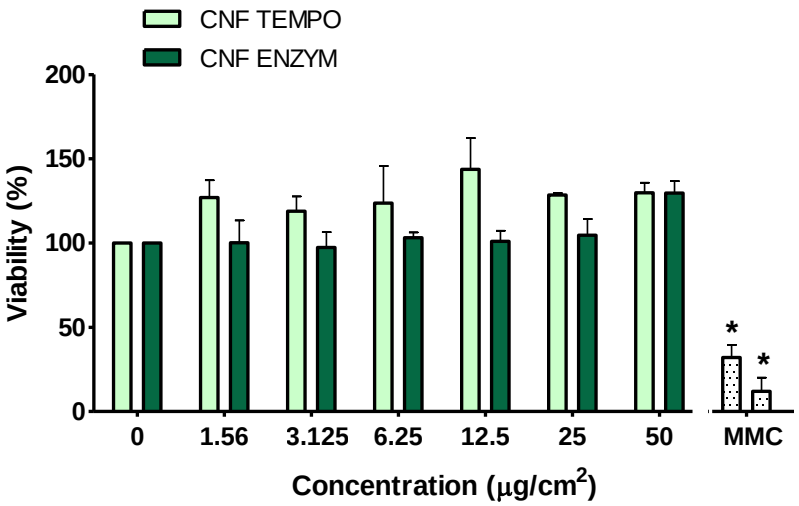

*Figure 4.9 - Results of the clonogenic assay in A549 cells after 8 days of exposure to CNF TEMPO and CNF Enzymatic. MMC was used as a positive control. * significantly different from control (p<0.05).*

4.2.3 Genotoxicity Assessment

The genotoxic effects of the studied CNFs were evaluated by the CBMN assay. Of the concentrations used in cytotoxic assays, the lowest four between 1.56 e 12.5 μg/cm^2 were selected. For both nanocelluloses two independent cell cultures were considered.

This assay allowed us to evaluate several endpoints, such as the number of binucleated cells containing micronuclei, proliferation index (CBPI) and replication index (RI). To determine these indices, the proportion of mono-, bi- and multinucleated cells was also taken in account (**figure 4.10**).

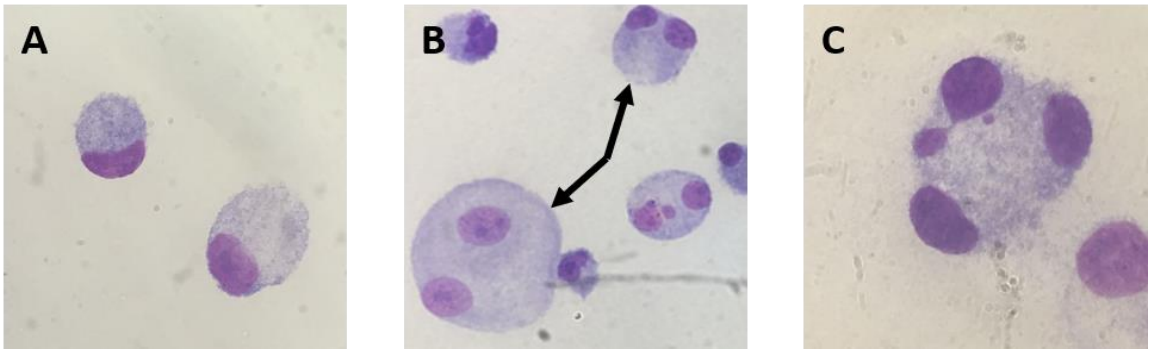

Figure 4.10 - Photographs of mononucleated (A), binucleated (B) and multinucleated (C) A549 cells. (Axioskop 2 Plus, Zeiss. 1000x Magnification).

Although exposure to CNF TEMPO had led to an increase in the frequency of micronucleated binucleated cells (MNBNC) at all concentrations tested, no value reached statistical significance (p>0.05). Similar results were observed in cells exposed to CNF Enzymatic, except for the concentration of 3.125 μg/cm^2, where a slightly lower value was recorded. The positive control used (MMC) recorded a significant increase. Results are plotted in **figure 4.11**.

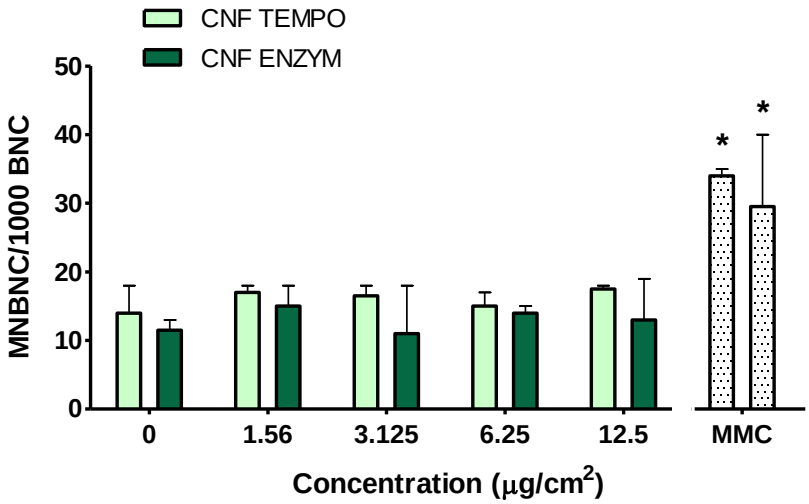

*Figure 4.11 - Frequency of micronucleated binucleated cells (MNBNC) per 1000 BN cells. The graph represents the output obtained in A549 cells exposed to CNF TEMPO and CNF Enzymatic. MMC was used as a positive control. * significantly different from control (p<0.05)*

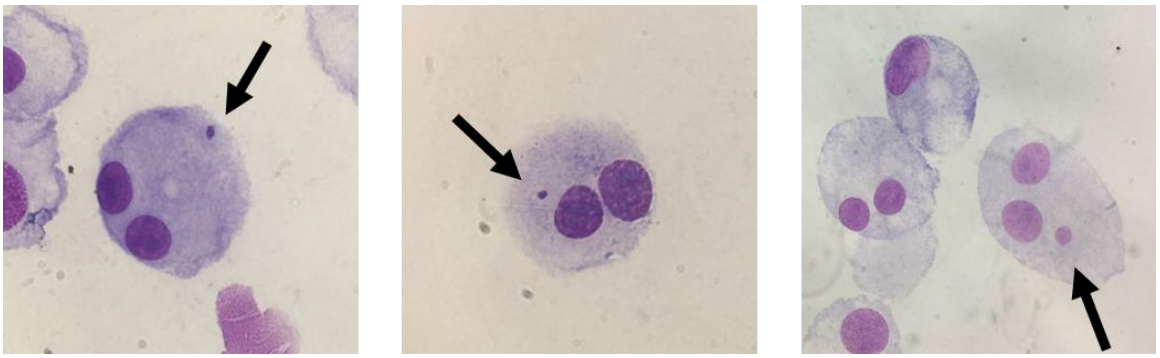

Figure 4.12 - Photographs of binucleated A549 cells with micronuclei (black arrows). (Axioskop 2 Plus, Zeiss. 400x and 1000x Magnification).

In addition to micronucleus analysis, other phenomena that can be observed in this assay, such as the formation of nuclear buds (NBs) and/or nucleoplasmic bridges (NBPs), have been considered and recorded. It is depicted in **figure 4.15**.

In terms of NBs frequency, shown in **figure 4.13**, none of the CNF TEMPO concentrations were shown to give rise to a significant number of bud-binucleated cells compared to the negative control. For CNF Enzymatic exposure, the highest values occurred at the lowest concentrations (1.56 e 3.125 μg/cm^2), although they were not statistically significant (p>0.05).

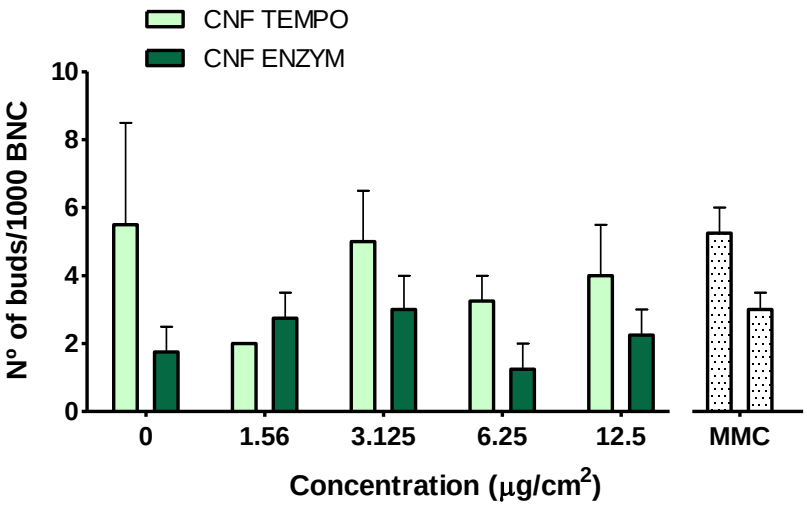

Figure 4.13 - Frequency of binucleated cells with NBs per 1000 BNC. The graph represents the output obtained in A549 cells exposed to CNF TEMPO and CNF Enzymatic. MMC was used as a positive control.

Regarding the frequency of NPBs in binucleated cells, as shown graphically in **figure 4.14** – except for the lowest concentration (1.56 μg/cm^2) at which a slight increase was recorded – exposure to CNF TEMPO did not induce the formation of nucleoplasmic bridges in A549 cells. In the case of the positive control (MMC) a significant increase was observed.

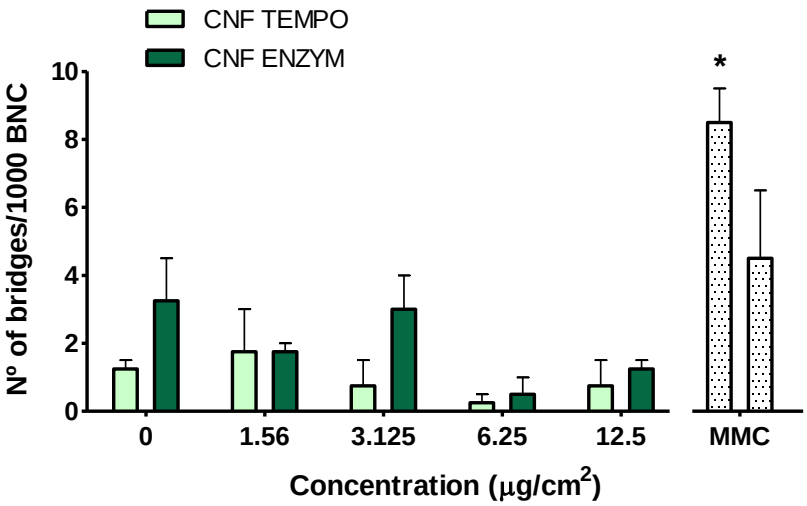

*Figure 4.14 - Frequency of binucleated cells with NPBs per 1000 BNC. The graph represents the output obtained in A549 cells exposed to CNF TEMPO and CNF Enzymatic. MMC was used as a positive control. * significantly different from control (p<0.05).*

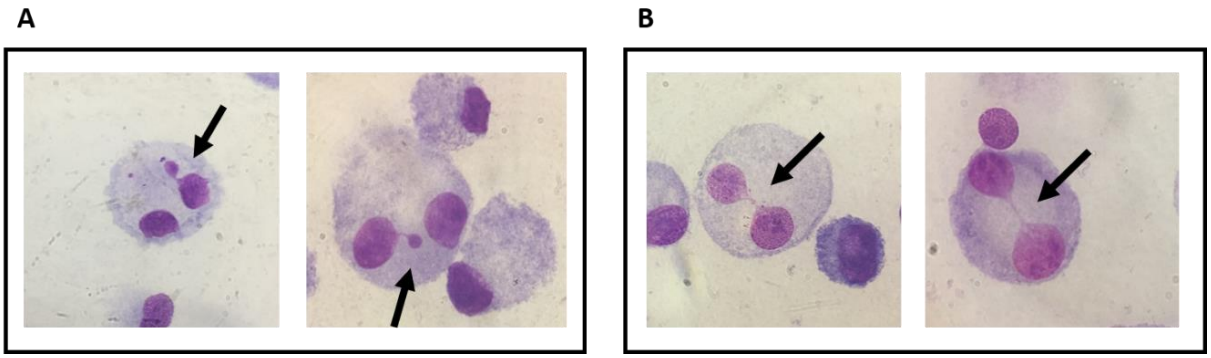

Figure 4.15 - Photographs of binucleated A549 cells with NBs (A) and NPBs (B). Note the similarity between buds and micronuclei, being distinguished by the existence of a bridge connecting to the main nucleus in the buds. The first cell also has micronuclei (Axioskop 2 Plus, Zeiss. 1000x Magnification).

The CBMN assay can also provide information on cytotoxic effects by analysing proliferation (CBPI) and replication (RI) indices (OECD, 2016).

Figures 4.16 and 4.17 show the values of CBPI and RI, respectively. CBPI allows to analyse changes in cell cycle progression, while RI allows to assess cellular capacity to enter mitosis. Treatment with CNF TEMPO appears to have significantly stimulated both cell proliferation and replication ($p<0.002$ and $p<0.007$, respectively, at a concentration of 12.5 µg/cm^2). Similarly, CNF Enzymatic seems to have slightly increased these parameters, especially at a concentration of 6.25 µg/cm^2 ($p<0.02$ and $p<0.02$, respectively). In contrast, at the lowest concentration there was a significant decrease in both indices ($p<0.001$).

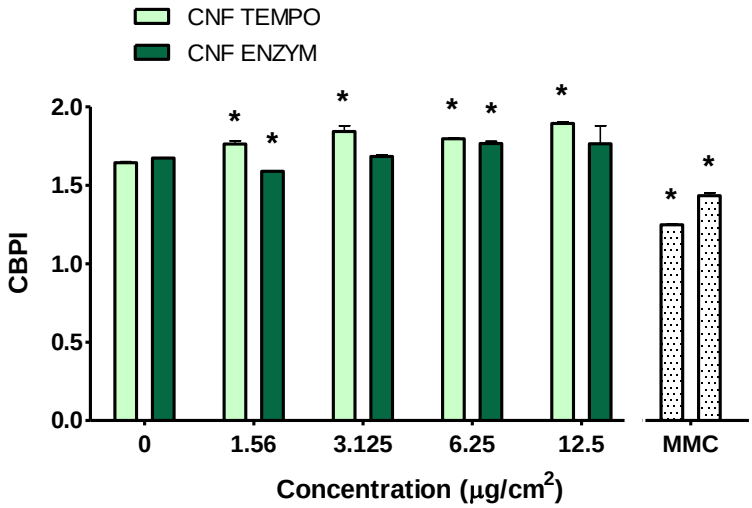

*Figure 4.16 - Estimated CBPI for A549 cells after treatment with CNF TEMPO and CNF Enzymatic. MMC was used as a positive control. * significantly different from control ($p<0.05$).*

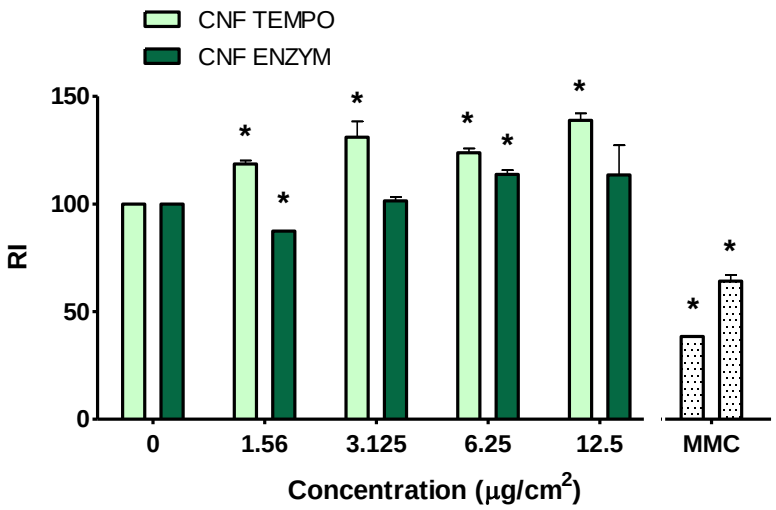

*Figure 4.17 - Estimated RI for A549 cells after treatment with CNF TEMPO and CNF Enzymatic. MMC was used as a positive control. * significantly different from control ($p<0.05$).*

5. Discussion

The use of NMs has led to a growing debate about their possible adverse effects on human health and the environment. Their inevitable presence in an occupational or environmental context justifies the need for their characterization. A good understanding of their entry routes and the consequent effects in our organism will help to better assess the risk associated with exposure to those NMs (Medina et al. 2007).

Due to their physicochemical properties and the vast applications for which they have been studied, especially those in the biomedical field, it is expected that the production and presence of nanocellulosic materials will be increasingly frequent. From their ability to incorporate various bioactive substances and form an essential matrix for wound healing, to the use of nanocellulose membranes for bone regeneration and possible application in sutures, due to the preservation of properties in moist environments (Halib et al. 2017), there is definitely plenty to explore about this nano-scale biomaterial.

Although cellulose nanofibrils have numerous advantages, they can trigger a sequence of harmful biological effects that had not been demonstrated in non-nanometric equivalent materials. Since CNFs have structural similarities to carbon nanotubes and asbestos, there are some studies that demonstrate their toxicity (Ilves et al. 2018). Thus, there is a need to ensure the development and application of safe CNFs for human health and the environment in order to reduce the possibility of unpredictable effects after their introduction in the market.

In vitro studies on cell lines allow us to reduce ethical problems and better control some variables that may influence the results. They may also provide information on the mechanisms underlying the effects of NMs on cells. The combination of different assays with specific targets within the cell is highly recommended as it provides a more reliable assessment of toxicity (Collins et al. 2017). For this purpose, different *in vitro* assays commonly used to evaluate relevant biological effects of nanofibrils were performed in this study.

The cell type under study also influences the methods chosen, as some are better suited for adherent cells and others for cells that grow in suspension (Magdolenova et al. 2014). For this study, epithelial cells covering the human respiratory tract (A549) were selected due to the fact that the major route of exposure to these nanofibers is through inhalation, especially in occupational settings, a close contact between the nanocelluloses and these cells within the body is likely to occur.

This study aimed to contribute to the safety evaluation of cellulose nanofibrils from *Eucalyptus globulus* by characterizing their toxicity in human cells, before their production is expanded.

5.1 Nanocellulose Production and Physicochemical Characterization

The characterization of the physicochemical properties of cellulose nanofibrils produced by different methods is of utmost relevance for practical studies and possible future applications. The amount of NM produced, the rheology of dispersion, particle size and size distribution (including aspect ratio), crystallinity and surface charge are key points that should be addressed (Kangas et al. 2014).

Following CNFs production, the physicochemical characterization showed that the CNF TEMPO reached a high degree of fibrillation (yield), with 100% of the sample in the nanometer scale. Their pre-treatment involves introducing a high number of carboxylic groups into the cellulose molecules, as can be confirmed by the CCOOH value (µmol/g) (**table 3.1**). The addition of negative charges causes repulsion between nanofibrils, facilitating the defibrillation process. Thus, subsequent high-pressure homogenization effectively separated and broke the fiber chains, producing a nanocellulose sample with low DP. Previous studies state that the higher the intensity of the associated mechanical treatment, the higher the percentage of fibrillated material obtained (Gamelas et al. 2015). The results are consistent with those found in the literature for CNF produced by TEMPO-mediated oxidation (Ventura et al. 2018; Lourenço et al. 2017; Saito and Isogai 2007) and suggest an inverse association between yield and quantity of carboxyl groups and the size of the fibrils. The same was not true for CNF Enzymatic, as there is a noticeable reduction in the efficiency of its production process. This resulted in an extremely low yield, meaning that most of the sample is at micrometer scale. It has been shown that physicochemical characteristics of nanofibrils, such as size, affect their behaviour in biological systems. The larger dimensions of CNF Enzymatic fibers may preclude the ability to cross the cell and/or nuclear membrane of cells. This limitation in obtaining good yielding CNFs through enzyme pre-treatments may be one of the reasons for the lack of published studies on its toxicity. The CNF Enzymatic presented a higher DP than the CNF TEMPO (**table 3.1**), therefore it can be concluded that the two samples differ in the fibrils thickness. As indicated in **table 3.1**, the number of carboxylic groups is substantially lower in the case of CNF Enzymatic. Consequently, due to the lower number of repulsive loads, there is an increase in the tendency of fiber aggregation.

In order to make the study more accurate and complete, other important parameters should be determined, such as sample surface charge or exact particle size. The first is obtained from the zeta potential, being the values for CNF TEMPO around -60mV and for CNF Enzymatic -20mV. Negative values are usually justified by the presence of carboxylates (COO-) on the surface of cellulose nanofibers, generated during oxidation pre-treatment with TEMPO or equivalent (Gamelas et al. 2015). For particle size and particle size distribution, there is still a gap in the availability of standard methods or validated techniques for the evaluation of nanomaterials with a high aspect ratio. Light scattering techniques, such as dynamic light scattering (DLS), can be used, however it is more suitable for spherical particles or close to it. Cellulose nanofibrils are closer to a cylindrical shape. Thus, microscopy techniques, such as high-resolution scanning electron microscopy, transmission electron microscopy (TEM) and atomic force microscopy (AFM) are most appropriate for visualizing CNFs. Nonetheless,

these methods are considered time consuming, very operator-dependent and only allow the analysis of small sample quantities. DLS, in addition to allowing the measurement of a significant number of fibrils, may also be useful in providing relevant information regarding size comparison between samples with similar structures (Gamelas et al. 2015). This technique can avoid most of the disadvantages of microscopy but is apparently affected by the shape of nanomaterials. For this reason, both methods can be considered complementary.

For CNFs with TEMPO-mediated pre-treatment, Ventura *et al.* (2018) determined the size of the fibrils using AFM and confirmed by DLS, detecting a diameter distribution between 20-25 nm. In the case of length, it could not be determined by AFM as they are several micrometers in length. Lourenço *et al.* (2017) based on the same methods, obtained a wider distribution of diameters between 5-40 nm. For enzyme pre-treated CNFs, Ilves *et al.* (2018) used fibrils with a width between 2-15 nm but with a micrometer length. The difference between our approximate values and those presented by these authors may be related to the origin of the CNF or the enzymatic treatment performed, given the lack of specifications.

5.2 Cellular Assays

5.2.1 Uptake Assessment

Studying the cellular internalization of particles is extremely important, as some toxic effects can occur only if they are internalized. In addition, for certain therapies, when a NM is designed to act as a dynamic drug delivery system, its uptake mechanism should be evaluated with the intention of not only relating possible toxic events to NM internalization but also to assess uptake efficiency of target cells.

In this context, A549 cell line was exposed to three different concentrations of CNF TEMPO and CNF Enzymatic. Assays were performed with labelled CNFs at two different times: before cells exposure and after incubation with cells. A qualitative analysis using fluorescence microscopy (**figures 4.1 and 4.2**), suggested that both types of CNFs apparently did not penetrate the cell nucleus and did not even appear to be present in the cytoplasmic compartment. However, 2D images do not allow distinguishing between intracellularly located and surface bound NMs.

To better clarify the possibility of nanocelluloses uptake, confocal microscopy studies were performed (**figures 4.3 and 4.4**) and showed that CNF Enzymatic seems more dispersed in the medium, being less present around cells compared to CNF TEMPO. The fact that the latter is visible near and around the cells, while CNF Enzymatic appears more distributed and in less quantity may confirm the hypothesis that smaller fibrils will produce greater interactions than larger ones.

The results of confocal microscopy show coherence with previous observations and appear to be

consistent with the hypothesis that particle size and shape have a major influence on their internalization. In the case of nanocelluloses, due to the higher heterogeneity of CNF Enzymatic fibers, it seems to have less interaction with cells. Despite the distinctions in some properties of the studied CNFs, this assay was not sufficient to detect possible differences between CNF TEMPO and CNF Enzymatic internalization.

Considering the surface charge, it is expected a greater internalization of positively charged particles considering the attraction exercised by the cytoplasmic membrane of negatively charged cells (Graça et al. 2017). Our results appear to be consistent with this information, as both CNFs used are negatively charged, so logically their internalization would be less facilitated.

It is also important to have in mind that higher cell surface adsorption may not mean a higher level of NM internalization, i.e., the fact that cell binding is verified may not represent better uptake efficiency. For a better distinction between internalized and adsorbed NMs on the cell surface, complementary techniques should be used, such as the use of Trypan Blue (TB) dye. This dye acts as a fluorescence blocker as it is unable to penetrate intact cell membranes. Subsequently, TB allows to avoid fluorescence background signals from extracellular NMs and to restrict data measurement to internalized NMs (Vranic et al. 2013).

Another approach of interest is to evaluate a larger range of CNFs concentrations or evaluate uptake efficiency at different exposure times, as it is likely that better results can be achieved over longer exposure periods. More important is to use higher resolution techniques, e.g., TEM to analyse if there is CNFs inside the cells.

Up to date, there are not many studies evaluating the internalization of nanocellulose derived materials. Still, some of the existing studies may provide information on the possible properties involved in cell internalization. CNFs cell uptake has recently been investigated by other authors.

Menas *et al.* (2017) found a significant difference in uptake of CNCs and CNFs by epithelial cells. Stained cellulose was used to visualize the presence of cellulose nanoparticles in cells after 72 hours of exposure. Interestingly, CNFs were seen outside, mainly at the limits of A549 cells, which means that no uptake of cellulose nanofibrils was observed, consistent with our results. However, CNCs of various types were clearly seen within cells, suggesting their cellular uptake. These results support the notion that size and shape play a critical role in determining the biological responses of nanocelluloses and their interactions with cells. These authors also revealed that both types of particles can induce distinct responses in A549 cells through different mechanisms. Although there was no absorption of CNFs, it showed cytotoxicity and increased oxidative stress. This finding is surprising considering that nanoparticle cytotoxicity is mostly correlated with cell uptake, suggesting that CNFs cytotoxicity may be caused by oxidative stress instead (Menas et al. 2017).

Furthermore, while Lopes *et al.* (2017) did not detect uptake of CNFs by macrophages *in vitro*, Catalán *et al.* (2017) administered *in vivo* TEMPO-oxidized CNF in rats and observed a dose-dependent accumulation in macrophages. Conversely to our work, *in vitro* studies using dendritic cells allowed to verify the partial internalization of CNFs. The authors stated that the interaction of CNFs and dendritic cells depended on the thickness and length of the material, highlighting the need for further studies to understand the predominant mechanism in biomaterial-cell interactions (Tomić et al. 2016).

Clift *et al.* (2011) demonstrated that cotton CNFs caused no form of frustrated phagocytosis in macrophage cells and was completely internalized within a vesicle, suggesting that they enter in these cells through a form of endocytosis, which usually culminates in the internalization of lysosomes. This observation can be attributed to nanofibrils with a relatively short length of 220 µm and a diameter of 15 nm, similar to that used by Pereira *et al.* (2013) between 85-225 µm and a diameter between 6-18 nm. Hence, it is possible that toxicity observed at higher concentrations may be due to damage in DNA, membrane or cell compartment. Possibly, increased exposure to this type of CNFs resulted in proportional increases in particle absorption. As a result, this can lead to lysosome saturation and ROS generation.

However, all authors mention the need for further studies to explore the functional interactions of different types of CNF with cells and to determine their mechanisms of action for inducing cytotoxicity (Pereira et al. 2013).

5.2.2 Cytotoxicity Assessment

According to the results obtained by the MTT assay, after a 24, 48 and 72 hours exposure to various concentrations of CNF TEMPO and CNF Enzymatic (**figures 4.5 to 4.7**), there was no significant cytotoxicity.

The reference value below which a NM is considered cytotoxic is 70% viability (ISO, 2009), which was not reached for any of the conditions tested. For cells treated with CNF TEMPO, the lowest viability values were around 90%, regardless the duration of exposure and no dose-response relationship was detected. Nevertheless, treatment with CNF Enzymatic caused more uniform decrease in cell viability, although values below 80% were not observed in any of the exposure times studied. The CNF Enzymatic used in this work was mostly in microfibrillated form, which can change their interaction with cells. Therefore, neither CNF TEMPO nor CNF Enzymatic were cytotoxic by the MTT assay.

Regarding the clonogenic assay (**figure 4.9**), cell viability was higher when compared to control once exposed to CNF TEMPO. The highest value of cell viability – 145%, was obtained with treatment at the concentration of 12.5 µg/cm^2. The CNF Enzymatic showed more constant values and closer to the control, except at the highest concentration (50 µg/cm^2) which showed a cell viability value of 130%. This indicates that both CNFs, regardless of their concentrations, stimulate cell proliferation and their

ability to form colonies. One possible explanation for this may be related to the ability of CNF to form an extracellular matrix that may promote cell attachment and growth (Halib et al. 2017).

Based on the two assays performed, it can be concluded that at both shorter (24 hours) and longer (8 days) exposures, none of the CNFs was cytotoxic.

Although there are already some studies that assess the short-term toxicity of CNFs, the current literature is still limited. In general, the absence of cytotoxicity obtained in this study is consistent with most published studies. However, it is important to consider that nanocelluloses can have different origins and physicochemical properties, often differing from study to study.

Our findings can be compared to those published by Yanamala *et al.* (2016) in which aqueous suspensions of different types of CNC and CNF (5 – 300 µg/mL) were exposed to A549 cell line or monocytes (THP-1) for 24 or 72 hours. The responses observed were cell type and material specific and therefore they considered the absence of correlation between cytotoxicity and surface properties. A similar study was published by Menas *et al.* (2017) where A549 cells were exposed for 24 or 72 hours to various forms of CNC or CNF (1.5, 15 and 45 µg/cm^2). Contrary to what we found, after 72 hours exposure to CNFs, cell viability decreased significantly at all concentrations. More recently, Ventura *et al.* (2018), aiming to better mimic the pulmonary environment, evaluated TEMPO-oxidized CNFs in a co-culture of A549 and THP-1 cells for 24 and 48 hours. At 24 hours they did not detect cytotoxicity, having been detected a more significant reduction in cell viability after 48 hours. The origin of the CNF used by these authors was the same as that of our study - *Eucalyptus globulus*.

For different cell lines, other authors also did not detect cytotoxicity after exposure to CNFs, similarly to the results obtained in this study. Vartiainen *et al.* (2011) observed no changes in the viability or morphology of human peripheral blood derived monocytes (PBMCs) and mouse macrophages. Cytotoxicity of CNFs was also evaluated by Alexandrescu *et al.* (2013) and Pitkänen *et al.* (2014) using mouse 3T3 fibroblasts and human cervical carcinoma cells (HeLa229), respectively. In mouse fibroblasts, the authors used two approaches (direct contact and indirect contact) to evaluate the behaviour of these cells incubated with wood CNFs. Both indicated that cell membrane integrity, mitochondrial activity and cell proliferation did not change during exposure, suggesting the non-toxicity of these biomaterials (Alexandrescu et al. 2013). Pitkänen *et al.* (2014) reported non-cytotoxic effects through the highest tolerated dose (HTD) test on HeLa229 cells exposed to thinner fractions of CNFs. However, by the total protein content (TPC) test, they reported inhibition of cell growth and decreased cell viability at the highest concentration for 24 and 72 hours exposures.

Nonetheless, Pereira *et al.* (2013) tested commercial needle-shaped cotton CNFs over a wide range of concentrations and confirmed that low concentrations (0.02 – 100 µg/mL) did not cause the death of bovine fibroblasts but concentrations above 200 µg/mL have led to significant inhibition in cell viability. The highest concentration used in our study was 50 µg/cm^2, equivalent to approximately 100 µg/mL.

Despite the different origin, Moreira *et al.* (2009) also observed no change in cell morphology and detected no more than 15-20% reductions in cell proliferation of mouse fibroblasts and Chinese hamster ovary cells after 72 hours of exposure to bacterial nanocelluloses at concentrations between 100 - 1000 µg/mL.

Hua *et al.* (2015) directly and indirectly tested the cytocompatibility of three types of wood CNFs in human dermal fibroblasts (HDF). In the indirect test, no cytotoxic effects were observed, while the direct contact test showed that modified cationic CNFs have a better cytocompatibility than unmodified and anionic modified CNFs. Later, they also reported the absence of cytotoxicity in THP-1 cells treated with the three types of functionalized CNFs for 24 hours (Hua et al. 2015). In the study by Čolić *et al.* (2015), a decrease in fibroblast cell proliferative capacity and dose-dependent metabolic activity was observed after 48 hours exposure to a high concentration of CNF (250 – 1000 µg/mL). However, as shown above, CNFs was considered non-cytotoxic as the reduction in cell proliferation was less than 30% and was not associated with oxidative stress or cell death. It should be noted that the range of concentrations tested was much higher than those used in the present work and therefore only includes the equivalent of the two highest concentrations analysed here (31.25 and 62.5 µg/cm^2), not including the one which increased cellular proliferation the most (12.5 µg/cm^2). Nordli *et al.* (2016) also observed no cytotoxic effects on human fibroblasts and keratinocytes. The same results were obtained by Rashad *et al.* (2017), which through indirect assays (crystal violet, MTT and LDH), incubated mouse fibroblasts with two types of CNF, oxidized by TEMPO and carboxymethylated, for 1, 4 and 7 days. Lopes *et al.* (2017) studied three CNFs with different surface functionalization incubated with HDF cells, THP-1 cells and pulmonary fibroblasts (MRC-5). The authors studied concentrations up to 500 µg/mL for 24 hours and reported no cytotoxic effects on all examined cell lines. In addition, they found that no internalization or morphological changes occurred in the THP-1 cells. Another study by Souza *et al.* (2018) also found no decrease in viability of like-fibroblast cells when exposed for 24h to a CNF produced from Curauá (*Ananas erectifolius*). However, a similar CNF had already been used by de Lima *et al.* (2012) and the results had pointed to the presence of cytotoxicity in mouse fibroblasts.

Considering the CNF Enzymatic, studies evaluating its cytotoxicity are scarcer. In a study by Ilves *et al.* (2018), four types of modified CNFs were tested in THP-1 cells and compared with conventional cellulose. The modification of the CNFs was made through different treatments, which include an enzyme-treated CNF. Contrary to our results, the authors found, through the LDH assay, that this type of CNF caused a significant decrease in cell viability. These effects were observed after 3, 6 and 24 hours of exposure from concentrations of 10 µg/mL. One of the reasons for this difference may be due to the origin of the CNF and the enzymatic treatment performed, given the lack of specifications. In addition, the CNF Enzymatic used in our study was mostly non-nanometric, which may change the interaction with cells.

On the other hand, stimulation of cellular metabolism after exposure to nanocellulose was also observed by different authors (Ventura et al. 2018; Rashad et al. 2017; Lopes et al. 2017; Nordli et al. 2016). This

may be due to the composition or structure of the CNFs used or the experimental conditions of the assays. As suggested by Ventura *et al.* (2018), another possible cause for the observed effect is the stimulation of cells by CNFs at low concentrations, thanks to their ability to mimic endogenous fibrous structures that facilitate cell adhesion and biocompatibility. At higher concentrations, CNFs may increase mechanical stress and therefore disrupt cell proliferation as observed in studies with 3D cell cultures by Nordli *et al.* (2016). The increased cell viability can also be explained by the interaction of nanofibrils in the mitotic spindle of cells, as described for asbestos fibers and carbon nanotubes with various diameters in different cell types, since the diameters are similar to those of CNFs. On the contrary, at higher concentrations the probability of agglomeration/aggregation occurrences is greater, reducing the amount of isolated nanofibrils that can be uptaken by cells and affect their proliferation. Some authors observed the induction of fibroblast proliferation after exposure to thin, coiled single-walled CNT, similar to one of the CNFs studied. This cellular response may be relevant in tissue fibrosis, which in turn may trigger a long-term carcinogenic effect (Ventura et al. 2018).

Based on the cytotoxicity classification defined by ISO (2009), the results of both assays showed that the studied CNFs, even at the highest concentration (50 µg/cm^2), do not appear to have potential toxic effects caused by any impact on metabolic activity or in the proliferation of treated cells, suggesting that CNFs are biocompatible. Nevertheless, the results should be interpreted with caution.

Therefore, further studies that focus on the properties that significantly influence biomaterial-cell interactions, in particular the presence of proteins on their surface (corona protein formation) are needed (Monopoli et al. 2011). As for CNFs, the use of very high concentrations in adherent cell assays may disrupt cell growth due to their kinetics and tendency to aggregate. In addition to making it difficult to measure actual concentrations used, it can influence the results.

5.2.3 Genotoxicity Assessment

In both cytotoxicity and genotoxicity analysis, it is important to include a simultaneous control sample in each assay performed during the study to check for large variations in experimental conditions that could have impacted the observed results. In the case of the CMBN assay, cells are often challenged with Mitomycin C, an MN-inducing agent (OECD, 2016).

The induction of genetic alterations is closely linked with the initiation and progression of neoplasias and, as such, genotoxicity assessment, *in vitro* and *in vivo*, is an indirect approach that allows the evaluation of carcinogenic potential. Assays to evaluate carcinogenesis in animals are costly in terms of time and resources, making direct evaluation difficult. For that reason, *in vitro* assays are widely used as they are simpler, faster and cost-effective (Collins et al. 2017). Among the various genotoxicity assays available, only a few were considered for use in nanoparticles, such as the micronucleus and the comet assay (Stone, Johnston and Schins 2009).

In this study, the evaluation of CNF genotoxicity in A549 cells was made by the CBMN assay, described by Fenech (2007), which allows to quantify the chromosomal changes induced by the studied nanofibrils.

Regarding the frequency of MNBNC (**figure 4.11**), only at the concentration of 3.125 µg/cm^2 of the CNF Enzymatic a slightly lower value compared to the control was obtained. Overall, all tested concentrations of both CNFs caused an increase in the frequency of unrepaired chromosomal abnormalities after A549 cell division, although not statistically significant. Nevertheless, the presence of a higher number of micronucleated cells may indicate the existence of alterations in the DNA that, since they were not repaired, remained after cell division and manifested as chromosomal breaks. In the case of the positive control used, a significant increase was confirmed due to the action of mitomycin on the cells. This can be explained by the difference in the dimensions of the two CNFs, which may suggest an absence of interaction between CNF Enzymatic and the cell genome.

Other parameters resulting from the assay used were evaluated. Regarding the number of buds found in binucleated cells (**figure 4.13**) no significant differences were observed in cells treated with CNF TEMPO or with CNF Enzymatic comparatively to controls. Considering the latter, we found a higher bud induction at the two lowest concentrations, which may be due to the presence of isolated nanofibrils, decreasing the likelihood of forming agglomerates and increasing their availability for interaction.

None of the CNFs was able to induce NPBs (**figure 4.14**). Finally, neither the CBPI nor the RI results showed a decrease in the ability of A549 cells to proliferate and replicate with higher doses of CNFs (**figures 4.16 and 4.17**) which is in line with the absence of cytotoxicity previously described.

In the current literature there are a limited number of studies that estimate the genotoxicity of CNFs. Moreover, different authors obtained conflicting results, which triggered very different opinions about the safety of its synthesis and use (Menas et al. 2017).

Possible explanations for differences in results may be based on the characteristics arising from the nanofiber synthesis process. For instance, differences in the type and quantity of functional groups or in their size and shape. It is usual for different types of nanofibrils to cause different genetic changes in the cells used. This difference is probably justified by the type of response caused by nanofibrils in each of the different cellular models (de Lima et al. 2012). Allegri *et al.* (2016) reported that a possible cause for the decreased toxicity observed in carbon nanotubes, is its functionalization, because it increases protein adsorption in biological media, leading to the formation of corona protein and promoting the agglomeration of NMs.

When NMs are more concentrated, there is a greater tendency for agglomeration or aggregation, decreasing their availability. In this sense, several studies have noted an inverse relationship between the genotoxicity of several NMs and their concentration, such as carbon nanotubes. (Ventura et al. 2018). A strong tendency of agglomeration of cellulose nanofibrils was also reported by de Lima *et al.*

(2012), especially in highly concentrated aqueous solutions, as they are highly hydrophilic. These authors state that agglomerated nanofibrils can cause different cellular effects compared to those caused by individual fibers, which could help to explain the different responses observed. On the contrary, Pereira *et al.* (2013) observed high concentrations of cotton CNF led to large CNF aggregates and increased cytotoxicity.

Although not statistically significant, the results of our study are in harmony with the conclusions made by Ventura *et al.* (2018), who observed an increase in the frequency of micronucleated cells at the lowest evaluated exposures – 1.5 and 3 μg/cm^2. The authors also showed that the nanocellulose under study induced a low but significant level of DNA damage in A549 cells at the highest concentration (25 μg/cm^2) in a co-culture with THP-1 cells. However, they suggest that given the rapid capacity of cellular repair systems, oxidative stress can be quickly repaired when exposure times are long. In this case, 24 hours may be too long to detect this kind of damage. These authors were also able to microscopically observe an agglomerate/aggregate of CNF in culture medium after 24 hours of incubation of the highest concentration studied (25 μg/cm^2), reinforcing the idea that decreased toxicity is related to lower availability of CNF in nanometric dimensions. A similar phenomenon was noted in the present study.

The findings of Catalán *et al.* (2017) allowed to confirm previous results through *in vivo* testing. Similarly to the case presented in this study, CNFs with TEMPO-mediated treatment were used and, through the comet assay, it was showed a significant increase in DNA damage in mouse lung cells exposed by pharyngeal aspiration at the two lowest concentrations studied (10 and 40 μg /mouse). However, they found no increase in the number of micronuclei in the bone marrow erythrocytes of these mice. One of the reasons given by the authors is that the study is conducted *in vivo*, therefore inhalation exposure for 24 hours may not be enough to transport nanocellulose to the bone marrow, its uptake and consequent development of systemic genotoxic effects, originating a greater number of MN. Other authors, such as de Lima *et al.* (2012), only detected cytotoxicity in CNFs from cuarará, while cotton CNFs were considered non-cytotoxic. Nevertheless, by comet assay, they observed genetic alterations in human lymphocytes after *in vitro* exposure to both nanocelluloses, obtaining low but significant values of DNA damage.

Several studies have stated that the CBMN assay is more sensitive to detect genotoxic effects of NMs than the comet assay (Louro et al. 2016). Indeed, this assay is considered suitable for testing the genotoxicity of various NMs, with the advantage of providing not only information on chromosomal damage, but also cell proliferation and cytostasis (Migliore, di Bucchianico and Uboldi 2014). There are, however, some additional factors that may contribute to the different sensitivity of these assays, such as the repair mechanisms of the target cell and their efficiency, the cell cycle stage and the time interval between exposure and analysis (Valentin-severin et al. 2003).

All the above results, both *in vitro* and *in vivo*, seem to be in agreement with the assumption that low concentrations of CNF seem to induce more toxic effects on lung cells than the highest. However, in the

present study it was concluded that, despite the differences described, none of the evaluated CNFs showed ability to induce chromosomal instability, which means that neither CNF TEMPO nor CNF Enzymatic revealed genotoxic effects.

Genotoxicity may be caused by primary mechanisms, by direct or indirect interaction with the genome, or secondary mechanisms, mediated by inflammation. *In vitro* assays using monolayer cell culture do not involve any inflammatory response, therefore, they are assumed to exclusively detect primary genotoxicity. Although not a variable studied in this work, there are immunotoxicity results obtained by other authors important to be mentioned. It is known that one of the routes of induction of genome damage is oxidative stress caused by overproduction of ROS (Magdolenova et al. 2014). The study by Stefaniak *et al.* (2014) demonstrated high intracellular ROS levels following exposure to a TEMPO oxidized CNF. Whereas, Lopes *et al.* (2017) did not observe increases in ROS production, suggesting that DNA lesions caused by CNFs may be other than intracellular ROS formation. These differences in toxicity may be justified by the different origins and functionalization of the CNFs produced, including the mechanical, chemical or enzymatic treatment that each one undergoes.

In conclusion, the results of this study showed that both types of CNFs do not appear to be uptaken by A549 cells and that there are no significant cytotoxic and genotoxic effects. Generally, exposure to CNF TEMPO has been shown to induce increased cell viability, whereas CNF Enzymatic did not seem to cause changes in cell viability and proliferation, except at the highest concentration which yielded contradictory results for these two parameters. CNF TEMPO had a more gel-like appearance with a higher tendency to form agglomerates/aggregates, which may reduce its bioavailability and internalization efficiency. CNF Enzymatic contained a greater number of larger fibrils, which may have influenced its interaction with cells. Neither CNF TEMPO nor CNF Enzymatic showed significant impact on chromosomal structure. Due to their size, it is not expected that these biomaterials can cross the pores of the nuclear envelope, which is in accordance with the absence of CNFs in the cell nucleus obtained in the preliminary results using microscopy techniques. For this reason, direct interaction between nanocelluloses and DNA is unlikely.

6. Conclusions and Future Perspectives

Due to the undeniable advantages that NMs present, their use for various purposes is expanding. However, associated with this expansion arises the need to understand their biological effects in order to ensure their safety for human health and to avoid adverse outcomes following their introduction into the market. The incorporation of NMs in the biomedical field may allow the improvement of existing materials and even the production of new ones. The vast number of applications of NMs currently exploited inevitably increases human exposure for workers, consumers and through the environment.

One of the most challenging issues for developing safer nanotechnology is the design and validation of appropriate methods for toxicity assessment of a NM. It is urgent to develop guidelines that can be adapted to the several types of nanomaterials and that are easily reproducible.

Nanocelluloses show characteristics much appreciated by the scientific community, such as biocompatibility, natural and renewable origin and low cost. In addition, their physicochemical properties give them great advantages.

This work was developed with the intention of contributing to an evaluation and characterization of biosafety of nanocelluloses, focusing on cellulose nanofibrils.

The results indicate that CNF TEMPO and CNF Enzymatic, both produced from *Eucalyptus globulus* by different pre-treatments, do not present cytotoxicity as they do not cause decreased viability of A549 cells exposed to different concentrations. No chromosomal changes were detected either, indicating that both CNFs are not genotoxic.

Nevertheless, there are some important considerations to retain. On one hand, due to their high tendency to agglomerate, it is necessary to explore methods for more effective dispersion of CNF gels. This phenomenon may alter the results as nanocelluloses may be being tested in a micrometer rather than nanometric form. On the other hand, when agglomerate formation occurs, the concentration of CNFs may not be as the one expected for the study. The evident stimulating action of the two CNFs on cell cycle progression and cell replication should not be ignored. This event may be related to the apparent biocompatibility of these materials but it can also be associated with tumour-promoting activity. Therefore, these effects need to be further explored.

The study of genotoxic effects performed in the present work helps to evaluate these two types of CNFs. However, for full characterization their potential effects should be explored through the use of different assays, which analyse other types of parameters, and representative cell lines of other tissues of interest. In this context, assays for detecting gene mutations or inflammatory potential are suggested. The latter is extremely relevant, since inflammation may lead to oxidative stress, which is associated with DNA damage. Considering the results obtained in this type of assay, the use of *in vivo* models may

be relevant not only to assess cytotoxic and genotoxic effects more closely to reality but also to find out the most relevant concentrations at which the target tissue is exposed.

Although further studies covering more essential factors are needed to confirm the biocompatibility and biosafety of CNFs, they have already proven to be a promising sustainable and economic alternative for future biomedical applications.

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8. Annexes

Annex I – Characteristics and effects of nanoparticles versus larger particles

The following table shows the comparison of characteristics and effects on the organism between nanoparticles and larger particles, with the respiratory tract as the portal of entry.

Table I.1 - Nanoparticles versus larger particles: characteristics, biokinetics and effects in the respiratory tract. Adapted from Oberdörster (2009).

General characteristics	**Nanoparticles (<100 nm)**	**Larger particles (>500 nm)**
Ratio: number/surface area per volume	High	Low
Agglomeration in air, liquids	Likely (dependent on medium: surface)	Less likely
Deposition in respiratory tract	Diffusion: throughout resp. tract	Sedimentation, impaction, interception; throughout resp. tract
Protein/lipid adsorption *in vitro*	Yes; important for biokinetics	Less effective
Translocation to secondary target organs		
Clearance	Yes	Generally not (to liver under 'overload')
Mucociliary	Probably yes	Efficient
Alv. macrophages	Poor	Efficient
Epithelial cells	Yes	Mainly under overload
Lymphatic circulation	Yes	Under overload
Blood circulation	Yes	Under overload
Protein/lipid adsorption *in vivo*	Yes	Some
Cell entry/uptake	Yes	Primarily phagocytic cells
Mitochondria	Yes	No
Nucleus	Yes (<40 nm)	No
Direct effects		
At secondary target organs	Yes	No
At portal of entry (resp. tract)	Yes	Yes
Inflammation	Yes	Yes
Oxidative stress	Yes	Yes
Primary genotoxicity	Some	No
Carcinogenicity	Yes	Yes

Annex II – Results of Cytotoxicity Assays

The following tables show the results obtained in the cytotoxicity assays, considering the viability and proliferative capacity of A549 cells after exposure to both types of CNFs.

Table II.1 – MTT assays for CNF TEMPO at three different exposure times.

		Cell Viability (%) (Mean ± SEM)		
	Concentration (µg/cm²)	**24h exposure**	**48h exposure**	**72h exposure**
CNF TEMPO	0	100.0±0.0	100.0±0.0	100.0±0.0
	1.5	104.1±5.1	100.8±3.6	105.6±6.4
	3.125	104.7±6.7	96.5±8.4	105.6±8.1
	6.25	109.9±3.2	91.5±3.7	108.3±9.5
	12.5	109.6±8.0	90.8±8.4	99.4±8.9
	25	96.5±9.5	87.7±9.2	94.0±11.4
	50	94.9±8.2	80.2±5.0	88.8±12.4
	SDS	5.5*±1.0	5.7*±0.7	4.3*±1.7

* significantly different from negative control ($p<0.05$).

Table II.2 – MTT assays for CNF Enzymatic at three different exposure times.

		Cell Viability (%) (Mean ± SEM)		
	Concentration (µg/cm²)	**24h exposure**	**48h exposure**	**72h exposure**
CNF Enzymatic	0	100.0±0.0	100.0±0.0	100.0±0.0
	1.5	105.3±3.1	110.5±12.1	112.9±12.4
	3.125	109.3±9.2	108.7±14.3	116.8±8.4
	6.25	103.1±6.9	113.3±11.3	112.0±3.7
	12.5	97.9±2.8	111.7±8.8	110.3±10.1
	25	95.7±2.4	96.9±11.0	101.0±8.7
	50	92.9±2.0	104.3±5.0	100.9±10.8
	SDS	4.9*±1.0	6.5*±1.0	6.2*±1.7

* significantly different from negative control ($p<0.05$).

Table II.3 – Clonogenic assays for CNF TEMPO and CNF Enzymatic, after 8 days of exposure.

	Cell Viability (%) (Mean ± SEM)	
Concentration (µg/cm²)	**CNF TEMPO**	**CNF Enzymatic**
0	100.0±0.0	100.0±0.0
1.5	100.2±13.2	105.6±6.4
3.125	97.3±9.1	105.6±8.1
6.25	103.1±3.2	108.3±9.5
12.5	101.1±6.3	99.4±8.9
25	104.6±9.5	94.0±11.4
50	129.6±8.8	88.8±12.4
MMC	11.9*±10	4.3*±1.7

* significantly different from negative control ($p<0.05$).

Annex III – Results of Genotoxicity Assays

The tables presented here show the results obtained in the Cytokinesis-Block Micronucleus Assay performed on A549 cells exposed to CNF TEMPO and CNF Enzymatic. For each CNFs, it was considered the MNBNCs, MNs, NBs and NPBs scores, as well as the CBPI and RI indices.

Table III.1 – CBMN assay for CNF TEMPO

	Concentration (µg/cm²)	Frequency of MNBNC/1000 BNC (Mean±SD)	Frequency of MNs/1000 BNC (Mean±SD)	Frequency of NBs/1000 BNC (Mean±SD)	Frequency of NPBs/1000 BNC (Mean±SD)	CBPI (Mean±SD)	RI (Mean±SD)
CNF TEMPO	0	14.0±4.0	8.5±1.0	5.5±3.0	1.3±0.3	1.64±0.01	100.0±0.0
	1.5	17.0±1.0	9.0±0.5	2.0±0.0	1.8±1.3	1.76*±0.02	118.6*±1.6
	3.125	16.5±1.5	9.8±1.8	5.0±1.5	0.8±0.8	1.84*±0.04	130.9*±7.4
	6.25	15.0±2.0	7.8±1.3	3.3±0.8	0.3±0.3	1.80*±0.00	123.8*±2.0
	12.5	17.5±0.5	9.3±0.8	4.0±1.5	0.8±0.8	1.90*±0.01	138.8*±3.3
	MMC	34.0*±1.0	19.5±0.0	5.3±0.8	8.5*±1.0	1.25*±0.00	38.5*±0.1

* significantly different from negative control ($p<0.05$).

Table III.2 –CBMN assay for CNF Enzymatic

	Concentration (µg/cm²)	Frequency of MNBNC/1000 BNC (Mean±SD)	Frequency of MNs/1000 BNC (Mean±SD)	Frequency of NBs/1000 BNC (Mean±SD)	Frequency of NPBs/1000 BNC (Mean±SD)	CBPI (Mean±SD)	RI (Mean±SD)
CNF Enzymatic	0	11.5±1.5	6.3±1.3	1.8±0.8	3.3±1.3	1.7±0.00	100.0±0.0
	1.5	15.0±3.0	8.3±1.8	2.8±0.8	1.8±0.3	1.6*±0.00	87.4*±0.1
	3.125	11.0±7.0	6.8±4.8	3.0±1.0	3.0±1.0	1.7±0.01	101.5±1.8
	6.25	14.0±1.0	7.8±0.8	1.3±0.8	0.5±0.5	1.8*±0.01	113.8*±2.0
	12.5	13.0±6.0	8.5±4.0	2.3±0.8	1.3±0.3	1.8±0.11	113.5±13.8
	MMC	29.5*±10.5	15.3±5.8	3.0±0.5	4.5±2.0	1.4*±0.02	64.7*±2.8

* significantly different from negative control ($p<0.05$).