

CARBON NANOTUBES AS DELIVERY SYSTEMS FOR CHEMOTHERAPEUTICS IN 2D AND 3D CELL MODELS

by

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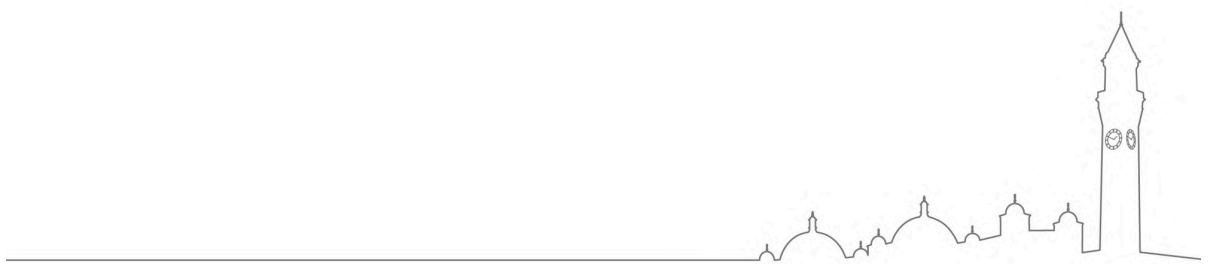
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ABSTRACT

Despite significant advances in recent years, cancer remains a major worldwide health problem. Traditional chemotherapeutics are very potent, yet their administration is accompanied by severe adverse effects due to low specificity, as well as the development of resistance. Therefore, in efforts to improve the success of current therapies, nanomedicine has presented revolutionary approaches in the delivery of anticancer drugs. In this context, carbon nanotubes have emerged as a promising drug delivery system due to their unique structure and physicochemical properties, offering a wide variety of opportunities for biomedical applications. This thesis presents the development of novel anticancer therapeutic modalities through the integration of multi-walled carbon nanotubes (MWNTs) with the first-line antineoplastic agents cisplatin (CDDP) or doxorubicin (DOX).

Thorough physicochemical characterisation of Pluronic-coated MWNTs, carboxyl-MWNTs and amine-MWNTs was performed to measure relevant aspects such as MWNT length, surface functionalisation and charge, concluding that the chemically functionalised MWNT were highly-dispersible and biocompatible. Furthermore, the anticancer activity of MWNTs + DOX and MWNTs + CDDP complexes was assessed on breast carcinoma (MCF7) 2D cells and 3D tumour spheroids, generated with a low-cost and reliable method. While the combination of MWNTs + DOX depicted a comparable efficacy of that observed with free DOX, MWNTs + CDDP complexes displayed a marked enhanced therapeutic efficacy compared to CDDP alone, resulting in higher cytotoxicity in monolayer 2D cultures as well as

greater growth inhibition and even disaggregation of the 3D spheroids. Further analysis revealed that carboxylated MWNTs + CDDP complexes increased the intracellular accumulation of CDDP, leading to oxidative imbalance, cell cycle arrest and apoptosis induction. This work highlights the development of a promising therapeutic modality for the treatment of breast cancer that demonstrated to not only efficiently chemo-sensitise cancer cells to CDDP, but also enhance the anti-tumour efficacy in 2D and 3D cellular models.

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ABBREVIATIONS AND ACRONYMS

ABC	ATP-binding cassette
ANOVA	Analysis of variance
ASR	Age-standardised rate
BBB	Blood brain barrier
BC	Breast cancer
BCRP	Breast cancer resistance protein
BIO	Biotin
CDDP	Cisplatin
CDKI	Cyclin-dependant kinase inhibitors
CM	Complete medium
CNTs	Carbon nanotubes
COOH-CNTs	Carboxylated carbon nanotubes
COOH-MWNTs	Carboxylated multi-walled carbon nanotubes
CVD	Chemical vapour deposition
DCIS	Ductal carcinoma in situ
DDS	Drug delivery system
diH ₂ O	Distilled water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EAD	Electric arc discharge

em	Emission
EPR	Enhanced permeability and retention
ER	Oestrogen receptor
ex	Excitation
F127	Pluronic F127
F127-MWNTs	Pluronic coated MWNTs
FBS	Fetal bovine serum
FDA	Food and Drug Administration of the United States of America
FITC	Fluorescein isothiocyanate
FTIR	Fourier-transform infrared spectroscopy
g	Standard gravity
GSH	Glutathione
h	Hours
HA	Hyaluronic acid
HIV	Human immunodeficiency virus
HMGB	Nonhistone chromosomal high-mobility group
HNO ₃	Nitric acid
IARC	International Agency for Research on Cancer
IBC	Inflammatory breast cancer
ICP-MS	Inductively coupled plasma mass spectrometer
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
LCIS	Lobular carcinoma in situ
LDH	Lactate dehydrogenase

MCTS	Multicellular tumour spheroids
MEM	Minimum Essential Medium
min	Minutes
mL	Millilitres
mLDH	Modified Lactate Dehydrogenase Assay
mM	Millimolar
MMR	Mismatch repair proteins
MPTP	Mitochondrial permeability transition pores
MRP	Multidrug resistant-associated protein
MTX	Methotrexate
mV	Millivolts
MWNTs	Multi-walled carbon nanotubes
NAD ⁺	Nicotinamide adenine dinucleotide
NER	Nucleotide excision repair proteins
NH ₃ ⁺ -CNTs	Aminated carbon nanotubes
NH ₃ ⁺ -MWNTs	Aminated multi-walled carbon nanotubes
nM	Nanomolar
nm	Nanometres
NOX3	Nicotinamide adenine dinucleotide phosphate oxidase 3
NPs	Nanoparticles
OCT	Organic cation transporters
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
OXA	Oxaliplatin

P-gp	P-glycoprotein
PAA	Polyallyamine
PARP	Poly ADP ribose polymerase
PBS	Dulbecco's phosphate-buffered saline
PCA	Poly citric acid
PEI	Polyethylenimine
PI	Propidium iodide
PLGA	Poly-lactic-co-glycolic acid
PR	Progesterone receptor
Pt	Platinum
Rf	Riboflavin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
SEM	Scanning electron microscope
SERD	Selective oestrogen receptor degrader
SERM	Selective oestrogen receptor modulator
SOP	Standard operating protocol
SSC	Saline Sodium Citrate buffer
TAT	Trans-activating transcriptional activator
TEM	Transmission electron microscope
TGA	Thermogravimetric analysis
TKI	Tyrosine kinase inhibitors
TLM	Transmitted Light Microscope

T _m	Thiamine
TNBC	Triple negative breast cancer
TOP2	Topoisomerase II
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labelling
WHO	World Health Organization
ZP	Zeta potential
$\Delta \Psi_m$	Mitochondrial membrane potential
μm	Micrometre
μM	Micromolar

CHAPTER 1

INTRODUCTION

1.1 General aspects of cancer

Cancer is a multifactorial genetic disease with a complex progression. It consists in the uncontrolled growth of cells capable to spread to adjacent organs and turn into tumours (1-3). Moreover, malignant cells can disseminate through the blood stream and lymphatic system, invading distant healthy tissues; this event is named metastasis (4, 5). Failing to control the disease can result in death. A variety of aetiologies account for the cause of cancer such as lifestyle, inherited genetic disorders, environmental exposures and errors in the deoxyribonucleic acid (DNA) among others (6, 7).

1.1.1 Cancer epidemiology

Any person can be affected by cancer at any point in their lifespan. The disease is considered a major international health problem regardless of the countries income level and human development index (8, 9). According to the World Health Organization (WHO) statistics, cancer is the second leading cause of death

(figure 1.1), causing more than 9.6 million deaths in 2018. These numbers represent approximately 16% of the total global deaths, of which 70% occur in low-income and developing countries (10). A 2017 analysis by the European Union Statistics revealed that 1.3 million people died due to neoplasms-related diseases (11). Particularly, the United Kingdom reports approximately 165,000 cancer deaths per year, representing 28% of the total deaths (12). More importantly, the International Association of Cancer Registries estimates an increase of deaths to 16.3 million by 2040 (13).

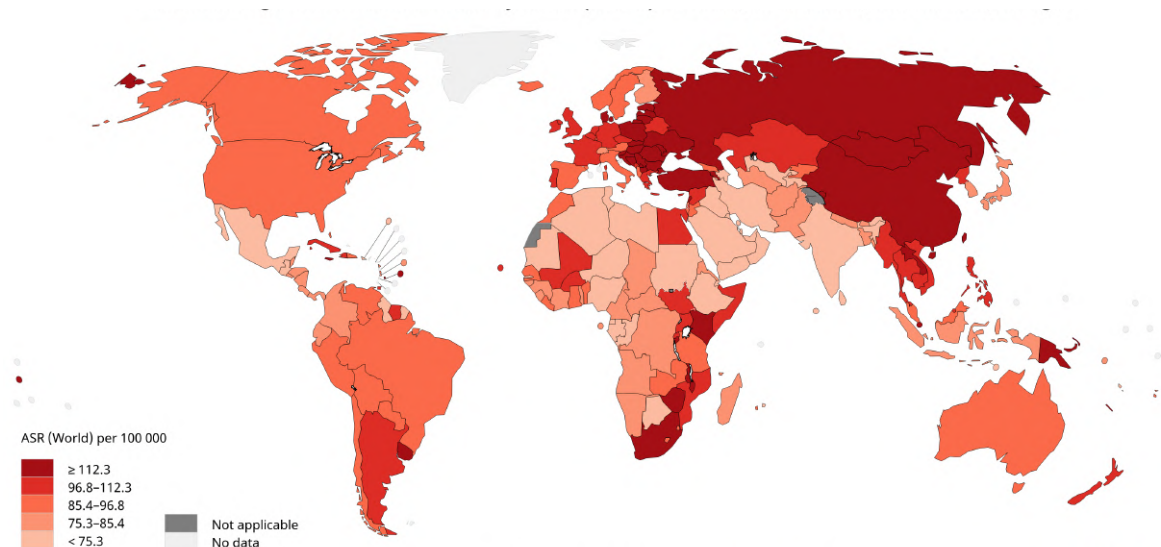


Figure 1.1 | Estimated age-standardised rate (ASR) mortality rates globally per 100, 000 individuals in 2018. The map considers all cancers, all ages and both sexes. Image adapted from (13).

GLOBOCAN reported that the most frequently diagnosed cancer in males was lung, closely followed by prostate and colorectum. On the other hand, breast cancer (BC) had the highest incidence in women and, with less than half the cases, colorectum and lung cancer followed the list (14) (figure 1.2).

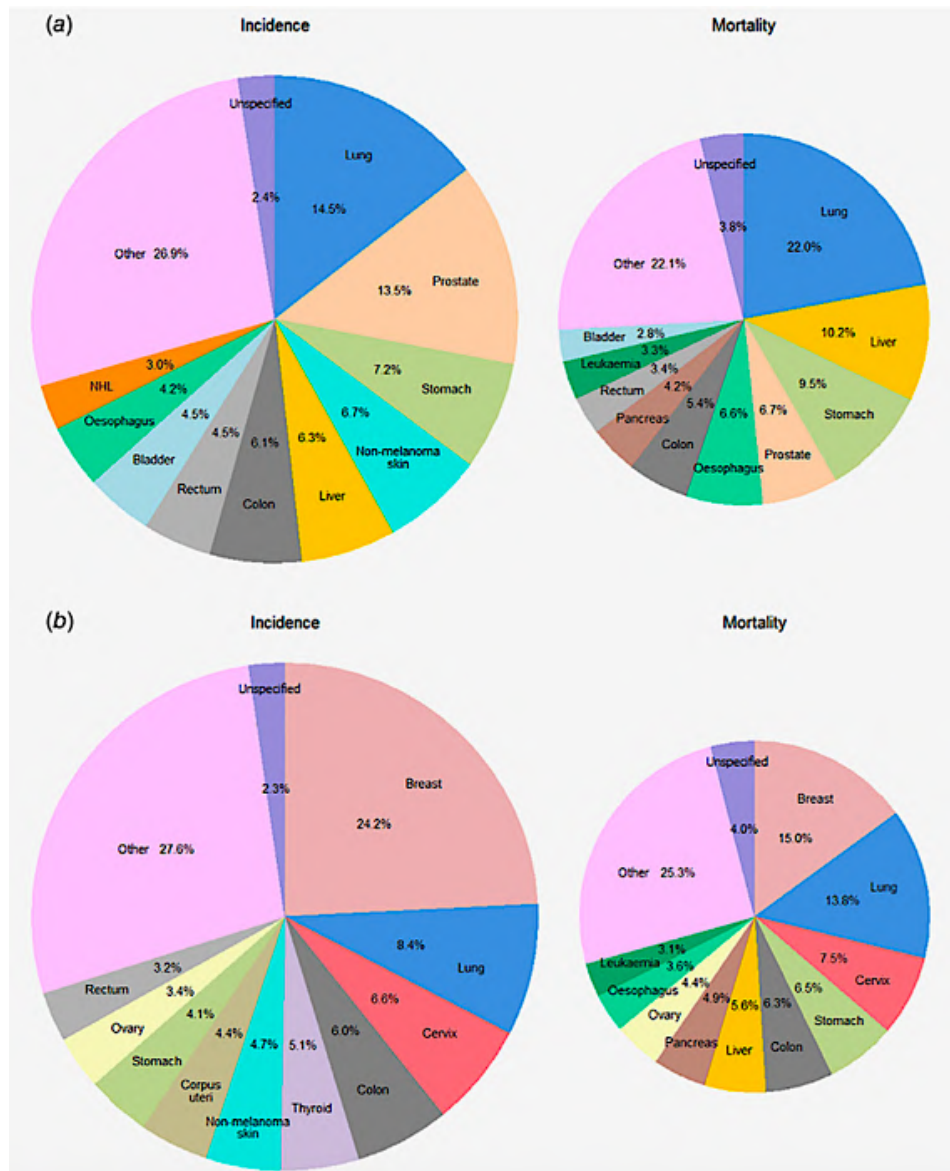


Figure 1.2 | Incidence and mortality distribution of estimated cancer cases in 2018. Each portion in the charts represents the percentage of the total number of cases of (a) males and (b) females. Image adapted from (15).

1.2 Breast cancer

BC is the malignant growth of cells that can initiate in the lobules, ducts or connective tissue of the breast. Breast carcinogenesis is mainly related to alterations in the control of cell division and death, environmental effects, hormone imbalance, and germline mutations.

1.2.1 Breast cancer epidemiology

With over 2 million new patients in 2018, BC is the most prevalent cancer in women (16) (figure 1.3). Although BC mortality is slowly declining in high-income countries, numbers are increasing in middle- and low-income nations. The projected 5-year survival in developed countries is 80%, while in less developed countries is 40% (17). Equally important is the fact that BC incidence is rising worldwide (18).

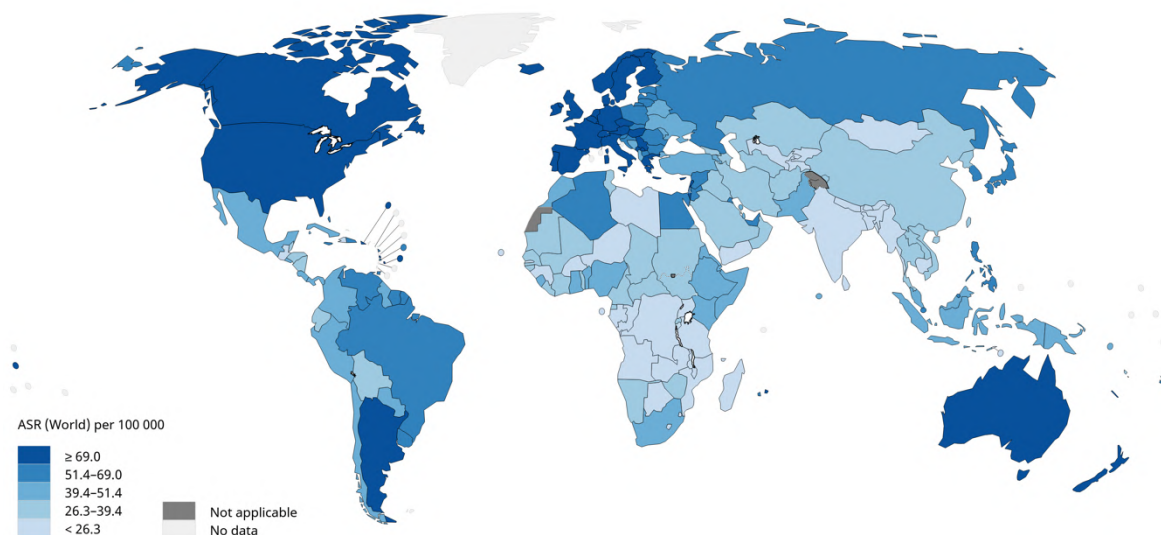


Figure 1.3 | Breast cancer incidence rates globally in 2018. The map depicts the ASR incidence of BC per 100, 000 females. Image adapted from (13).

Specifically, the United Kingdom reported 55,176 cases and 11,399 deaths in 2015-2017 (19). Furthermore, in 2020, 13.4 new cases per 100,000 people are expected in Europe (20). According to the International Agency for Research on

Cancer (IARC) extrapolations, the number of BC patients will grow to 3,059,829 by 2040 (13).

1.2.2 Histopathological classification

There are various histologic types of BC, mainly classified into two groups: in situ and invasive carcinomas (21, 22). In situ carcinomas are cancers contained within a tissue, being the most representative the ductal carcinoma in situ (DCIS). Also known as intraductal carcinoma, this non-invasive cancer is enclosed in the lining of the breast ducts; however, if ignored, it can progress to invasive cancer. 13% - 50% of the initial diagnoses evolve over 10 years. Since it is an early stage of the disease, it is highly treatable. An average of one in five patients will present DCIS (23-25) (figure 1.4).

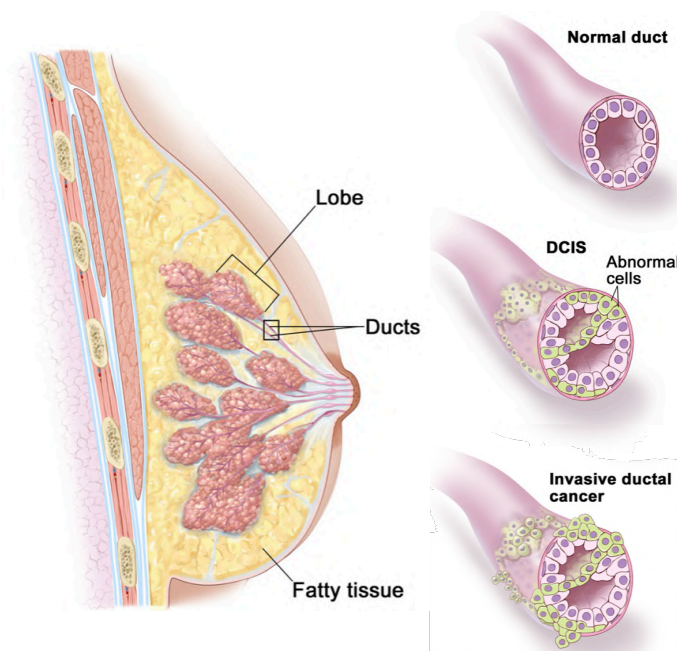


Figure 1.4 | Ductal carcinoma progression. On the left, the anatomy of the breast, depicting the breast lobes and ducts as common sites of carcinogenesis. On the right, DCIS progression to IDC. Image adapted from (26, 27).

Another type of non-invasive BC is the lobular carcinoma in situ (LCIS), which refers to the cancer that starts in the breast lobules, at the final portion of the ducts and has not affected any surrounding tissue. It is considered of moderate risk and rather a rare condition (28, 29) (figure 1.5).

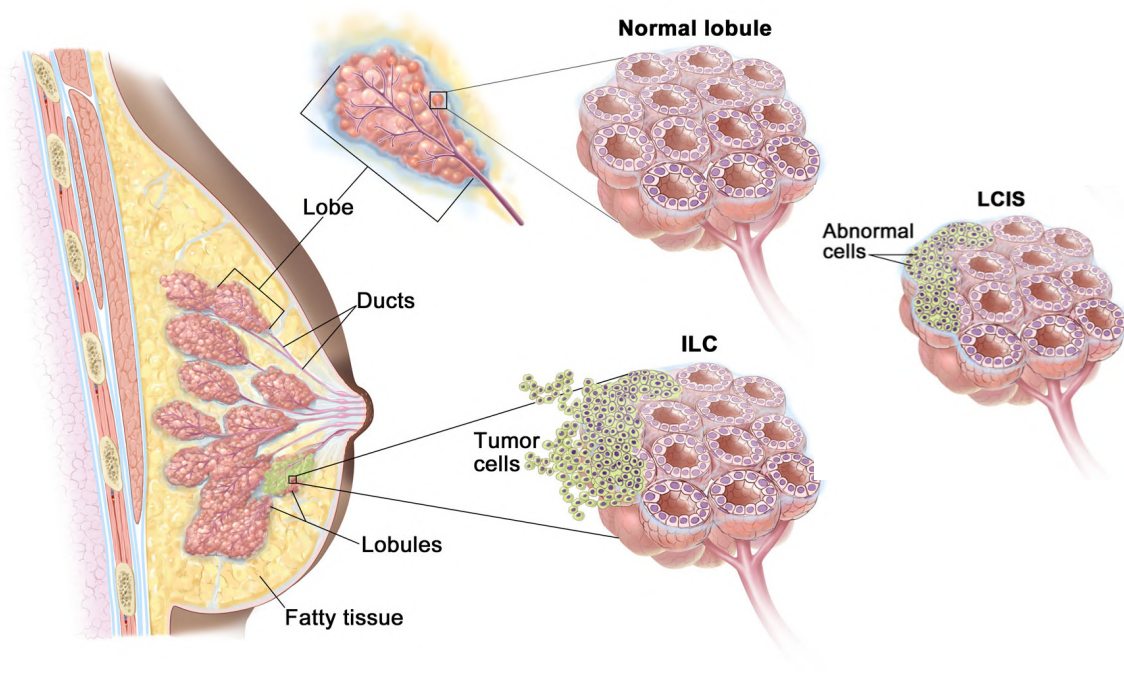


Figure 1.5 | Lobular carcinoma progression. Healthy breast lobule cells can become malignant and turn to contained LCIS. Progression of these lesions can develop to ILC, where abnormal cells invade adjacent tissues. Image adapted from (26, 27).

The most frequently detected breast carcinomas are invasive. They occur when abnormal cells propagate out of the tissue of origin to the proximities. The invasive ductal carcinoma (IDC) or infiltrating ductal carcinoma is the most common BC, making approximately 80% of the total diagnoses. IDC begins in the breast ducts; nonetheless, malignant cells manage to invade nearby tissues

and can metastasise other body organs. It is also the most common BC type in men. Most of the affected patients are older than 55 years old (30-32).

The invasive lobular carcinoma (ILC) reports 5% to 15% of total BC cases, positioning it as the second most common. This malignancy initiates in the breast glands; however, cells have broken out of the lobule to surrounding tissues and can potentially spread to distant parts of the body. Commonly affecting patients in their 60s, ILC has been described as a BC difficult to detect by palpation and mammogram (33-36).

There are other less common invasive breast cancers, making about 5% of total incidence rates. Medullary and mucinous carcinomas of the breast are subtypes of IDC. The medullary carcinoma produces a soft tumour that resembles the medulla of the nervous system. It usually affects young women in the age of 40 to 50 years old. This cancer is not considered aggressive as its development is slow and does not infiltrate the lymphatic system, facilitating the treatment (37, 38).

The characteristic feature of the mucinous carcinoma is the over production of mucin. Under the microscope, the cancer cells look immersed in gel pools of the glycosylated protein (39). While about 2% of the total BC cases are considered pure mucinous carcinomas, 5% are found within another tumour (40). On average, patients affected with this cancer are in their late 60s and tend to respond well to treatments (39, 40).

Inflammatory breast cancer (IBC) is an aggressive and fast developing cancer. As few as 2% of the total BC patients are detected with IBC, but it is responsible of 7%-10% of deaths. In this malignancy, the IBC cells infiltrate and block the breast lymphatic vessels, producing a characteristic red, tender, and swollen skin. Due to the rapid progression of the disease, IBC often does not produce any detectable lumps (41, 42).

Paget's disease of the breast, also defined as Paget's disease of the nipple, is an unusual BC type. The tumour starts in the nipple ducts to then spread to the nipple and areola skin. Researchers and clinicians associate this disorder with DCIS or IDC as most patients present one or more tumours in the same breast (43, 44). According to statistics from the USA National Cancer Institute, most women are diagnosed in their early 60's, while the neoplasm can be discovered one decade later in men (45, 46).

Phyllodes tumours are unusual fibroepithelial carcinomas with high risk of local recurrence. It causes less than 1% of the BC incidence. The distinctive feature about the phyllodes carcinomas is their leaflike growth (47, 48).

Triple negative breast cancer (TNBC) refers to tested malignant cells that do not express any of the three important growth receptors: oestrogen (ER), progesterone (PR) and human epidermal growth factor (HER2). The study of these receptors is relevant to determine the therapy to follow as no hormone-

based treatments nor compounds targeting ER and PR would be efficacious. Approximately 15% of the BC cases are TNBC, affecting mainly young patients. Usually, TNBC has a poor prognosis due to their aggressive behaviour, treatment limitations, and ability to spread and recur (49-51).

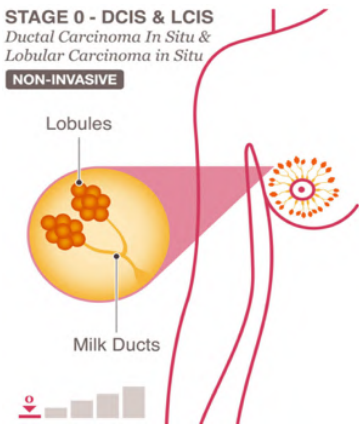
1.2.3 Breast cancer grade and stage

Once a malignancy has been confirmed, it is critical to assess its grade and stage. These factors are essential to determine the treatment and prognosis. The Union for International Cancer Control recommends the use of the TNM grading system. In this scheme, the letter T describes the size of the main tumour; N means the number of lymph nodes infiltrated, while M states the presence or absence of metastasis. From 2018, new measurements are also considered: tumour grade, ER, PR and HER2 levels, menopausal status and general health (52-54).

Likewise grading, carcinomas staging describes valuable characteristics to be accounted when evaluating the outcome of the disease. As described in table 1, BCs are classified in a scale of 0 to IV.

Table 1 | Breast cancer stages. Confirmed breast malignancies are classified into IV stages and sub-stages following the guidelines established in 2018 by the American Joint Committee on Cancer. Information obtained from (55); images adapted from (56).

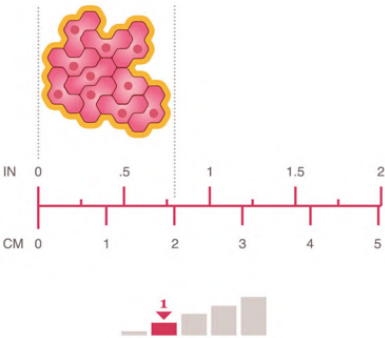
Stage 0
Carcinomas in situ



- Abnormal cells are contained in the tissue of origin
- DCIS, LCIS and Paget's disease
- Highly treatable

Stage I
Invasive carcinomas

STAGE 1
Has Not Spread
INVASIVE



Stage IA

- Tumour measures up to 2cm
- Microscopic invasion; no lymph infiltration

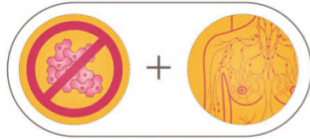
Stage IB

- Presence or absence of main tumour of less than 2cm
- Groups of cancer cells no larger than 2mm in near lymph nodes

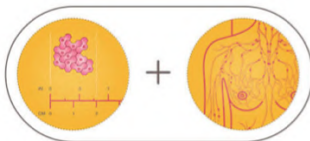
Stage II

Growing invasive carcinomas, still contained in the breast

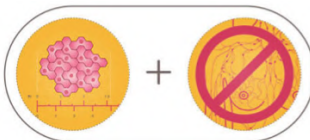
Stage IIA



- Absence of main tumour; less than 4 near lymph nodes infiltrated

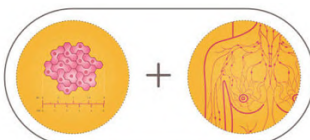


- Tumour smaller than 2cm; less than 4 near lymph nodes infiltrated



- Tumour measures 2-5cm; no lymph infiltration

Stage IIB



- Tumour measures 2-5cm; less than 4 near lymph nodes infiltrated



- Tumour larger than 5cm; no lymph infiltration

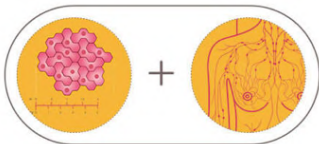
Stage III

Spread cancer to nearby tissues, possible nodes infiltration and muscle invasion

Stage IIIA



- Tumour of any size; 4-9 near lymph nodes infiltrated

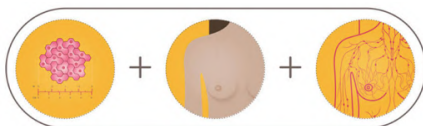


- Tumour larger than 5cm; presence of cancer clusters smaller than 2mm in near lymph nodes



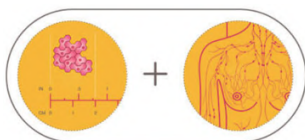
- Tumour larger than 5cm; cancer spread to up to 3 axillary lymph nodes or close to breastbone

Stage IIIB

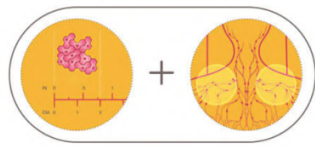


- Tumour of any size; cancer spread to chest wall or breast skin; up to 9 near lymph nodes infiltrated

Stage IIIC



- Absence or presence of any size tumour; cancer spread to chest wall or breast skin; 10 or more axillary lymph nodes infiltrated

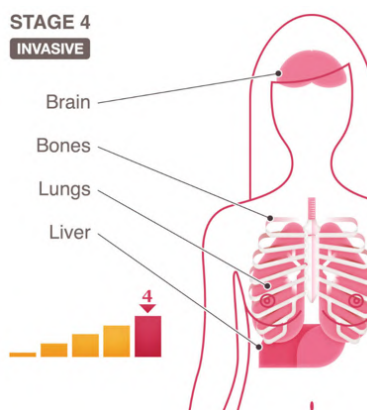


- Absence or presence of any size tumour; cancer spread to collarbone lymph nodes



- Absence or presence of any size tumour; cancer spread to axillary and breastbone lymph nodes

Stage IV Metastatic cancer



- Cancer invaded distant organs, mainly brain, bones, lungs and liver
- Incurable; however, current treatments can improve the life expectancy and quality

Even though important advancements have been accomplished in therapy and life quality of patients, BC remains a priority in global health research, considering it is undoubtedly the most frequent and deadliest malignancy in women (57).

1.2.4 Risk factors

Nowadays, on average, there is 12% probability that a woman will be diagnosed with invasive BC before 80 years old (58). Therefore, it is important to determine relevant risk factors that can influence the development of cancer.

- **Age.** Advanced age is a key risk factor. Every decade older increases approximately 1% the probability of developing BC. The median age at detection is 61 years old (59, 60).
- **Race.** Caucasian women have higher possibilities to develop BC than any other race. However, women of black race tend to present more aggressive carcinomas (61, 62).
- **Genetic mutations and family history.** First-degree relatives diagnosed with BC or ovarian cancer increases twofold the possibilities of developing a malignancy (63, 64). Estimations show that 5% - 10% of BC are associated with inherited genetic alterations. For example, BRCA1 and BRCA2 germline mutations have demonstrated a strong relationship with BC (65, 66).
- **Breastfeeding.** Breastfeeding has proved a crucial protective effect against hormone receptor-negative BC (67, 68).

- **Unhealthy life style.** Studies have shown that sedentariness, overweight and obesity as well as a poor diet, smoking and recurrent alcohol drinking are important risk factors (69, 70).
- **Hormone replacement therapy.** The use of oestrogen- and progesterone-based replacement therapies prescribed for menopause have been linked to BC development and late-stage detections (71, 72).

1.2.5 **Diagnosis**

BC can be diagnosed using multiple tools. Clinicians often declare the presence of carcinomas based on various confirmatory tests.

- **Diagnostic mammogram.** It is a detailed x-ray analysis performed to elucidate unclear screening mammograms or after the discovery of BC signs such as lumps or visible abnormalities (73).
- **Breast ultrasound.** Scan used to evaluate the internal breast tissue in detail. Particularly useful to determine the density of any abnormal structure (74).
- **Magnetic resonance imaging.** Body scan that produces multiple axial images. The results are valuable to establish the presence and degree of carcinomas (75).

- **Biopsy.** Surgical procedure that involves the liquid aspiration or tissue removal of suspicious areas (76). Obtained cells from biopsies are pathologically analysed to confirm cancerous lesions (77).

Furthermore, along with plasma, biopsies are used to test for molecular markers and tumour heterogeneity. These biomarkers are typically overexpressed in the existence of cancer. The ones that have proven clinical value are cancer antigen 15-3, cancer antigen 27.29, ER, PR, HER2, carcinoembryonic antigen, urokinase plasminogen activator, plasminogen activator inhibitor 1, human epididymis protein 4, p53, cathepsin D, cyclin E and nestin (78, 79). Markers are useful to diagnose early stages of BC, both de novo and recurrent, predict metastasis and determine suitable treatments.

1.2.6 Breast cancer standard treatment

Several factors are considered when selecting the optimal therapy such as menopause status, histological type, stage and tumour markers. Nevertheless, most patients are treated with combinations of surgery, radiotherapy, hormone therapy or chemotherapy.

1.2.6.1 Surgery

Surgical treatment is the main option for early-stage, localised BC; it may refer to a lumpectomy or mastectomy.

- **Lumpectomy.** Also known as conserving surgery as it removes the smallest possible portion of the breast tissue. The objective is to extract the tumour and adjacent healthy tissue (80). Currently, lumpectomy is considered the first approach to patients with non-advanced BC (81).
- **Mastectomy.** These surgical procedures can be partial or radical. A partial mastectomy involves the removal of a larger portion of tissue than the lumpectomy, which may include parts of the chest lining and muscle. On the other hand, the radical mastectomy implicates the ablation of the whole breast and axillary lymph nodes (82). Modified skin- and nipple-sparing mastectomies are less aggressive options, in which parts of the breast skin and chest wall are preserved (83). These surgeries can be followed by a breast reconstruction procedure.

1.2.6.2 Radiation therapy

Radiotherapy means the use of targeted high-power rays to kill malignant cells or decrease the size of tumours. There are two types of radiation therapy, external beam radiation and brachytherapy.

- **External beam radiation.** It is the most common type of radiotherapy. Often used as supportive treatment after a lumpectomy in order to destroy any remaining cancer cells (84). It can also be used to irradiate the chest wall after mastectomy for the removal of advanced and invasive tumours. Depending on the treatment plan, the external beam radiation may last from 3 days to 6 weeks (85).
- **Brachytherapy.** This type of radiotherapy involves the implantation of an internal radioactive source, which can be injected using needles, catheters or during surgery (86, 87). Brachytherapy allows the administration of higher doses in smaller areas. However, its use is limited upon consideration of tumour size and location.

Radiation therapy is usually well tolerated with sides effects limited locally. It includes bruised skin and irritation, breast swelling and heaviness as well as fatigue (88).

1.2.6.3 Hormone therapy

The objective of these adjuvant therapies is to inhibit the oestrogen-dependant pathways of hormone-fuelled carcinomas. This can be achieved by either decreasing the circulating hormones or administering ER- and PR-blocking treatments (89). There are several options to reduce the concentration of oestrogen, which is mainly produced in the ovaries. Surgical procedures

(oophorectomy) and radiological-induced ablation can be performed to block the production. Additionally, drug treatments such as the luteinising hormone-releasing hormone decreases the oestrogen and progesterone levels in the body. Aromatase inhibitors like anastrozole and letrozole, prevent the conversion of androgens into oestrogen (90).

On the other hand, there are two types of receptor blockers: selective oestrogen receptor modulators (SERM) and selective oestrogen receptor degraders (SERD). SERMs are able to block the effect of oestrogen by binding to the ER, producing inactive complexes. Examples of widely used SERMs are tamoxifen, raloxifene and toremifene (91). SERDs, while having a similar mechanism, the result is different. The binding of SERDs to ER leads to proteasomal degradation of the receptor (92). Fulvestrant is the most used SERD, which is only prescribed to postmenopausal women.

1.2.6.4 Targeted therapy

These adjuvant treatments are meant to target specific characteristics only expressed in cancer cells, especially those that promote abnormal cell division. Examples of targeted therapies for BC are monoclonal antibodies, tyrosine kinase inhibitors (TKI), mTOR inhibitors, cyclin-dependant kinase inhibitors (CDKI), and poly ADP ribose polymerases (PARP) inhibitors.

- **Monoclonal antibodies.** This therapy includes laboratory-developed antibodies to specifically bind cell receptors overexpressed in tumour cells. Trastuzumab is the most used antibody treatment, which binds HER2 and stops the proliferation signal. Another example is pertuzumab for the treatment of inflammatory and metastatic BC (93). Also, antibody-drug conjugates like ado-trastuzumab emtansine and sacituzumab-govitecan are used for the therapy of recurrent carcinomas and advanced triple-negative BC respectively (94).
- **TKIs.** These molecules inhibit the phosphorylation of signalling pathways known to promote cell growth (95). The most representative TKIs are lapatinib and neratinib, regularly used in HER2-positive malignancies that have developed resistance to trastuzumab (96). TKIs are administered with other anticancer drugs.
- **mTOR inhibitors.** These inhibitors specifically block the mammalian target of rapamycin, disrupting the PI3K pathway (97). Such signalling pathway plays a key role in cell metabolism, proliferation and angiogenesis. Everolimus is a relevant mTOR inhibitor for the treatment of HER2-negative malignancies and hormone-receptor-positive advanced BC with poor response to other therapies (97).
- **CDKI.** These inhibitors target specific proteins of the cyclin-dependant kinase family, arresting the progression of the cell cycle (98). Some

examples of CDKIs are palbociclib and ribociclib, which, used in combination with other therapies, are prescribed in cases of HER2-negative metastatic BC and recurrent carcinomas (98). Abemaciclib is a good option for patients with HER2-negative, hormone-receptor positive metastasis (98).

- **PARP inhibitors.** PARP are enzymes that repair single-strand DNA breaks. PARP inhibitors block the action of such proteins, promoting cell death (99). Olaparib and talazoparib are promising PARP inhibitors for the treatment of HER2-negative advanced BC with germline BRCA1 and BRCA2 mutations (99). Their efficacy as adjuvant therapy administered with antineoplastic drugs and radiation as well as in triple-negative BC is under study (99, 100).

Due to their specificity, these therapies have shown less severe adverse effects than regular chemotherapy. Nonetheless, their administration is limited to the presence of the appropriate target and cell resistance development.

1.2.6.5 Chemotherapy

Chemotherapy is the systemic administration of small molecules with cytotoxic activity aimed to kill or inhibit the proliferation of cancer cells. Conventional chemotherapeutics include alkylating drugs, intercalating agents, antimetabolites, antimitotic drugs and topoisomerase inhibitors. There are

several factors to consider when selecting the right therapy such as BC type, grade and stage, receptor and invasive status, and lymph node infiltration (101). It is important to define that adjuvant chemotherapy is typically prescribed after an invasive tumour ablation to reduce the possibilities of recurrence, whereas neoadjuvant chemotherapy is administered to reduce the size of tumours before surgery. Chemotherapy is the first-line treatment for advanced-stage BC (102).

Some of frequently used anticancer drugs approved for BC are anthracyclines, taxanes, 5-fluorouracil, platinum (Pt) agents, cyclophosphamide, gemcitabine, methotrexate and mitomycin C (103, 104). Particularly, anthracyclines is a family of chemotherapeutic drugs widely used for the treatment of virtually all types and stages of BC (105). The leading anthracycline compound is doxorubicin (DOX).

1.3 Doxorubicin

DOX is an anticancer antibiotic approved for the single use or in combination with other drugs for the treatment of several cancers such as leukaemia, gastric, ovarian, lymphoma, sarcomas, thyroid and BC among others (106) (figure 1.6). DOX is used as adjuvant therapy to prevent BC recurrence, while neoadjuvant therapy helps on the management of advanced-stage/metastasis and large tumours (107). DOX is part of the WHO model list of essential medicines (108), demonstrating its importance in the cancer therapy.

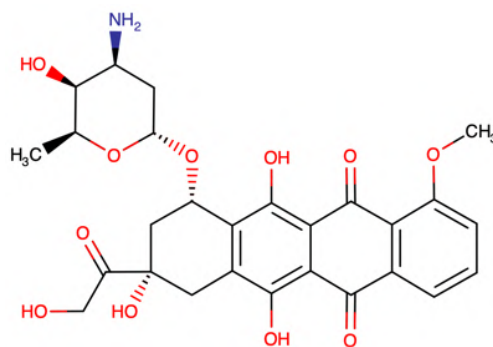


Figure 1.6 | Doxorubicin chemical structure. DOX is a tetracyclic anthraquinone linked to a sugar moiety. The amino group is the most reactive group of the molecule. Image obtained from (109).

DOX was originally isolated from *Streptomyces peucetius* var. *caesius* in the 1960s (110), and approved by the Food and Drug Administration of the United States of America (FDA) for medical use in 1974. The cytotoxicity mechanism of DOX is not totally understood; however, there are some accepted theories.

1.3.1 Doxorubicin mechanism of action

DOX diffuses inside the cell and binds to the proteasome, which translocates the drug into the nuclei (111). The anthraquinone structure has a higher affinity with the DNA than the proteasome (111). Hence, DOX intercalates among base pairs, preferably to adjacent guanine-cytosine. Hydrogen bonds and electrostatic forces stabilise the complex DOX-DNA-topoisomerase II (TOP2), while the drug's sugar moiety interacts with the minor groove, leading to DNA fragmentation and DNA and RNA synthesis blockade (112, 113). Failure to repair the damage causes cell cycle arrest in G1 and G2 phases, triggering apoptosis (111).

Other DOX effects are inhibition of macromolecule synthesis, DNA unwinding, energy depletion, autophagy and necrosis (111). Studies suggest that the cell death mechanism may depend on the DOX accumulation, treatment period and cancer type (111) (figure 1.7).

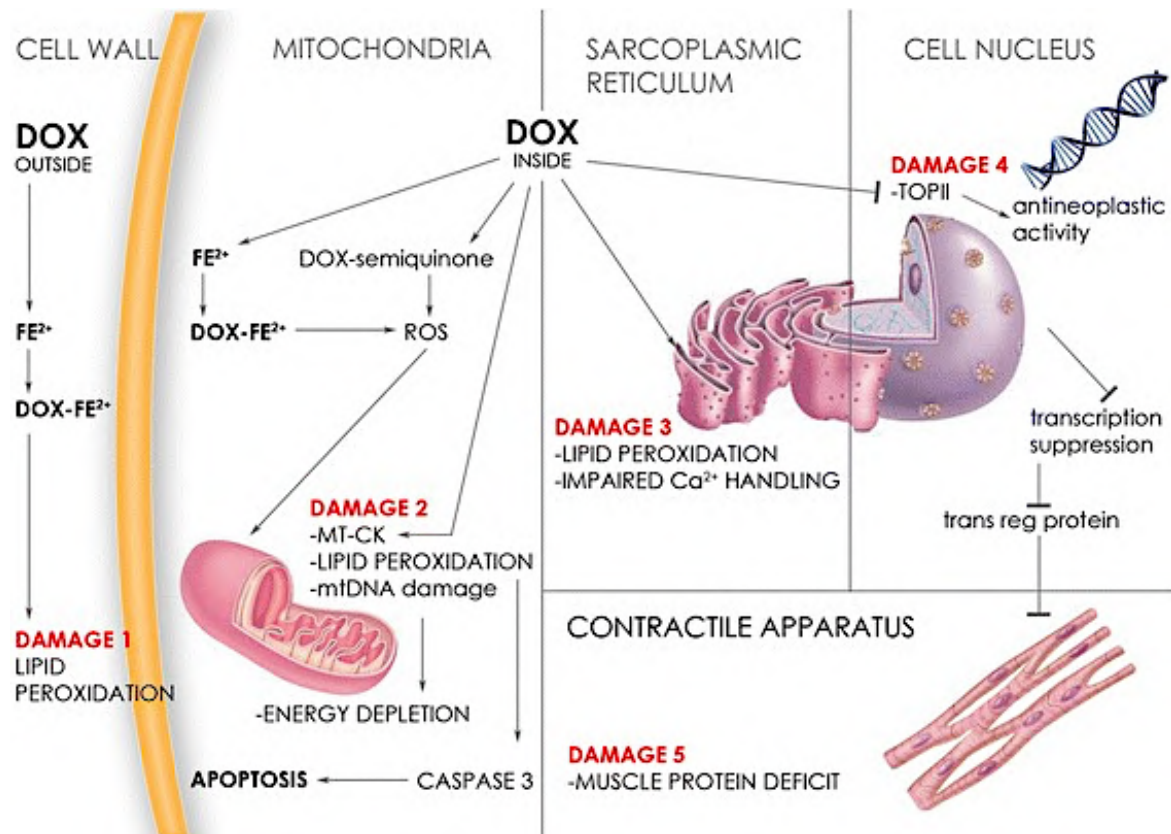


Figure 1.7 | Proposed mechanism of action of Doxorubicin. There are various mechanisms of action proposed for the DOX cytotoxicity. Recognised DOX actions include lipid peroxidation, energy depletion and formation of DOX-DNA-TOP2 complex, leading to cell death. Image obtained from (114).

Another theory proposes that DOX is metabolised to semiquinone by oxide reductases in a reversible reaction called redox-cycling. The reaction can occur in the cytoplasm, endoplasmic reticulum, mitochondria (NADH dehydrogenase) and nucleus, with the inherent production of reactive oxygen species (ROS) (115, 116). In addition, semiquinone can be oxidised, returning to the DOX molecule

with the sub-product of superoxide species in a non-enzymatic perpetual reaction (116, 117). ROS imbalance is related to membrane and DNA damage, lipid peroxidation, oxidative stress and ultimately apoptosis (115).

Finally, DOX can also complex with molecular iron, resulting in toxic reactive nitrogen species (RNS) and ROS (115). Regarding pharmacokinetics, DOX presents a fast plasma clearance with high tissue concentration (118). 50-85% of DOX binds to serum albumin (119). The drug is enzymatically oxidised in the liver, producing the important free radicals involved in its toxicity. It has an average half-life of 30h (119). More than half of the drug is excreted in the bile and only a tenth in the urine (120).

1.3.2 Doxorubicin adverse effects

Even though DOX is one of the most used chemotherapeutic agents, its clinical administration is limited due to severe adverse effects. One single dose has demonstrated to cause severe damage in some organs (121) (figure 1.8).

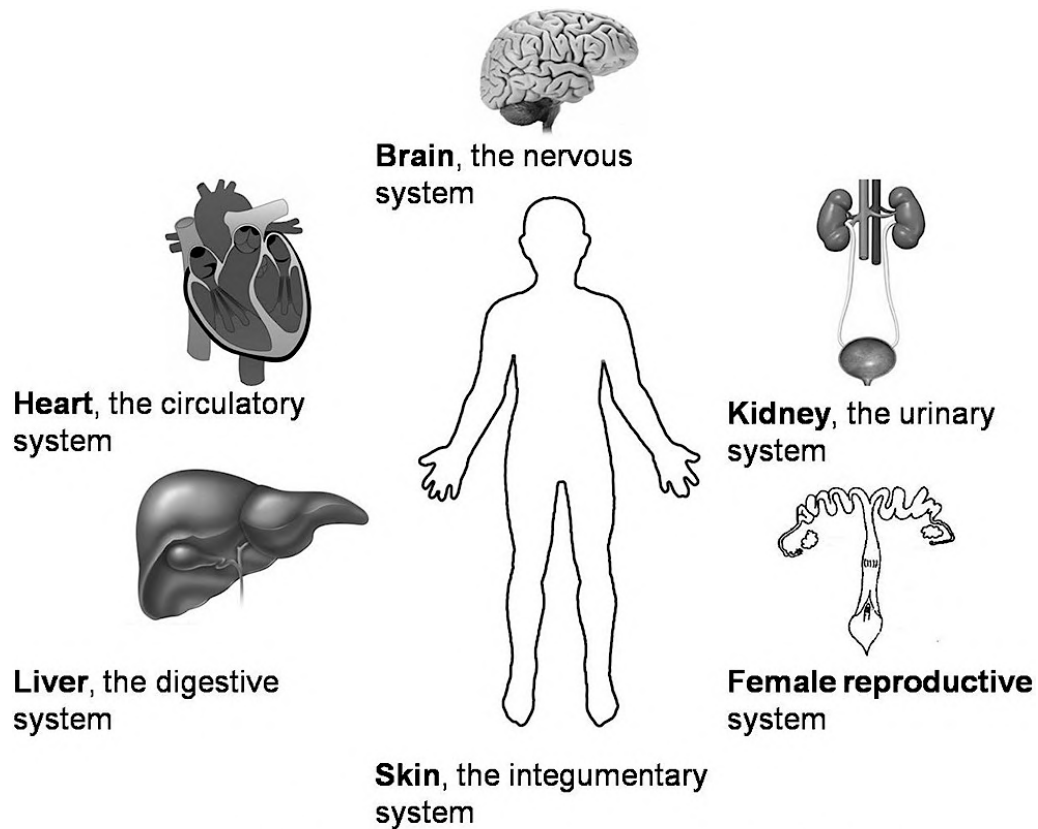


Figure 1.8 | Organs affected by the administration of doxorubicin. Doxorubicin side effects range from mild to severe, being the most frequent emesis, nephrotoxicity and cardiotoxicity. Image adapted from (122).

- **Cardiotoxicity.** The most studied disadvantage is the chronic cardiotoxicity. Incidence rates vary from 5% to 48% risk in cumulative doses of 400-700 mg/m² (123). The myocardial damage can range from arrhythmias and myocarditis to severe ventricular dysfunction and congestive heart failure (123). Patients at advanced age and those with pre-existing cardiac disease are at higher risk. It is suspected that oxidative stress and energy imbalance due to mitochondrial dysfunction are decisive factors on the cardiotoxicity development (124). The strategies to diminish the risk of cardiotoxicity are limited. Although low doses of DOX have proven to cause damage to some extent, clinical

studies recommend limiting the cumulative dose to 450 mg/m² (125). Prolonging the drug's infusion time over 6h and weekly administrations have showed good results (126). Probably the best options to mitigate the cardiotoxicity is the use of cardio protective therapies such as iron-chelating agents, angiotensin-converting enzyme inhibitors, β -blockers, angiotensin receptor inhibitors, statins and antioxidants (125, 126).

- **Neurotoxicity.** DOX affects the brain indirectly through a pathway that involves ROS. The drug promotes the production of tumour necrosis factor alpha; this factor activates the signal so microglial cells produce inflammatory cytokines (111, 127). This process will increase the levels of RNS which will trigger the production of ROS and eventually apoptosis and autophagy (111). As high as 75% of patients can show cognitive impairment; however, most of them fully recover within one year after the treatment finished (127). The prevention includes physical exercise, cognitive behavioural therapy, anti-cholinergic agents and selective serotonin reuptake inhibitors (127).
- **Hepatotoxicity.** Approximately 40% of people treated with DOX suffer from liver damage (128). The injury is due to the metabolism of the anthracycline, a process that generates oxidative stress, AMP, ADP and ATP depletion, and cell death. DOX has not showed to cause acute liver failure or chronic hepatitis (128, 129). Studies claim the improvement of

hepatotoxicity with the use of flavonoids (130), carotenoids (131), and natural products (132).

- **Nephrotoxicity.** DOX is known to damage the glomerular podocytes, causing proteinuria and glomerulosclerosis (111). The cause is believed to be due to the drug's disruption of the electron transport chain of the mitochondria and the following ROS production and lipid peroxidation (111). The oxidative kidney injury could be alleviated with the use of natural products like vitamin E, flavanones, flavonoids, N-acetylcysteine and propolis derivatives (133, 134). There are other promising components under preclinical studies such as theanine (135) and nicotinamide (136).

Despite the fact that DOX is widely used to treat BC and other malignancies, there are further antineoplastic drugs that have proven excellent results, such is the case of Pt agents.

1.4 Cisplatin

Cisplatin (CDDP), also known as cis-diammineplatinum (II) dichloride, is prescribed both as single and combinatory therapy in more than half of human cancers. It is effective to treat lung, gastric and bladder cancers as well as gynaecological and germ cell malignancies to name a few (137, 138). Moreover, the antineoplastic drug is frequently incorporated in treatment schemes, both as

adjuvant and neoadjuvant therapy, for primary BC, in addition to recurrent, advanced and metastatic tumours (139).

CDDP was the first member of the Pt-based drug family and to date, the most extensively used (140). Serendipitously discovered in 1965 by Barnett Rosenberg, CDDP was approved for antitumoral use in 1978 (141) (figure 1.9).

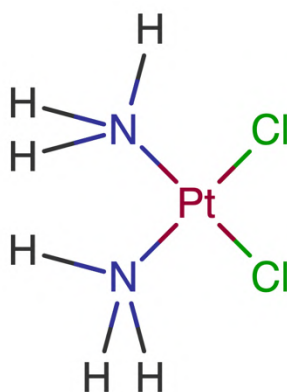


Figure 1.9 | Cisplatin chemical structure. CDDP chemical conformation consists in a square-planar molecule, comprising a Pt (II) central atom coordinated with two ammonia and two labile chlorine ligands in a cis arrangement. Image obtained from (142).

1.4.1 Cisplatin mechanism of action

The relatively inert alkylating agent enters the cell through passive diffusion, facilitated transport with the help of organic cation transporters (OCT), and active transport through transmembrane copper transporters CTR1 and CTR2 (137, 143). Inside the cell, CDDP is hydrolysed, displacing the chloride molecules and resulting in a more reactive positively-charged complex. This reaction is promoted by the low chloride concentration in the cell (144, 145) (figure 1.10).

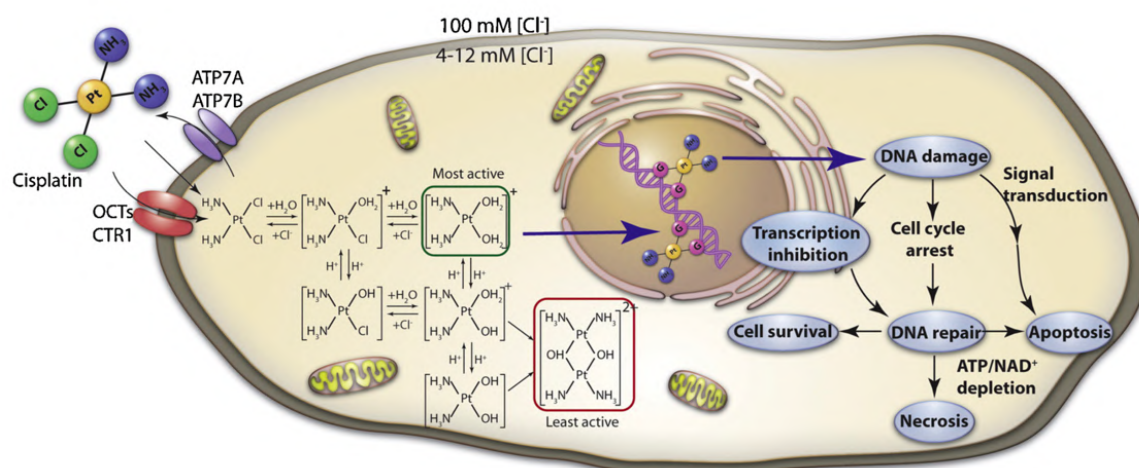


Figure 1.10 | Mechanism of action of cisplatin. CDDP is intracellularly activated due to the transmembrane difference of chloride concentrations. Bis-aquated CDDP promptly binds DNA, blocking DNA transcription and replication, which leads to cell cycle arrest and cell death. Image obtained from (146).

The aquated CDDP translocates into the nucleus to covalently bind imidazole groups of purine bases present in the DNA major groove (147). The drug preferably binds to guanine, causing a high number of DNA adducts, intrastrand crosslinks and interstrand crosslinks (147, 148) (figure 1.11). CDDP can also interact with RNA, mitochondrial DNA, peptides like glutathione (GSH), sulfhydryl moieties in proteins, phospholipids and disrupt the cytoskeleton (149).

DNA adducts are responsible for most of the CDDP cytotoxicity mechanisms, which include DNA unwinding, suppression of DNA replication, RNA transcription inhibition, cell cycle arrest and ultimately cell death (150). The DNA damage activates a signal transduction cascade that affects the cell from different fronts. Adducts are detected by the mismatch repair (MMR) complex, the nonhistone chromosomal high-mobility group (HMG) 1 and 2 and nucleotide excision repair (NER) proteins (150, 151). MMR and NER are in charge of restoring the function

of DNA by cutting and removing the affected DNA sites; failing to do it causes G2/M phase arrest of the cell cycle and triggers apoptosis. In contrast, HMG proteins promote toxicity progression by protecting damage from repair; HMG also stimulates the action of p53 (150, 151). Besides, in efforts to repair DNA breaks, PARP cleaves nicotinamide adenine dinucleotide (NAD^+), blocking the glycolytic synthesis of ATP. Depletion of ATP can lead to necrosis and autophagy (152, 153).

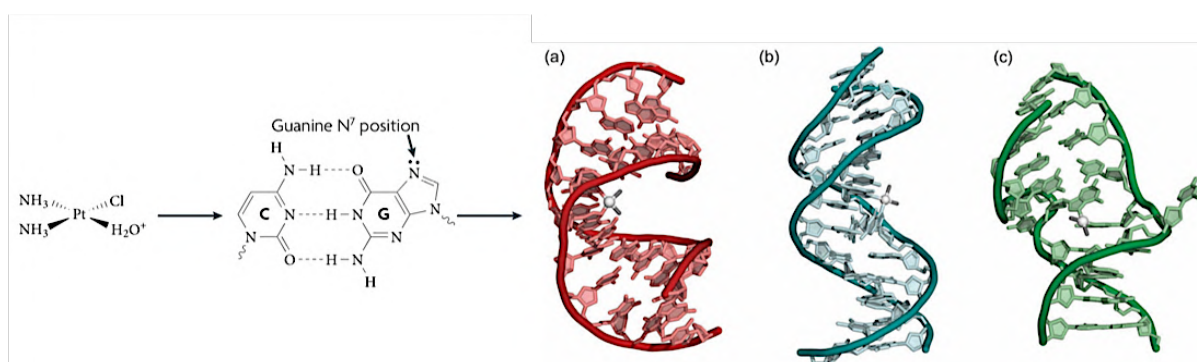


Figure 1.11 | Cisplatin effect on DNA. Activated bis-aqua CDDP binds to DNA, preferentially to the N7 position of guanines, causing DNA bending and unwinding. It is calculated that (a) 1,2-d(GpG) intrastrand crosslinks accounts for approximately 60-65%, 20-25% of the adducts are 1,2-d(ApG) intrastrand crosslinks (not shown), (b) 5-10% are 1,3-d(GpNpG) intrastrand crosslinks and (c) only 1-3% of the adducts are interstrand crosslinks. Image adapted from (154, 155).

Independently of DNA damage, ROS generation is also an important mechanism of CDDP antitumoral activity. ROS imbalance is linked to changes in the membrane fluidity due to the CDDP binding with phospholipids and fatty acids (156). Moreover, CDDP can alter the redox balance as a result of its interactions with thiol group of peptides and proteins, which produce superoxide formation (156).

Finally, the drug damages the mitochondria, inducing oxidative stress, lipid peroxidation, mitochondrial calcium flux inhibition and disruption of membrane potential (156-158). All these related mechanisms will lead cells to apoptosis and necrosis.

After intravenous diffusion, CDDP rapidly complexes with serum proteins. It is calculated that more than 90% of the drug binds permanently to serum albumin (142). Clinically, the Pt agent presents a half-life of 14-49min and 11-12 L/m² volume of distribution, with highest concentrations observed in liver, kidneys and intestines. 15-30% of the drug is eliminated in the first 2-4h, the rest can take up to 24h (142, 159, 160).

1.4.2 Cisplatin adverse effects

Toxicity is a major obstacle in the general use of CDDP. Adverse effects range from mild to life threatening and can be transient or permanent. Mild effects are normally limited to injection site hypersensitivity, skin rash, dry mouth and skin and loss of appetite (161). Nonetheless, a high percentage of patients suffer from severe side effects (figure 1.12).

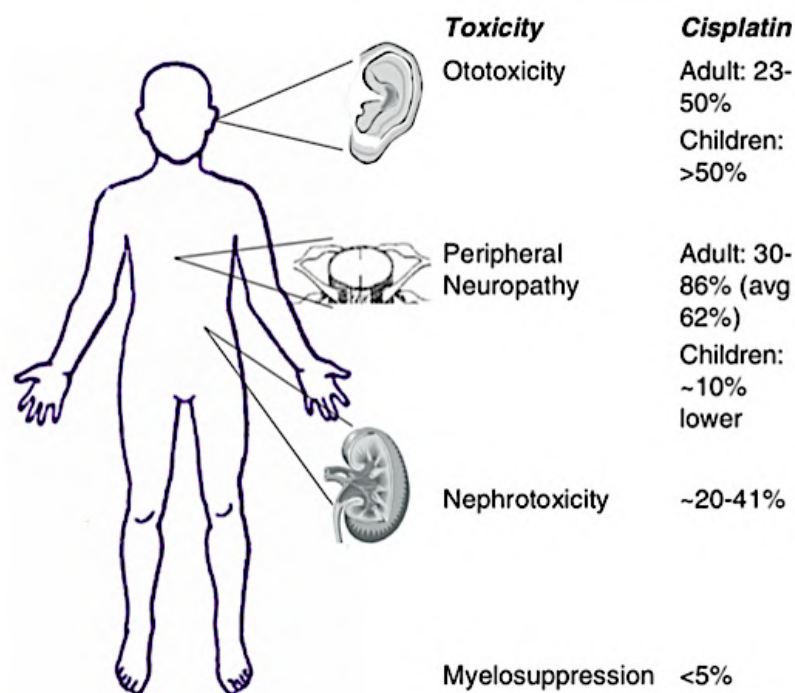


Figure 1.12 | Toxicities associated with the administration of cisplatin. Regular CDDP side effects are myelosuppression, neuropathy, nephrotoxicity and ototoxicity, being the latter remarkably more frequent in infant patients. Image adapted from (162).

- Nephrotoxicity.** Kidney injury is a major CDDP disadvantage, affecting 20-41% of treated people (162). Normal renal excretion promotes the drug accumulation in the kidney, reaching concentrations several times higher than any other body tissue. In hepatocytes, cytoplasmic GSH binds to CDDP. The complex is pumped out of the cell and transferred for elimination. However, the conjugate can be enzymatically cleaved, leaving CDDP free and active in the kidney (163). Evidence suggests that nephrotoxicity is caused due to mitochondrial oxidative stress, causing pro-inflammatory cytokines, apoptosis and necrosis (162-164). Renal damage can be prevented with hydration and mannitol before, during and after chemotherapy administration (165, 166), using anti-oxidants such as

N-acetylcysteine, lycopene and vitamins C and E (167) and forced diuresis (168). It is important to note that the extent of damage depends on the frequency, time and dose of the drug. Thus, the importance of managing an appropriate treatment scheme.

- **Ototoxicity.** As high as 54% adult and 77% infant patients suffer from ototoxicity, making CDDP one of the most ototoxic drugs in medical practice (169). Both ears can be permanently affected. It is believed that tinnitus and hearing loss is related to ROS production. As explained previously, CDDP induces the production of oxidative stress, which is closely related to cell death pathways. Cochlear cells present a high expression of the NADPH oxidase 3 (NOX3), which is known to readily produce superoxide radicals (162). Ototoxicity treatment is limited and usually ineffective; damage can be alleviated with lower dose and repeated administration of the antineoplastic and the use of thiol antioxidants, vitamin E and anti-inflammatory agents (162, 170, 171).
- **Neurotoxicity.** An average of 62% of CDDP treated patients suffer from peripheral neuropathies characterised by sensory loss in hands and feet, proprioception deficit and ataxia in severe cases (162, 172). CDDP affects the central nervous system by damaging the myelinated fibres and decreasing the sensory nerve conduction potential and velocity (172). Chronic neurotoxicity may be caused by the accumulation of CDDP and apoptosis of dorsal root ganglia neurons, sodium and potassium channels

block, calcium chelation, oxidative stress and mitochondria disruption (173, 174). While vitamin E and erythropoietin have showed neuroprotective benefits (162), studies claim protective properties of sulphur-amino acids (175), progesterone (176) and the natural compound thymoquinone (177).

- **Myelosuppression.** Leukopenia, thrombocytopenia and anaemia are associated with CDDP inhibition of bone marrow cells (178). Haematological toxicity usually aggravates with combinatory chemotherapy. Palliative treatments include blood transfusion, erythropoietin and colony-stimulating factors (178, 179).
- **Gastrointestinal toxicity.** CDDP attacks the lining cells of the gastrointestinal tract, inducing nausea and emesis in virtually all patients. Ranging from moderate to very intense, these adverse effects are considered some of the most common causes of denial to undergo chemotherapy (180). Nevertheless, there are effective treatments to control emesis, for example, dexamethasone and serotonin and neurokinin-1 receptors antagonists (181).

1.5 Resistance to chemotherapy

Another important fact to consider in the clinical practice is the cellular resistance to chemotherapy, which is pointed as the main reason of therapy failure.

Generally, tumour cells have the ability to develop resistance, which can be intrinsic or acquired. Several mechanisms underlying resistance can be involved as shown in figure 1.13. Cancer stem cells present in tumours may be responsible for the intrinsic resistance due to their increased DNA repair response, death resistance and high expression of efflux proteins (182, 183). Therefore, cellular resistance would be present from carcinogenesis. Other explanations are associated with the cancer environment characteristics and developed resistance, such as tumour heterogeneity, mutations that increase protective pathways, failed response to apoptosis and drug inactivation (183, 184). Overall, frequent chemotherapy resistance mechanisms are presented as follows.

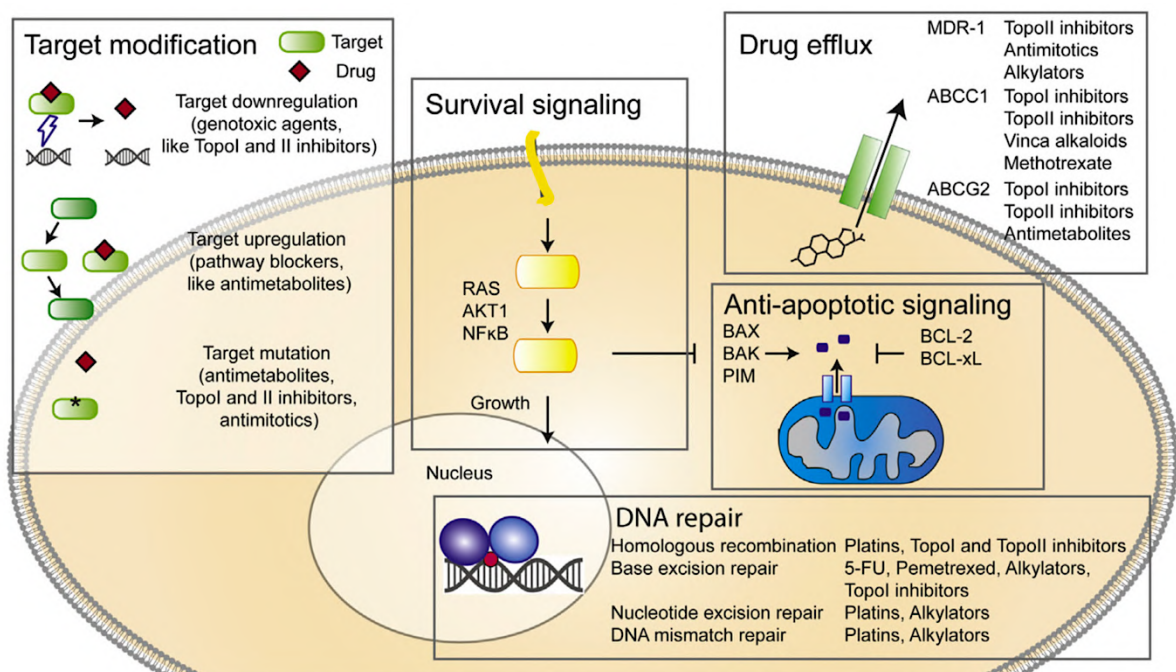


Figure 1.13 | Cellular mechanisms associated with chemotherapy resistance. Intrinsic or developed resistance to chemotherapeutic drugs frequently involve the inhibition of apoptotic pathways and increased DNA repair machinery, which promotes apoptosis evasion. Additionally, the overexpression of efflux proteins leads to ineffective intracellular drug concentrations; the upregulation of growth signalling pathways stimulates the uncontrolled division of malignant cells, whereas mutation of drug targets avoids the correct action of therapies. Image obtained from (185).

- **Multidrug resistance transporters.** These proteins are members of the efflux ATP-binding cassette (ABC) transporter family, normally expressed in all cells, with higher levels in kidneys, intestines, placenta, liver and blood-brain barrier (186, 187). They are responsible to cause the inability of the drug to accumulate in the target organelles, preventing cell death. Today, the ABC family comprises 49 different transporters. Nonetheless, the most relevant are multidrug resistance protein 1 (MDR1), multidrug resistant associate protein (MRP) and breast cancer resistance protein (BCRP) (186). MDR1 is the most studied efflux transporter, which is capable to reduce the intracellular accumulation of anthracyclines, taxanes and Pt agents among many others (185). BCRP is an important efflux protein often overexpressed in BC and related with numerous drug interactions such as DOX and CDDP (187, 188).
- **Topoisomerase 2 beta overexpression.** Several anticancer agents complex with TOP2 alpha isoform in order to exert their cytotoxic mechanism. Researchers suggest that anti-tumour efficacy could be diminished in case of TOP2 beta overexpression due to lack of specificity (189, 190). Studies have found that BCs with high expression of TOP2 alpha are associated with good treatment response, while TOP2 alpha deletions have the opposite outcome (191, 192).

- **Increase damage repair response.** The MMR and NER complexes are important mediators of the DNA damage repair. Variations in these proteins, along with ERCC1, a relevant resistance biomarker, frequently occur in cancer and are closely related to the repair of CDDP crosslink DNA lesions (154, 193). Increase in damage repair leads to downregulation in apoptosis.
- **Apoptosis inhibition.** The programmed cell death plays a very important role in DOX and CDDP cytotoxicity. While apoptosis maintains a strict equilibrium in normal conditions, disruption in the pathway can lead to chemoresistance. Particularly important are mutations or deletions of p53, present in many cancers, overexpression of Bcl-xL, aberrant Bcl2, and alterations in the tumoral necrosis factor family (194, 195).
- **Drug inactivation.** Antineoplastic compounds can be inactivated in the cytoplasm, blocking their cytotoxic action. In the case of CDDP, GSH and metallothioneins among others are responsible for binding the drug, solubilising it, and excreting it out of the cell with the help of efflux transporters (196, 197). Increased concentrations of sulphur-containing molecules have been associated with resistance in ovarian cancer (198), lung cancer (199) and BC (200) among others.

1.6 Limitations of oncology practice

Cancer therapies have made a great progress over the years, offering a wide range of treatment options. With the support of biomarkers and precise diagnoses, cancer prognosis is typically good in early stages. Nevertheless, survival rates in advanced stages are still low. Conventional antineoplastic drugs remain as the gold standard treatment in advanced malignancies, but they are far from ideal. Their administration is limited due to their low specificity, commonly leading to severe toxic side effects that may be permanent and risk patients' lives. Furthermore, cellular resistance is a major problem as it impedes the success of therapies and brings the need of combining other drugs with their respective adverse effects. Therefore, researchers are focusing efforts to find alternatives that improve current treatments and overcome the mentioned problems. In this context, nanomedicine has offered revolutionary approaches with a novel generation of therapeutic systems.

1.7 Nanomedicine

Nanomedicine involves the synthesis, manipulation, control, measurement and application of nanoscale materials for medical purposes. Nowadays, nanomedicine has showed promising results in multiple areas. The aim of complexing active molecules with nanoparticles (NPs) is to achieve unmet needs in the clinical field.

Major objectives are delivering compounds that could not be administered freely like poor-water soluble molecules and improving the therapeutic efficacy of drugs while reducing the toxicity. Also, to modify unfavourable drug's pharmacokinetics, combine different mechanisms of action and control the release and target of the active molecule (201). With current technology, nanostructured materials can be synthesised with precise characteristics like size, shape, coating and chemical ligands (202, 203). Applications of nanomedicine can be classified in three main areas: diagnostics, regenerative and drug delivery.

Table 2 | Classification and applications of nanoparticles.

	Nanoparticle	Application
Diagnostics	Iron oxide	Image-guided cancer therapy (204)
	Silver	Enhanced cancer theranostics (205)
	Gold	Photoacoustic imaging (206)
	Silica	Optical imaging (207)
Regenerative	PLGA	Bone regeneration (208)
	Gold	Bone regeneration (209)
	Glass-gold	Skin wound regeneration (210)
	Extracellular micro-vesicles	Tissue regeneration (211)
Drug delivery	Liposomes	Chemotherapy (212-214)
	Chitosan	Antimicrobial (215)
	Graphene oxide	HIV inhibitors (216)
	Carbon nanotubes	Chemotherapy (217-219)

Poly-lactic-co-glycolic acid (PLGA); human immunodeficiency virus (HIV).

As observed previously, several types of NPs have been used as delivery vehicles. Possibly, the most representative DDS is the first approved for human use Doxil. Also known as Caelyx, the nanoformulation consists in a pegylated-liposomal system that encapsulated DOX. It is prescribed as adjuvant and neoadjuvant treatment for various malignancies, including ovarian cancer, Kaposi's sarcoma, and BC.

Doxil has demonstrated advantages over conventional therapies. In preclinical models, the liposomal formulation showed improved therapeutic efficacy and higher median lethal dose compared to free DOX (220). Moreover, clinical studies demonstrated that cumulative doses of 500 mg/m² or more of Doxil reduced considerably the risk of developing cardiomyopathy, an adverse effect generally associated with free DOX (221). Studies have demonstrated that the PEG-coating of liposomes prevents the recognition of the endoplasmic reticulum, which prolongs the circulation half-life, reduces the volume of distribution, and slows the clearance rate in comparison to free DOX (222). Such pharmacokinetic properties in addition to the nanometric particle size of the formulation, provide advantages at passively increasing the drug accumulation in tumours while modifying the toxicity profile (222). Nonetheless, a meta-analysis of randomised clinical and preclinical studies that compared the anticancer efficacy of conventional and liposomal DOX concluded that no significant difference was observed (223). The therapeutic efficacy, overall survival, and progression free survival of patients was no different between the two formulations (223).

Despite such results, the improvement of adverse effects that Doxil has demonstrated are encouraging and should be considered as an important benefit in clinical use. Additionally, these findings stress the importance of thorough assessment of not only biological responses of nanoformulations like the carbon nanotube-based systems presented herein, but also of the pharmacological properties.

Based on the chemical composition, NPs are categorised in metal, ceramic, semiconductor, polymeric, lipid-based NPs and carbon-based nanomaterials (224, 225). The latter ones and particularly carbon nanotubes (CNTs) have attracted the scientific attention due to their distinctive structure, exceptional characteristics and ability to attach a wide range of active molecules.

1.8 Carbon nanotubes

CNTs are graphitic-derived, hollow seamless cylinders comprised of hexagonal sp^2 hybridised carbon atoms, members of the fullerene family (226). They are the result of the plication of graphene sheets and can be simply classified in single-walled carbon nanotubes (SWNTs) when a single graphene sheet is involved and multi-walled carbon nanotubes in particles constructed of multiple sheets (227). These differences confer an average diameter of 0.4 to 2nm for the SWNTs, while MWNTs can range from 1.4 to 100nm diameter (228) (figure 1.14).

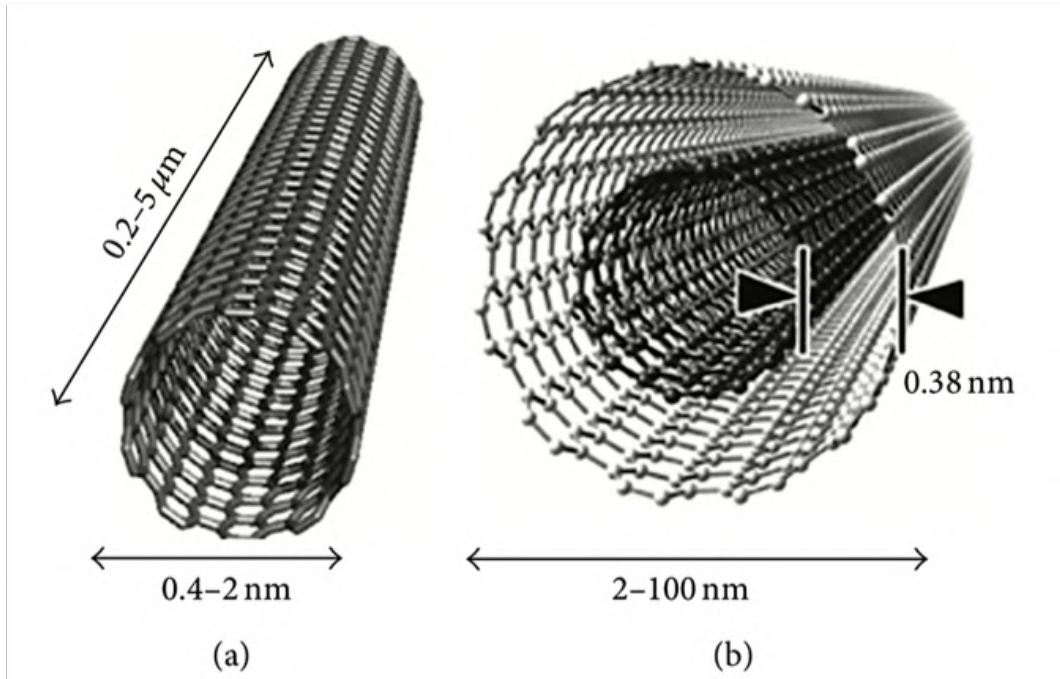


Figure 1.14 | Carbon nanotubes structure. CNTs are graphene sheets rolled up into empty cylinders. CNTs are classified in (a) single-walled carbon nanotubes and (b) multi-walled carbon nanotubes based on the number of graphene layers. Image obtained from (229).

Discovered by Sumio Iijima in 1991 (230), CNTs have demonstrated unique properties. The high aspect-ratio nanomaterial is lightweight; possesses elevated stiffness and tensile strength values as well as exceptional elastic buckling ability (231-233). In addition, CNTs present great adsorption capability and thermal stability (234, 235). Depending on the atom arrangement and geometry, CNTs can have metallic or semiconducting electronic characteristics (236).

Table 3 | Summary of single-walled carbon nanotubes and multi-walled carbon nanotubes characteristics. Table adapted from (237).

SWCNT	MWCNT
Single layer of graphene.	Multiple layer of graphene
Catalyst is required for synthesis.	Can be produced without catalyst.
Bulk synthesis is difficult as it requires proper control over growth and atmospheric condition.	Bulk synthesis is easy.
Not fully dispersed, and form bundled structures.	Homogeneously dispersed with no apparent bundled formation.
Resistivity usually in the range of 10^{-4} – 10^{-3} Ω -m.	Resistivity usually in the range of 1.8×10^{-5} – 6.1×10^{-5} Ω -m
Purity is poor. Typical SWCNT content in as-prepared samples by chemical vapour deposition (CVD) method is about 30–50 wt%.	Purity is high. Typical MWCNT content in as-prepared samples by CVD method is about 35–90 wt%.
However high purity up to 80% has been reported by using arc discharge synthesis method.	
A chance of defect is more during functionalization.	A chance of defect is less especially when synthesized by arc-discharged method.
Characterization and evaluation is easy.	It has very complex structure
It can be easily twisted and are more pliable.	It cannot be easily twisted.

1.8.1 Carbon nanotubes synthesis

The most frequently employed methods to produce CNTs are electric arc discharge (EAD), laser ablation, gas-phase catalytic growth from carbon monoxide and chemical vapour deposition (CVD) from hydrocarbons (238).

- **Electric arc discharge.** This synthesis process is the most used. The reaction is performed in a pressured chamber containing two pure graphite electrodes, under constant argon, helium or organic atmospheres, which promotes the carbon deposition (figure 1.15) (239, 240). An electric arc is produced applying 20V direct current and approaching together the electrodes to about 1mm apart. While the anode is pulled to the cathode, the temperature rises to $>3,000^{\circ}\text{C}$, vaporising the carbon atoms (240). Once the reaction is stabilised, MWNTs are deposited on the negative electrode and a fullerene soot is collected in the chamber. SWNTs can be

produced by adding metal catalysts to the graphite such as Ni, Co, Pd, Ag and Cu (239-241).

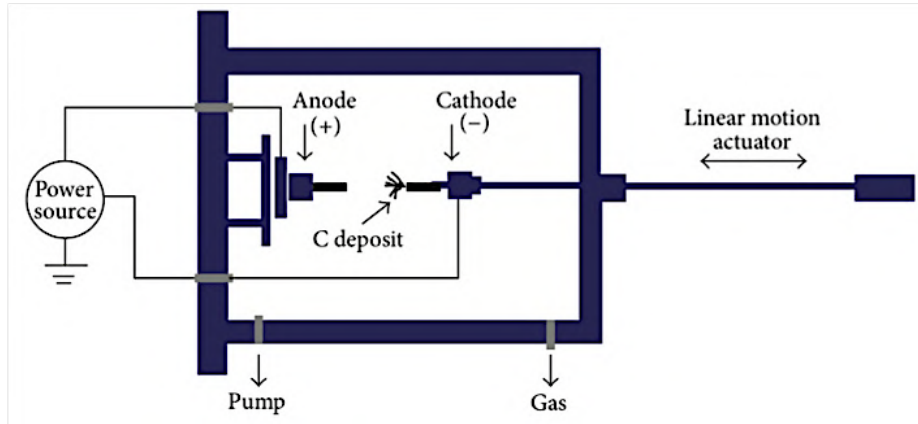


Figure 1.15 | Arc discharge method. Schematic illustration of the arc discharge method performed to synthesise CNTs. Image adapted from (242).

Although this technique is the easiest to implement and produces high yield of low structural defects CNTs, there are important disadvantages to consider. Along with CNTs, amorphous carbon forms are obtained, reducing the purity of the product (243). Moreover, this method limits the control of the nanomaterial structure, resulting in heterogeneous nanoparticles (243).

- **Laser ablation.** This preparation method involves the ablation of graphite using krypton fluoride laser pulses (244). Here, the carbonaceous material is mixed with approximately 1% of Ni and Co metal catalysts and placed in a quartz chamber (245). The reactor is heated to 1,200°C with constant argon flow while the laser is targeted (244). Finally, a soot containing CNTs is deposited in a cooled collector (figure 1.16).

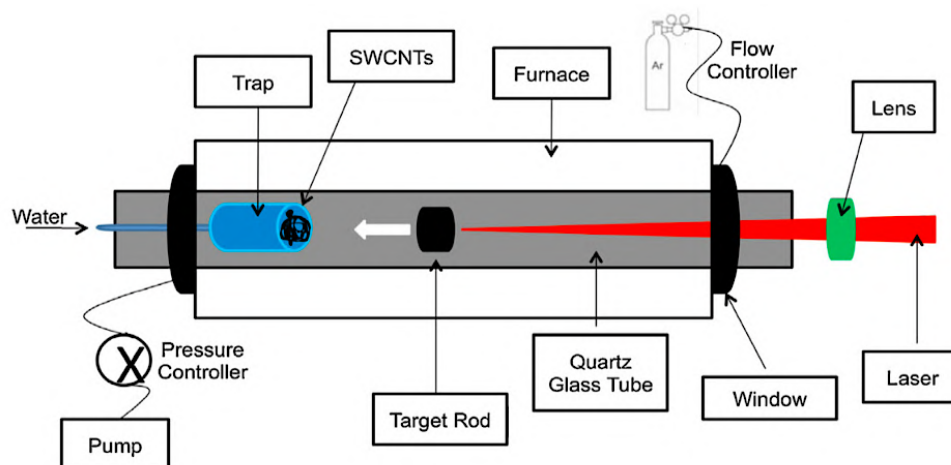


Figure 1.16 | Laser ablation method. Schematic example of the laser ablation method performed to synthesise SWCNTs. Image obtained from (240).

The most important variables in this technique are the composition of the target material, temperature, gas flow rate and nucleation substrate (246). This technique provides highly graphitised and pure nanostructures (246).

- **Chemical vapour deposition.** CVD is the choice method for industrial synthesis of CNTs due to facility to scale up the production. In this technique the source of carbon material is vaporised, regularly a hydrocarbon like methane or acetylene mixed with an inert gas (247). The volatile compound is transported in a quartz tube connecting two hot furnaces, where the hydrocarbon is decomposed (240) (figure 1.17). CNTs are deposited on a substrate which acts also as metallic catalyst. The substrate is usually composed of Ni, Co or Fe nanoparticles (240, 247).

Numerous CVD variations have been developed in the last years with the objective of increasing the yield. The most relevant methods are plasma-enhanced CVD, laser-assisted thermal CVD and high-pressure catalytic decomposition of carbon monoxide (248).

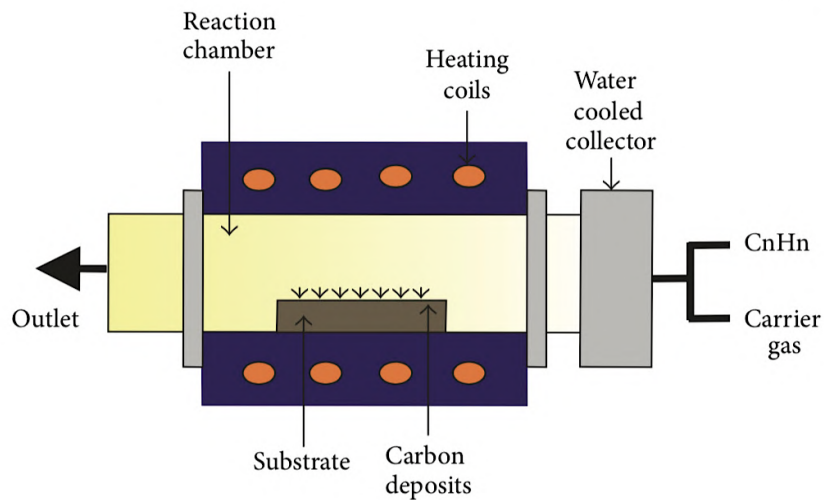


Figure 1.17 | Chemical vapour deposition method. Schematic illustration of the CVD technique performed to synthesise CNTs. Image adapted from (242).

CVD is a high-yield low-cost process; it is performed with lower temperatures than other methods and allows to control the nanomaterial morphology. However, it also produces CNTs with considerable structural defects (249).

1.8.2 Purification of carbon nanotubes

CNTs synthesis is constantly accompanied with impurities that if ignored can affect their properties and applications (figure 1.18). Thus, the importance of removing any associated soot, residual catalysts and support material (table 4).

Table 4 | Frequent contamination associated with carbon nanotubes synthesis. Table obtained from (250).

Composition of raw nanotubes		Residual material		
Nanotubes, with varying lengths		Metals	Organic impurities	Support materials
SWCNTs	MWCNTs			
~1–3 nm diameter They often form bundles	~10–100 nm diameter	Co, Fe, Ni, Mo nanoparticulate carbon encapsulated oxides	Amorphous carbon, microstructured carbon (carbon black, onions, fibers etc.)	Fine alumina, magnesium oxide, silica, or zeolite

The levels of impurity have been inversely correlated with CNTs diameter reduction. Therefore, several purification techniques has been employed; nonetheless it is important to consider the possible damage to the CNTs structure (237, 250).

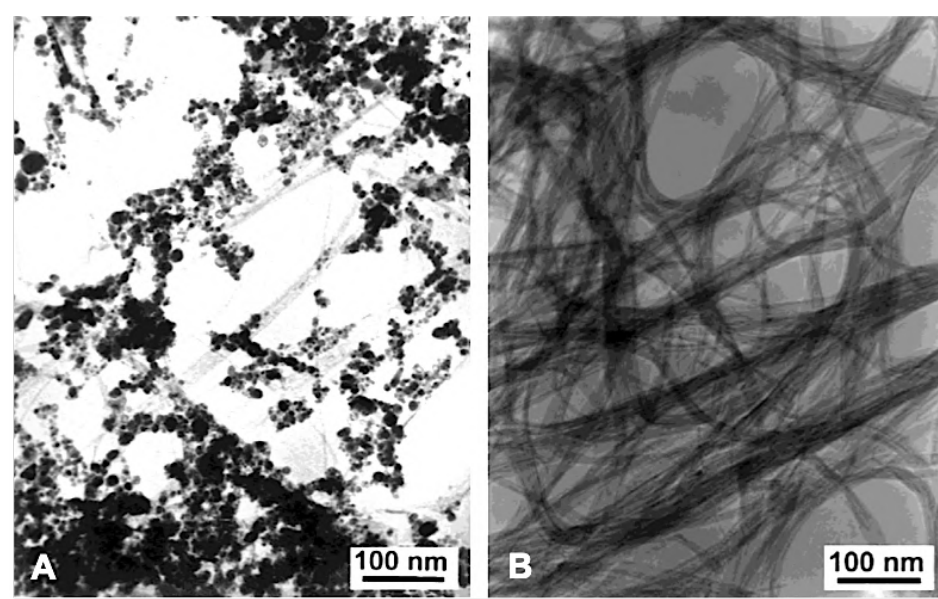


Figure 1.18 | Carbon nanotubes impurities. TEM image of SWNTs (A) before and (B) after purification. Image obtained from (251).

The commonly used purification methods can be divided in three categories: chemical oxidation, physical separation and a combination of the two (252). Further information of these methods is explained in table 5.

Table 5 | Summary and characteristics of common purification processes of carbon nanotubes. Table adapted from (237).

Process	Description
Gas phase oxidation	(i) In general, chemical oxidation includes gas phase oxidation (using air, O₂, Cl₂, H₂O, etc.), liquid phase oxidation (acid treatment and refluxing, etc.), and electrochemical oxidation. (ii) Tubes are heated at a controlled rate either in wet air or vacuum or other oxidizing agents for an extended time at about 330°C. (iii) This is a good way to remove carbonaceous impurities. (iv) The disadvantages of this method are that it often opens the end of CNTs, cuts CNTs, damages surface structure and introduces oxygenated functional groups (-OH, -C=O, and -COOH) on CNTs. Metal particles cannot be directly removed, and further acid treatment is needed.
Liquid phase oxidation (acid treatment)	(i) Usually, the acid treatment will remove the metal catalyst and some fullerenes. (ii) Reflux in HNO₃, HCl, or other acid for period of time ranging from 4 to 48 hours. (iii) HNO₃ is the only acid that does not cause degradation to tubes (unless left in HNO₃ for extended time frames, usually >16 hrs). (iv) The metal catalyst is solvated while the CNTs remain in suspended form.
Electrochemical oxidation	(i) CNTs with fewer defects show higher electrochemical oxidation resistance than CNTs with more defects. Suitable for purifying CNT arrays without destroying their alignment. (ii) The CNTs electrode is immersed in 0.2 M HNO₃ solution or 0.2 M HCl (purging with N₂ for 20 min prior to use) and the potential was cycled between +1.00 and +2.00 V at a scan rate of 50 mV s⁻¹. (iii) Increases the specific area of nanotubes by cutting off the nanotube tips and by converting the surface property of nanotubes from the hydrophobic state to the hydrophilic state.
Centrifugation	(i) Centrifuge at 7,000 g or more for 30 min to 3 hours. Often supernatant is removed after one run and centrifuged again. (ii) This removes nanospheres, metal nanoparticles, other carbon particles, but some methods produce a low yield of nanotubes, especially when centrifugation is repeated many times.
Filtration	(i) This technique is often used in conjunction with oxidation. The acid decomposition products are highly soluble in basic solution and CNTs are not and are separated using a basic solution of pH 11 and filtered using 3–5 µm filter, often under vacuum. (ii) This removes nanospheres, metal nanoparticles, polyatomic carbons, and fullerenes, without any noted negative effects.
Ultrasonication	(i) CNTs are suspended in distilled water, toluene, or acid solution and sonicated for 5–30 minutes. (ii) This separates tubes from attached particles, creating dispersion of nanotubes and other particles for better centrifugation. (iii) The separation of the particles is highly dependable on the surfactant, solvent, and reagent used. The solvent influences the stability of the dispersed tubes in the system.
Magnetic	(i) CNTs is suspended in soap or toluene and nanoparticle powder (ZrO₂, NHCl₄, CaCO₃, diamond) is added. This particles will attach to the CNTs and make them magnetic. (ii) The slurry is sonicated for 2 hr and subsequently the magnetic particles are trapped using permanent magnetic poles. (iii) This is then followed by chemical treatment. (iv) This removes catalyst materials and small inorganic particles. Few to no known negative effects.
Microwave purification	(i) CNTs are sonicated, then diluted in HNO₃ (or other acid). (ii) Microwaved at 100–200 W, and microwave is ramped up to ~200°C over 30 min. (iii) Microwave is then held at temperature 200°C for 30–90 minutes. This removes amorphous carbon, metals, and other nanoparticles, with no known negative effects.

1.8.3 Carbon nanotubes functionalisation

CNTs are considerably stable and poorly soluble. Thus, CNTs are usually functionalised or derivatised. The process involves the addition of molecules to the nanostructure surface, mainly with the objective of increasing their dispersibility and reactivity, modulating their properties and coupling active compounds (253). Equally important, CNTs agglomeration has been associated with higher toxicity (254). Modifications of the nanomaterial are divided in four main categories: covalent functionalisation, non-covalent functionalisation, endohedral filling and external decorations (255) (figure 1.19). The next paragraphs contain descriptions of these approaches.

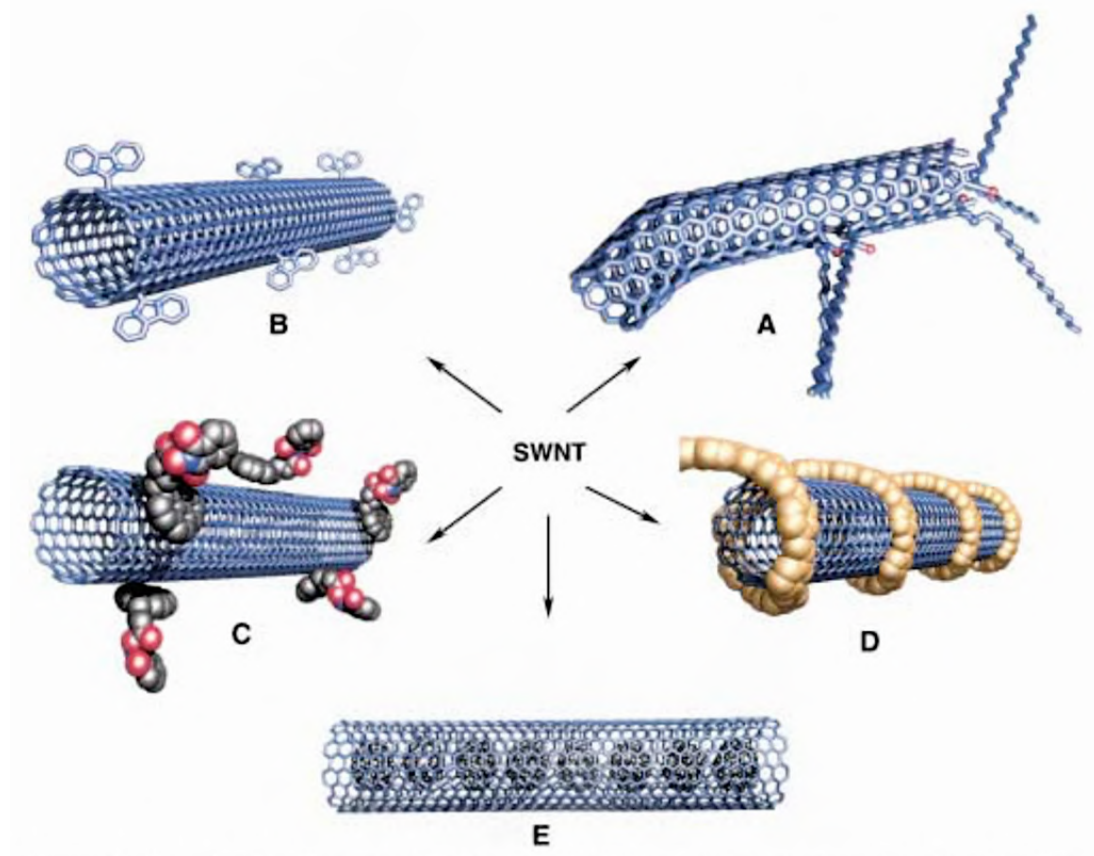


Figure 1.19 | Carbon nanotubes functionalisation. Schematic representation of possible types of CNTs covalent functionalisation: (A) defect-group and (B) sidewall functionalisation; and non-covalent functionalisation using (C) surfactants and (D) polymers. (E) endohedral functionalisation. Image obtained from (255).

- Covalent functionalisation.** It refers to the chemical binding of moieties to the ends and sidewalls of the nanomaterial. Figure 1.20 depicts examples of covalent functionalisations, being the most used methods oxidation, cycloaddition reaction and sulfoxides reaction, resulting in carboxylated CNTs (COOH-CNTs), aminated CNTs (NH_3^+ -CNTs) and sulfhydryl functionalised CNTs respectively.

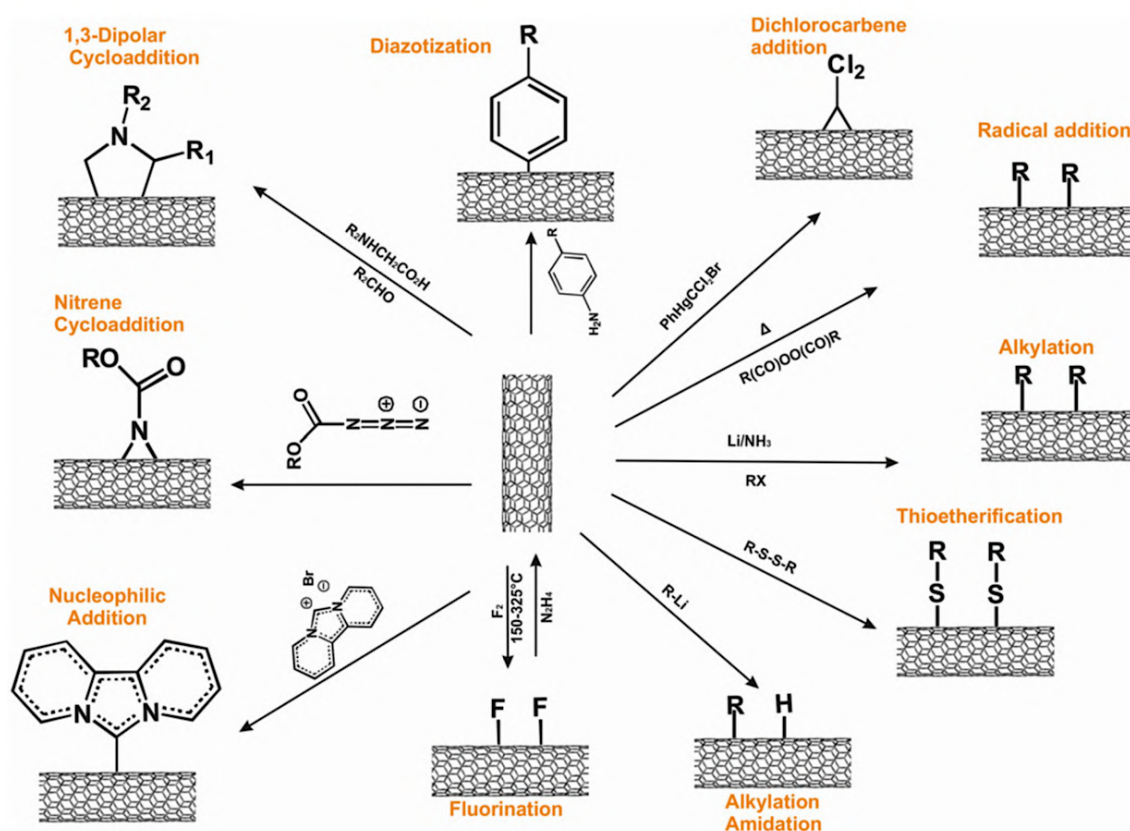


Figure 1.20 | Covalent functionalisation of carbon nanotubes. Schematic diagram of frequent chemical sidewall functionalisations. Image obtained from (256).

Covalent derivatisation is the most stable modification and offers the opportunity for further modifications; however, it disrupts the aromaticity of the structure, diminishing some of the nanomaterial properties. (257, 258).

- **Non-covalent functionalisation.** It comprises the wrapping and adsorption of molecules onto the nanostructure skeleton (figure 1.21). Non-covalently techniques improve the dispersibility of CNTs and allow their application in aqueous mediums while avoiding disruptions to the carbon configuration (259). The nanomaterial can be derivatised adsorbing aromatic molecules through hydrophobic interactions, particularly π - π stacking, electrostatic interactions and CH- π interactions (259, 260). Three main types of moieties are commonly used for non-covalently functionalisation: polymers, biomolecules and surfactants (246).

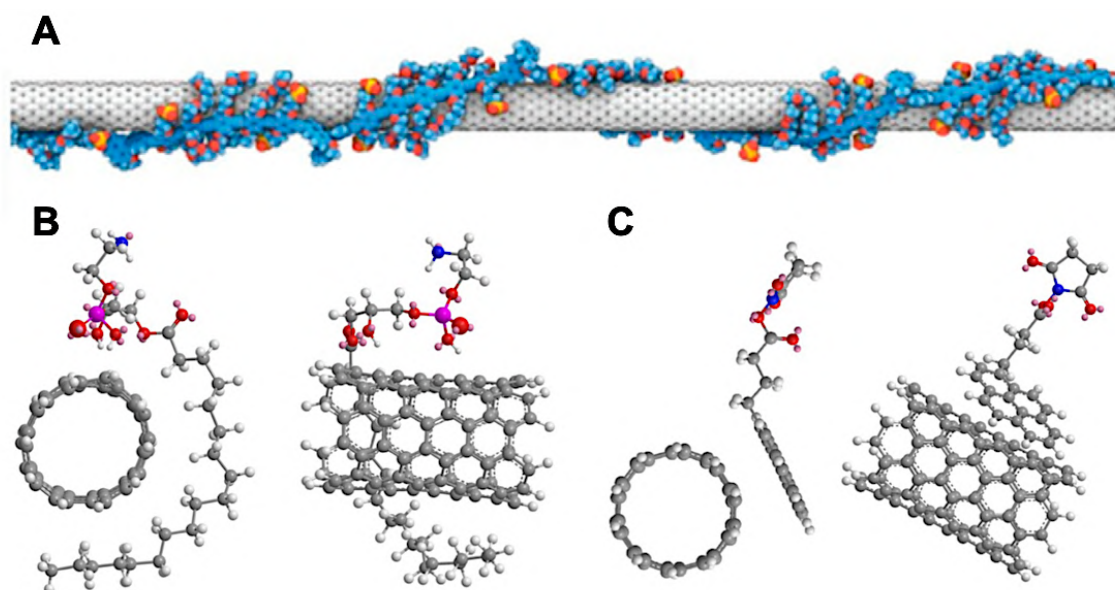


Figure 1.21 | Non-covalent functionalisation of carbon nanotubes. Schematic illustration of common non-covalent functionalisations for CNTs. (A) polymer functionalisation, (B) hydrophobic interactions and (C) π -interactions. Image adapted from (261, 262).

1.8.4 Carbon nanotubes characterisation

A collection of physical and chemical analysis is used to evaluate intrinsic features of CNTs. Characterisation can be generally classified into three classes: imaging, spectroscopy and thermal techniques. The most relevant analysis methods include transmission electron microscope (TEM), scanning electron microscope (SEM), Raman spectroscopy, thermogravimetric analysis (TGA), Fourier-transform infrared spectroscopy (FTIR) and zeta potential.

- **Transmission electron microscope and scanning electron microscope.** These optical techniques are conventionally used to obtain an insight of the nanomaterial morphology such as size and shape (figure 1.22).

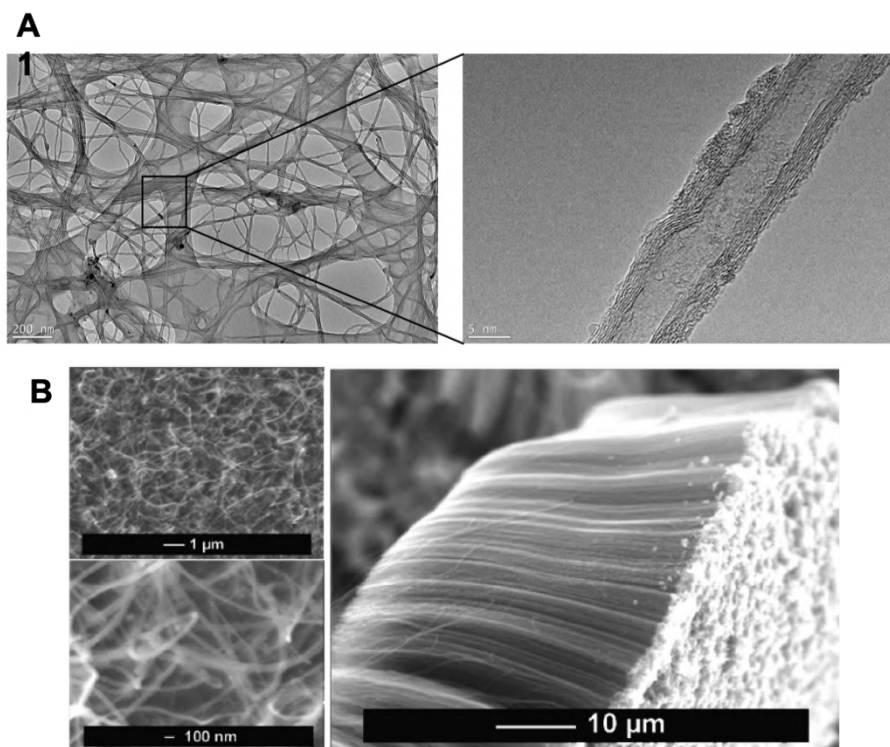


Figure 1.22 | High resolution optical techniques to analyse CNTs. (A1) and (A2) are TEM sequential magnifications, (B) SEM images of MWNTs. Image adapted from (263, 264).

While TEM offers the unique opportunity to detect associated carbonaceous materials and cellular uptake, SEM is the only method that provides information about metallic impurities (265-267).

- **Raman spectroscopy.** Raman spectroscopy is considered a fundamental highly sensitive technique to determine defects, functional groups, number of walls and conductivity of CNTs (268). Raman spectra result in peaks or bands related to the electronic and structural configuration of CNTs; defining peaks are the D-band, G-band and G'-band. The D-band is a peak around $1,350\text{cm}^{-1}$ and corresponds to the vibrational hybridisation of carbon atoms (269). It is frequently associated with sp^2 network disorder and defects in the nanotubes structure. The G-band is a peak around $1,580\text{cm}^{-1}$; it is distinctive feature of graphite; thus, the band is a good indicator of graphitisation level (270). It originates from the tangential vibrations of the structural atoms and stretching of C-C bonds. Finally, the G'-band is a peak observed at $2,600\text{cm}^{-1}$, which is a second-order, two-photon process characteristic signature of all graphitic structures (269). Additionally, a series of low-frequency bands can be found at $<200\text{cm}^{-1}$ defined as radial breathing mode. These peaks are exclusive to SWNTs and are related to the contraction and expansion of the nanostructures (269, 271) (figure 1.23).

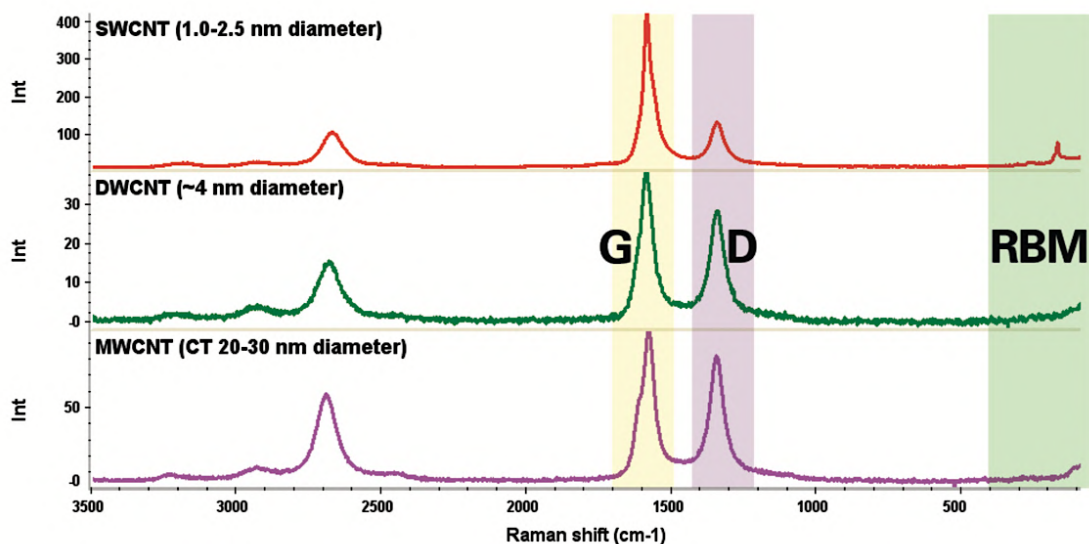


Figure 1.23 | Raman spectroscopy analysis of carbon nanotubes. Representative diagram of Raman spectra of SWNTs, double-walled carbon nanotubes (DWNTs) and MWNTs. Image obtained from (272).

- Thermogravimetric analysis.** TGA is a technique used to determine the purity, homogeneity and thermal stability of CNTs based on the weight loss of a sample as the temperature increases (269, 273) (figure 1.24). The analysis can be sectioned in three parts: initiation temperature, oxidation temperature and residual mass. At the start of the study, the temperature is set to increase gradually; initiation temperature refers to the point where a material commences to decompose (269). This is followed by the oxidation temperature or thermal stability which defines the temperature necessary to produce the maximum weight loss. Finally, the residual mass is the material that remains after the process has finished. In the case of CNTs, the residual mass usually contains metal impurities from the synthesis (269). It is known that carbon impurities such as amorphous and graphitic carbon result in lower resistance to oxidation (269, 273). Moreover, defects in CNTs have been associated with a reduction in the

oxidative stability (274). Therefore, the weight loss curve is an important parameter to determine the quality of the tubes.

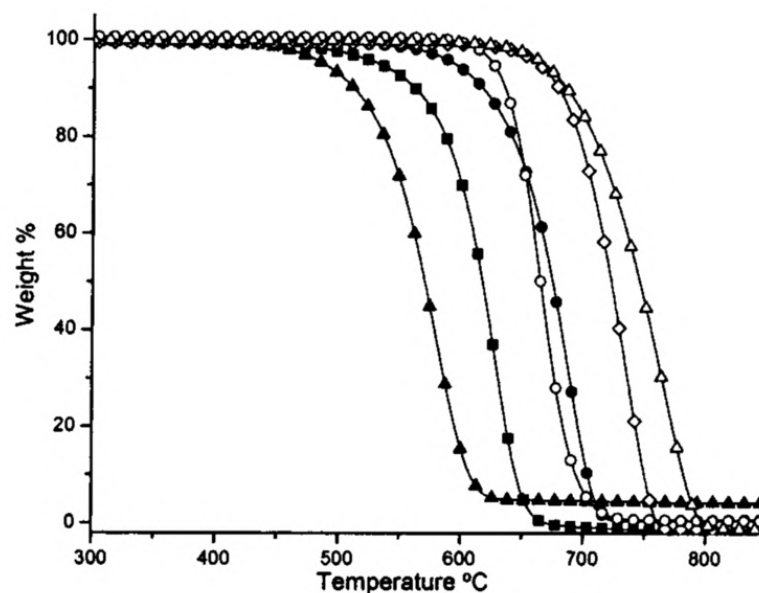


Figure 1.24 | Thermogravimetric analysis of carbon nanotubes. TGA spectra comparing the weight loss of ▲ pristine MWNTs, ■ diamond, ○ annealed diamond, ● graphite, ◇ annealed MWNTs and △ annealed graphite. Image obtained from (273).

- Fourier-transform infrared spectroscopy.** FTIR is a simple, low cost analytical technique to evaluate the surface chemistry of materials and detect alterations (275) (figure 1.25). Resulting spectra represents the frequency of the interactions between infrared radiation and atom bonds. Thus, FTIR is especially useful to identify the presence of functional groups on CNTs after derivatisation (275, 276). For example, the hydroxylic group show characteristic dominant peaks at $1,600\text{cm}^{-1}$ and $3,450\text{cm}^{-1}$; carboxylic groups depict definitive bands at $1,584\text{cm}^{-1}$ and a group of small peaks at $3,000\text{cm}^{-1}$, whereas a small peak at $1,445\text{cm}^{-1}$ is unique to MWNTs (269).

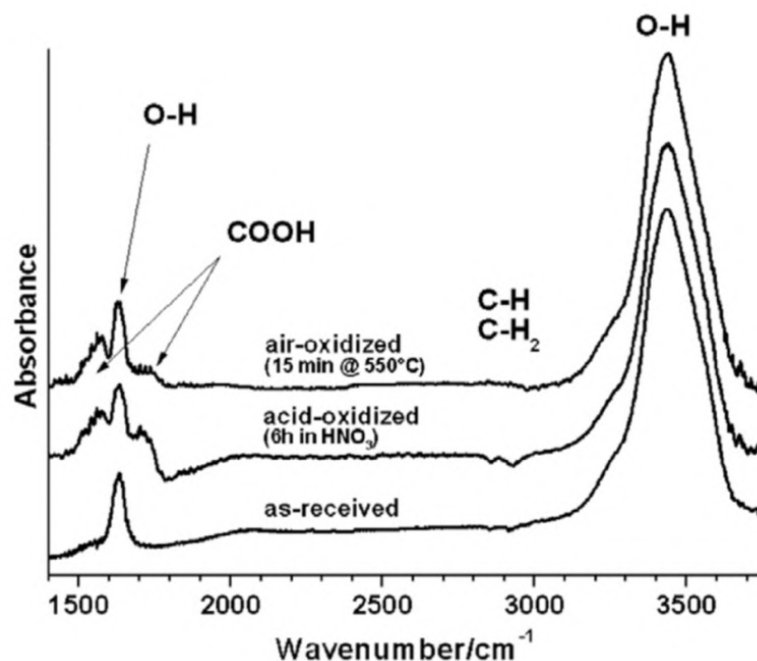


Figure 1.25 | Fourier-transform infrared analysis of carbon nanotubes. FTIR spectra comparing three types of MWNTs: as-synthesised pristine MWNTs, acid-oxidised and air-oxidised MWNTs. Spectra illustrates characteristic peaks for the presence of -COOH and -OH functional groups. Image obtained from (269).

- Zeta potential.** Superficial charge is a measurement of the magnitude of surface electrostatic interactions between particles in colloidal dispersions (277). Several studies have mentioned the relevance of this technique on the analysis of surface acidity of functionalised CNTs and as an indicator of their stability in water and colloidal dispersions (278). It is important to note that factors such as pH and electrolytes can affect the zeta potential of NPs besides the functionalisation (277, 278).

1.8.5 Carbon nanotubes applications

CNTs are considered a definitive nanomaterial in the nanotechnology research, expected to revolutionise science and technology. As mentioned previously, CNTs' remarkable properties have practical applications in a wide variety of science and technology fields (figure 1.26). Some applications are described in the following paragraphs.

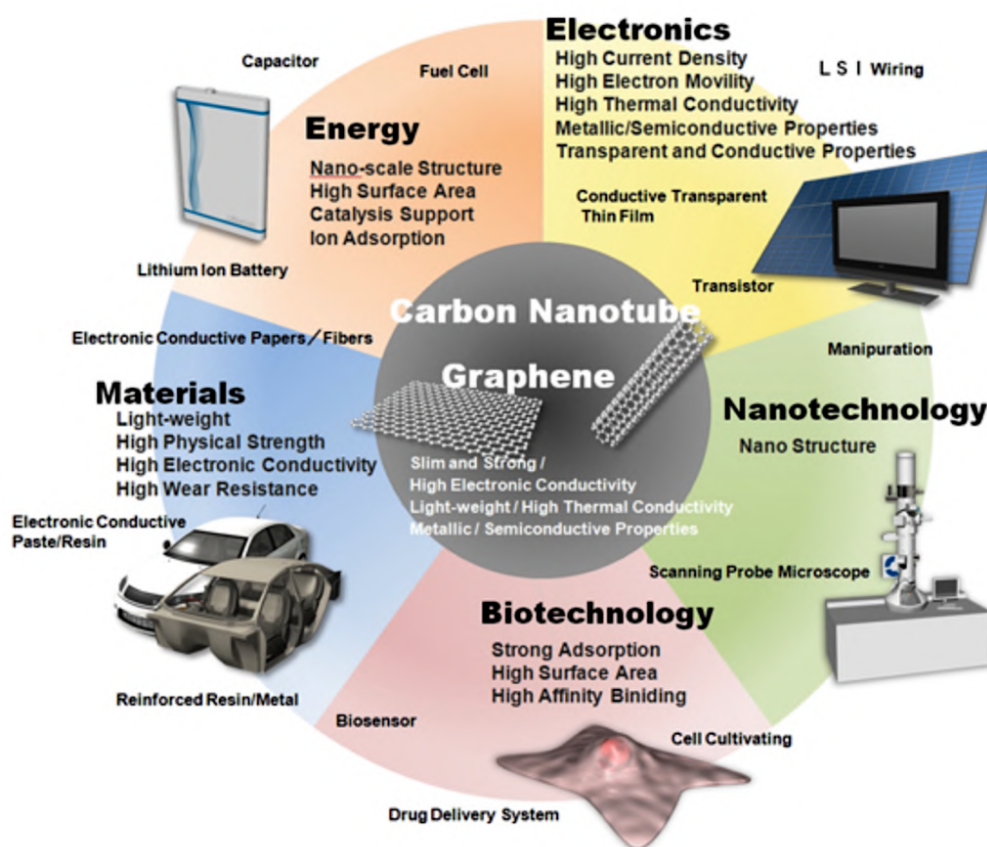


Figure 1.26 | Applications of carbon-based nanomaterials. Schematic illustration of graphene and CNTs current applications on different scientific and technology fields. Image obtained from (279).

- **Sensors and probes.** CNTs are used to detect the attachment of molecules onto their surface and external stimulus by measuring variations

in the vibration frequency and resistance or other physical properties (280, 281). Considered the most successful industrial application, CNTs have been used for the production of computer transistors (282). Moreover, due to their metallic and semiconductor properties, CNTs are being studied to design resistive sensors to measure temperature (283), pressure (284), gas (285), and pH (286, 287). Similarly, CNTs are used in biosensing devices to detect analytes and produce biological responses (288, 289).

- **Energy storage.** CNTs and derived composites have arisen as potential base to develop power cells, photovoltaic devices, and supercapacitors due to their ability to adsorb ions, donate and accept electrons as well as electrochemical and electromechanical characteristics (290, 291).
- **Coatings and films.** CNTs are under evaluation to be added into paints to increase the coating strength against corrosion while reducing the environmental impact (292). In addition, the nanomaterial has been incorporated in the production of transparent conducting films, film heaters and emission displays (293).
- **Environment.** Taking advantage of their high aspect ratio, well defined porous cylindrical structure, and adsorption properties, CNTs offer environmental remediation to different pollution problems (294). Researchers have documented the use of CNTs in air pollution control (295, 296), water purification (297, 298), heavy metal ions and

radionuclide adsorption (299-301), in addition to organic pollutants and antibiotic removal (302-304).

- **Biomedicine.** In recent years, researchers have explored a variety of CNTs-based diagnostic, monitoring, and therapeutic tools for the medical field (305, 306). In the biosensing area, CNTs have been found useful in probes to detect biological analytes such as glucose and insulin (307-309), cancer biomarkers (310, 311), neurotransmitters (312), and immunosensors (313, 314) among others.

Additionally, CNTs have been examined in other biomedical applications such as imaging (315, 316), solubility enhancement (317), surgical aids (318), tissue engineering and regeneration (319, 320), vaccines (321, 322), gene carriers (323), hyperthermia therapy (324) and anti-bacterial (325, 326). More importantly, CNTs have also gained particular attraction as drug delivery systems (DDS). The nanomaterial can entrap therapeutically active molecules through endohedral functionalisation or conjugate them via surface functionalisation. Hydrophobic compounds and aromatic-structure molecules can be adsorbed onto the nanomaterial surface through Van der Waals and π - π stacking (327-329). CNTs are widely investigated for targeted drug delivery (330-332) and controlled release (333, 334), aiming multiple locations like ophthalmic (335), lymphatic (336), transdermal (337, 338), central nervous system and brain (339-341), showing capability to cross the

blood brain barrier (BBB). Above all, one of the most promising uses of CNTs is their ability to attach, target, and deliver chemotherapeutics.

1.9 Carbon nanotubes in cancer

The main objective of CNTs as DDSs against cancer is to increase the antitumour drug accumulation in malignant cells while diminishing the intrinsic systemic toxicity. An important advantage of CNTs as nanocarriers is their rich chemistry and dimensions, giving the opportunity to produce stable conjugates with high amounts of active moieties and even combine therapies for simultaneous drug delivery. Additionally, the CNTs-drug complexation modifies the cell uptake of the single drug, which could also be protected from degradation and efflux mechanisms (342, 343). Several studies have demonstrated that functionalised CNTs are biocompatible and non-immunogenic nor carcinogenic (344, 345).

Moreover, in efforts to increase the tumour accumulation and specificity, CNT-based DDSs have been covalently and non-covalently conjugated with targeting compounds such as antibodies or magnetic NPs; the latter bring the possibility to direct the nanoconstructs with external magnetic fields (346).

The significance of CNTs in cancer is under intense exploration, aiming to determine the therapeutic efficacy, cytotoxicity, cell interactions, and mechanism of action. A summary of relevant studies is presented in table 6.

Table 6 | Summary of CNT-based delivery systems for cancer therapy. Description of functionalised CNTs conjugated with anticancer molecules and evaluated on different cancer models.

Cancer type	CNT system		Bioactive component	Cancer model	Study results	Ref.
	Type	Functionalisation				
Breast cancer	MWNTs	Pluronic F127 (F127)	DOX	MCF7	F127-MWNTs-DOX showed significantly improved cytotoxicity compared to free DOX. F127 depicted no cytotoxic effect	(347)
Breast cancer	SWNTs	Oxidation	Paclitaxel (PTX)	MCF7 SK-BR-3 BALB/c nude mice	Oxidised SWNTs enhanced the PTX cytotoxic effect in hypoxic cultures, reducing 10%-25% the cell viability compared to free PTX. Moreover, the nanosystem decreased the tumour weight 20%-25% more than the free drug	(348)

Breast cancer	MWNTs	Riboflavin (Rf) Thiamine (Tm)	PTX	MCF7	PTX-Rf-MWNTs and PTX-Tm-MWNTs decreased approximately 80% and 50% the IC ₅₀ respectively than free PTX in 48h-studies	(349)
Breast cancer	MWNTs	Oxidation	Hybrid Pt-acridine	MDA-MB-231 MDA-MB-468 MDA-MB-436 BT20	Nanoconstruct demonstrated non-apoptotic, cell death potentiation, arresting cells in S-phase.	(350)
Lung cancer	SWNTs	Amination Polyethylene glycol (PEG)	Gemcitabine (GEM)	A549 MIA PaCa-2 B6 athymic nude mice	SWNTs-GEM and SWNTs-PEG-GEM tested at 24h, 48h, and 96h showed higher cytotoxicity than free GEM. In vivo studies depicted a marked tumour size reduction of the groups treated with the conjugates compared to free GEM	(351)

Lung cancer	MWNTs	PEG	Anti-Bcl-2 compound ABT737	A549	PEG-MWNTs-ABT737 showed (352) increased anticancer efficacy compared to free ABT737. The nanosystem led to intrinsic apoptosis via increased drug accumulation, disrupting the mitochondria and downregulating Bcl-2
Lung cancer	MWNTs	Polyethylenimine (PEI) Polyallylamine (PAA)	Plasmid DNA pCMV-βGal	A549	PEI-g-MWNTs and PEI/PAA-g- (353) MWNTs increased the gene expression of β-galactosidase compared to the free plasmid. Plasmid internalisation was proved using trafficking experiments
Ovarian/lung cancer	MWNTs	Hyperbranched poly citric acid (PCA)	PTX	SKOV3 A549	MWNTs-g-PCA-PTX resulted in (354) 13.3% enhanced pH-responsive-

cytotoxicity compared to PTX alone.

An increased cellular accumulation was suggested

Cervical cancer	MWNTs	Hyaluronic acid (HA)/fluorescein isothiocyanate (FITC)/PEI	DOX	HeLa L929	pH-responsive MWNTs/PEI-FITC-HA/DOX showed biocompatibility and target CD44 receptors. The construct resulted in 7-fold higher uptake and 50% increased cytotoxicity in HeLa cells compared to L929 cells	(355)
Brain cancer	MWNTs	Trans-activating transcriptional activator (TAT)/PEI/biotin (BIO) copolymers	Oxaliplatin (OXA)	HBMEC/C6 co-culture	TAT-PEI-BIO-MENTs-OXA showed higher cell recognition and improved OXA penetration in BBB. Calculations demonstrated almost 3-fold lower IC ₅₀ value than free OXA.	(356)

Colon cancer	MWNTs	HA/PEG	GEM	HT-29 Sprague Dawley rats	GEM/HA-PEG-MWNTs	acid- (357) responsive complex depicted lower haemolytic toxicity and higher cytotoxicity than free GEM. In vivo studies showed increased efficacy of the nanoconstruct, reducing significantly the tumour size, while improving the survival rate in comparison to the unbound drug

1.10 Carbon nanotubes in biological systems

Cancer growth normally follows an inconsistent tumour microenvironment, characterised with erratic angiogenesis, faulty vasculature, and fenestrations. These issues result in increased extravasation, slow venous flow return and decreased lymphatic drainage (358). In this scenario, CNTs and other nanocarriers exhibit a passive targeting action and preferential accumulation in cancer tissues defined as enhanced permeability and retention (EPR) effect (358-360) (figure 1.27).

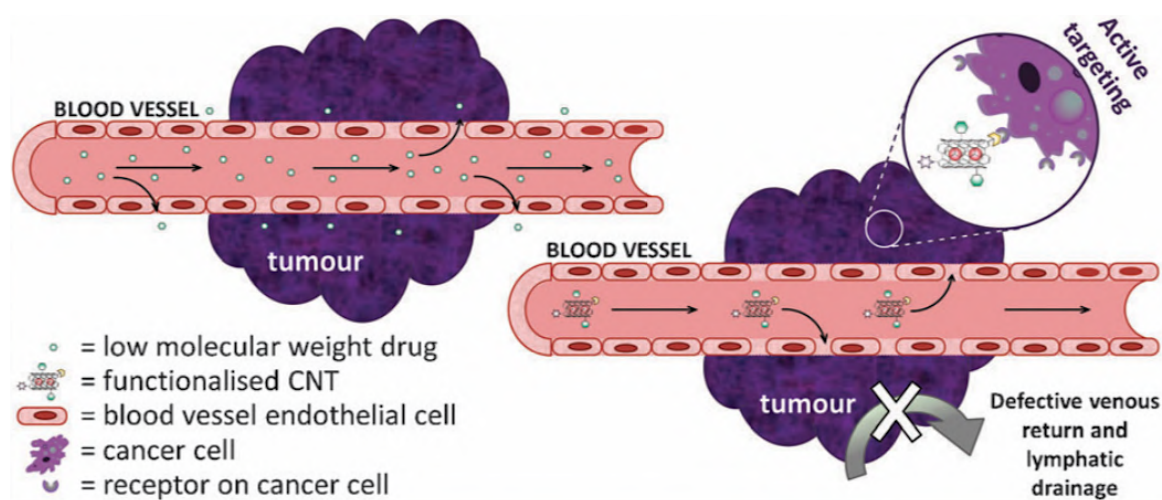


Figure 1.27 | Enhanced permeability and retention effect. Schematic illustration of CNTs targeting actively cancer cells while accumulating passively in tumours due to the EPR effect. Image obtained from (358).

Different studies have documented the CNTs internalisation into cells through endocytosis and nanoneedle penetration (359). In this context, three main uptake mechanisms have been studied: membrane wrapping of individual CNTs, internalisation of nanomaterial bundles within vascular compartmentalisation, and membrane translocation of single CNT (361, 362). Furthermore, studies

showed that the CNTs uptake process is fast, resulting in observable cellular trafficking in one hour (361).

Initially described in 2004 by Pantarotto et al., the nanoneedle mechanism consists in the ability of CNTs to cross biological membranes by piercing the cell in a passive diffusion manner (363) (figure 1.28). Such process avoids the vesicle formation and interaction hindering between CNTs and the inner of cells (364, 365).

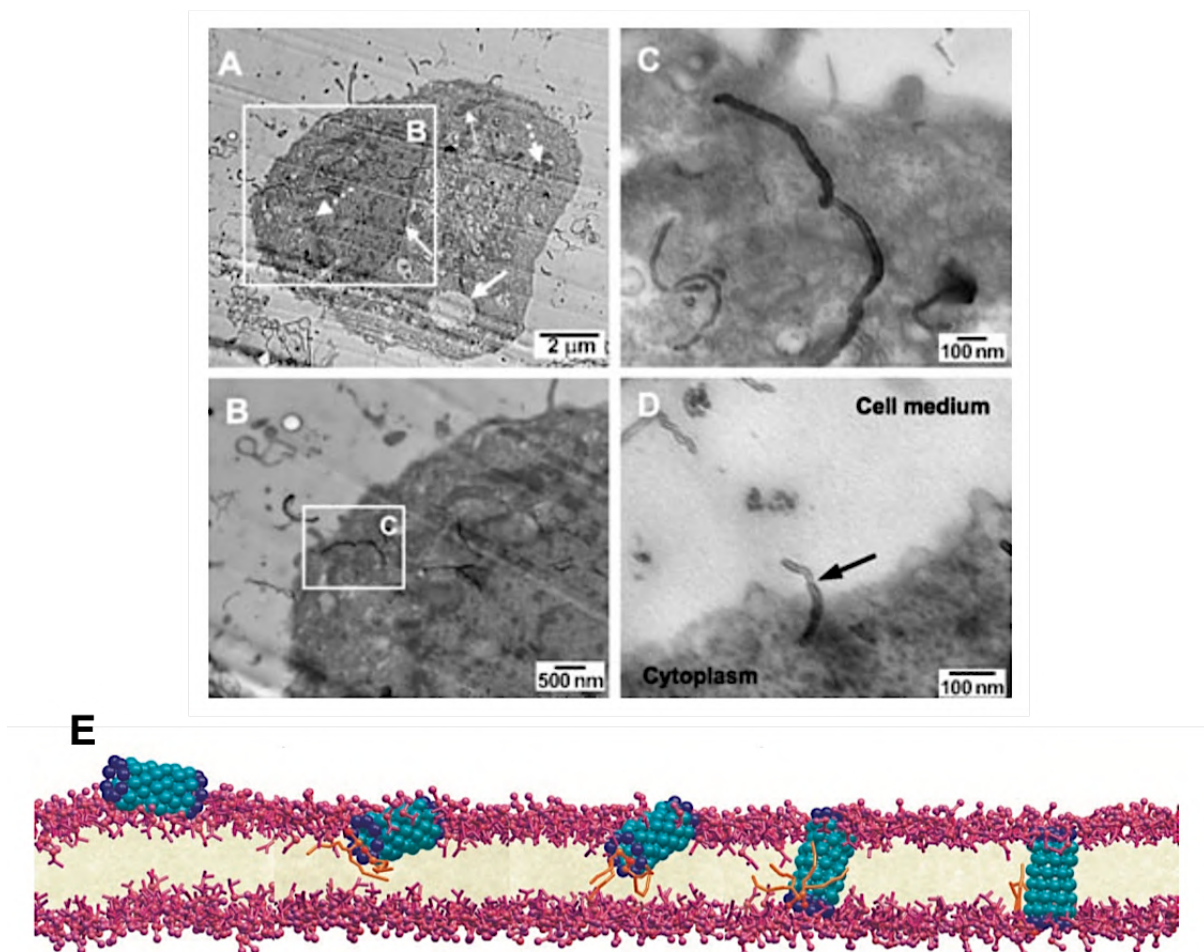


Figure 1.28 | Cellular internalisation of carbon nanotubes. TEM images of ultrathin sections of HeLa cells treated with functionalised MWNTs. (A) HeLa cell, (B) and (C) are subsequent magnifications; (D) MWNT piercing the cell membrane, (E) molecular dynamics simulations of the nanoneedle mechanism. Image adapted from (363, 366).

According to insertion molecular dynamics simulations, once in touch with cell membranes, CNTs are partially immersed parallelly to the membrane (366). Due to the hydrophilic nature of the nanomaterial, the tube ends interact with the hydrophobic core of the biomembrane, producing a spontaneous perpendicular reorientation (366). CNTs finally cross the lipid bilayer resembling a needle and acting as transmembrane pore for hydrophilic molecules like water, which enhances the tubes translocation (366, 367).

Besides, CNTs may enter the cell by energy-dependant active transport, which can be receptor or non-receptor mediated (242). This mechanism favours functionalised CNTs and involves the engulf of nanoparticles in vacuoles, usually leading to lysosomal degradation (242, 368). Endocytosis process encompass different pathways, including clathrin-mediated endocytosis, caveolae-mediated endocytosis, micropinocytosis and phagocytosis (368) (figure 1.29).

CNTs internalisation pathway is determined by the size and length, functionalisation type and surface moieties, and degree of dispersion (242). Likewise, CNTs physicochemical features and purity are essential determinants for their biocompatibility, biodistribution and toxicity (369, 370). The possibility of controlling these characteristics suggests the ability to modulate their biological outcome.

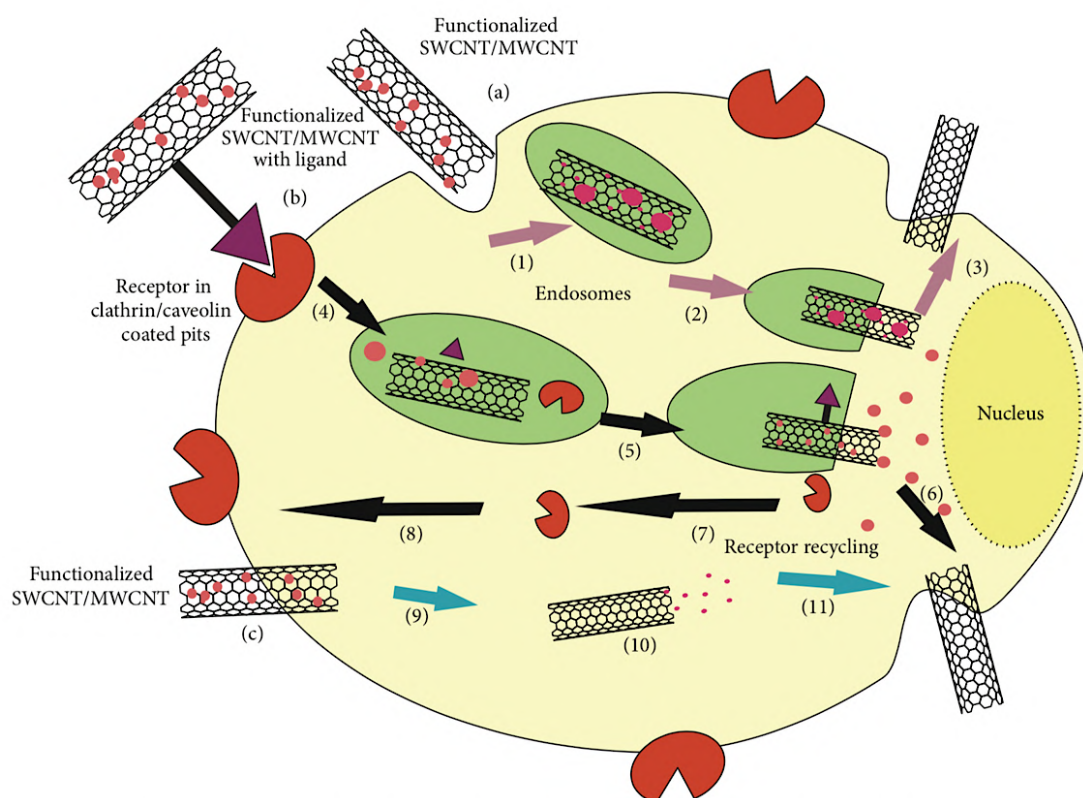


Figure 1.29 | Pathways of cellular uptake of carbon nanotubes. (a) endocytosis mediated pathway: (1) CNTs are compartmentalised in endosomes and (2) released into the cytoplasm, (3) followed by the delivery of the cargo. (b) receptor mediated pathway: (4) targeted CNTs are recognised by membrane receptors and internalised in endosomes; (5) the CNTs and cargo are released and (6-8) the receptor regenerated to active form. (c) nanoneedle mechanism: (9) CNTs passively penetrate into the cell, (10) releasing the drug directly in the cytoplasm. Image obtained from (242).

CNTs biodistribution and pharmacokinetic profiles are still under extensive evaluation. Systemically administered pristine CNTs in mice were found in the liver and spleen from 1 day after administration to 28 days after (371). Nonetheless, in vivo studies using NH_3^+ -CNTs demonstrated a liver retention time as low as 0.5h (371). Other scientific reviews described the use of radio-labelled SWNTs and MWNTs to trace their biodistribution. Results showed a rapid distribution and clearance, a half-life of 3.5h, 94% excretion of CNTs through urine and the rest through faeces with no retention in reticuloendothelial

organs; a preferred accumulation was observed in the kidney and bone with no tissue damage registered (229, 372). Researchers have concluded that, generally, non-covalently functionalised PEG-CNTs present longer half-lives, lower reticuloendothelial system uptake, and increased elimination time (373). Enzymatic systems like horseradish peroxidase system, human myeloperoxidase and peroxidases as well as radical intermediates like hypochlorite are responsible for the CNTs biodegradation (374, 375). These enzymes acid-attack the walls of CNTs, starting in defective sites and resulting in oxidised aromatic fragments. Due to their multi layered structure, MWNTs degradation takes longer than SWNTs (374, 375) (figure 1.30).

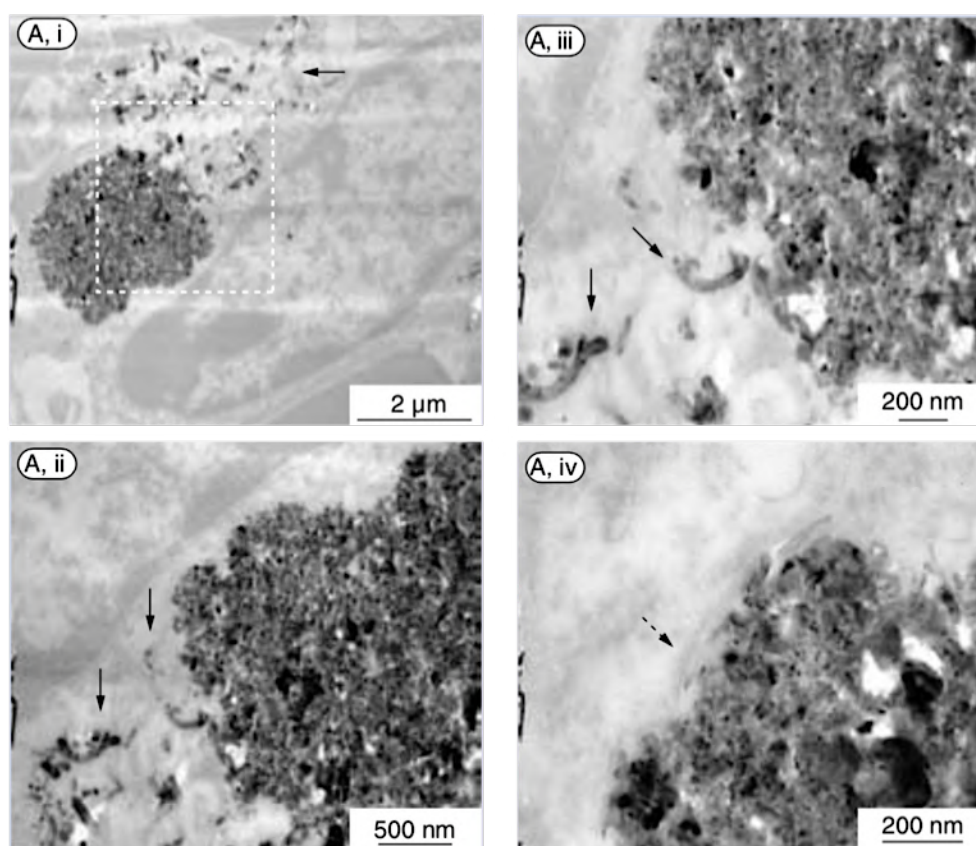


Figure 1.30 | Carbon nanotubes biodegradation. TEM images depicting (A,i) NH_3^+ -MWNTs clusters in microglia cells; (A,ii) and (A,iii) are subsequent magnifications from the dashed box. Solid arrows represent intact MWNTs, while the dashed arrow in (A,iv) shows MWNTs losing tubular structure, evidencing biodegradation. Image adapted from (376).

1.10.1 Carbon nanotubes toxicity

Toxicity of CNTs is still unclear and contradictory. CNTs and asbestos have been widely compared due to their fibre-like structures. In one hand, different researchers and organisations have warned about possible risks for human health. In vivo studies have associated the CNTs with mesothelioma, fibrosis and lung and liver damage (377, 378). Moreover, a 2014 WHO evaluation left open the possibility that inhalation of long, stiff MWNTs, also called MWNTs-7, could potentially be carcinogenic to humans (379). Suggested mechanisms related to CNTs toxicity include oxidative stress due to lipid peroxidation (380) and interactions with DNA and tubulin as result of destabilisation of the double helix and mitotic disruption respectively (381).

Nevertheless, there is inconclusive and narrow evidence to define all types of CNTs as genotoxic. In March 2020, a group of almost 40 internationally recognised researchers in the nanotechnology field published a manuscript to show discontent regarding a previous correspondence from the International Chemical Secretariat about the addition of CNTs to the “Substitute It Now” list. In this list, CNTs were recommended as a whole class “to be restricted or banned in the European Union”. However, long-experienced researchers explained that such actions would be unjustified and would have a negative impact on research and development as it is irresponsible to encompass the many different CNTs equally toxic under the current understanding (382).

It is important to note that there are well known factors that contribute to the CNTs' toxicity. The most important are metal catalyst contaminations from the CNTs synthesis, nanotubes geometry, functionalisation type, dispersion degree and aggregation (383, 384).

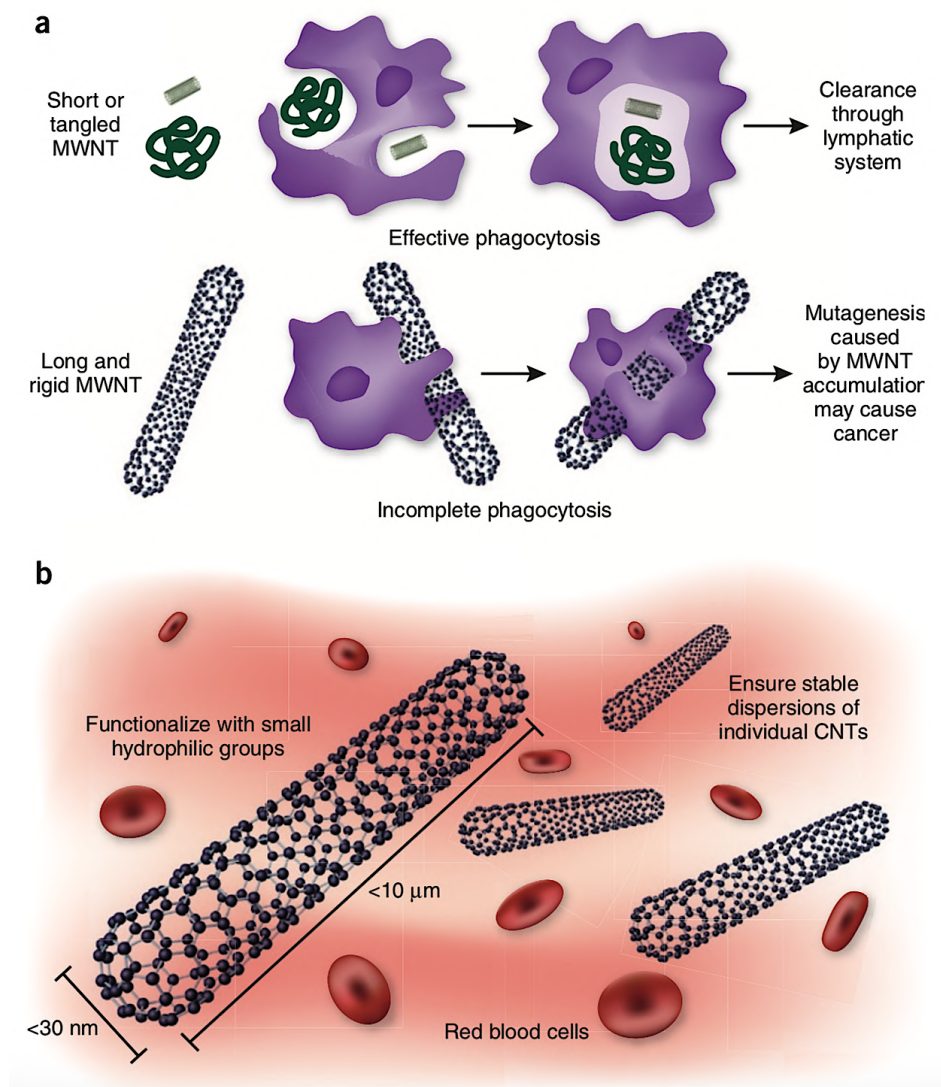


Figure 1.31 | Determinant factors associated with Carbon nanotubes safety. (a), the length of CNTs plays an important role in the phagocytosis, degradation and elimination, whereas (b) the surface functionalisation dictates the dispersibility and individualisation of the nanomaterial. Both are some of the most significant characteristics to determine the in vivo safety. Image obtained from (384).

A definitive feature of CNTs' associated toxicity is their geometry; studies have showed that lengths similar to the size of macrophages affect the phagocytosis (385) (figure 1.31). Equally important is the nanomaterial functionalisation; surface modifications will determine most of the interactions between CNTs and the biological milieu. To exemplify the importance of functionalisation, a study from Zhou and collaborators compared the IC_{50} of pristine, -OH and -COOH functionalised MWNTs. The values resulted in 255.2 $\mu\text{g/mL}$ of the non-functionalised nanomaterial, 2,198 $\mu\text{g/mL}$ of OH-MWNTs and 2,456 $\mu\text{g/mL}$ of COOH-MWNTs (386). CNTs' toxicity is also linked to the route of exposure, being the pulmonary exposure the one with highest risk (387).

All considered, CNTs are complex structures with exceptional characteristics, subject to different structural and chemical modifications that can be used to modulate their behaviour. Therefore, it is impossible to simply define all different CNTs as potentially harmful. Toxicity must be balanced over the biomedical benefits offered, just like any other therapy. Biomedical and toxicological research must continue alongside, generating further comprehension and delineating unbiased guidelines and regulations for the use of this promising nanomaterial.

1.11 Aims and objectives

In recent years, CNTs have attracted enormous attention as new tools to integrate and deliver a number of chemically-different active molecules. The

unique properties of the nanomaterial and ability to enter cells have opened promising possibilities of new antitumour therapies. Therefore, this thesis proposes the development of new MWNTs-based nanosystems for the transport and delivery of CDDP and DOX in an in vitro breast cancer model. In order to achieve this goal, this work was developed following established objectives:

- Develop MWNTs-based nanocomplexes and perform an exhaustive physicochemical characterisation.
- Determine the cytotoxic profile and biocompatibility of the DDSs.
- Assess the anticancer efficacy in relevant 2D and 3D breast cancer models.
- Investigate the mechanistic processes involved in the DDSs' anticancer activity.

CHAPTER 2

MATERIALS AND METHODS

Methods and techniques carried out routinely are detailed in this chapter along with the materials used to perform them.

2.1 Multi-walled carbon nanotubes

95+% pure pristine MWNTs with nominal dimensions of 20-30 nanometres (nm) diameter and 10-30 micrometres (μm) length were obtained from Nanostructures & Amorphous Material Inc. (Houston, TX, USA). The nanomaterial was used for all further functionalisations.

2.1.1 Pluronic F127 MWNTs coating

Pluronic F127-coated MWNTs were prepared by mixing 20 milligrams (mg) of pristine MWNTs into a 1% solution of Pluronic F127 (Sigma-Aldrich. St. Louis, MO, USA) in sterile distilled water (diH_2O) (w/v). To fully disperse the nanomaterial, the mixture was sonicated with an ultrasonic cleaner (VWR International. Malaysia) for 20 minutes (min) at room temperature (RT).

2.1.2 Carboxyl MWNTs functionalisation

Carboxylated MWNTs were prepared via covalent derivatisation. The reaction was performed by adding of 20mg of pristine MWNTs into 15 millilitres (mL) of a mixture of sulfuric acid (Sigma-Aldrich. Munich, Germany) and nitric acid (HNO₃) (VWR International. Edmonton, Canada) in a proportion of 3:1 (v/v) respectively. The resulting dispersion was sonicated for 12 hours (h). Then, the acid mixture was neutralised with sterile diH₂O and centrifuged at 9,000 revolutions per minute (rpm) using a Mega Star 600R centrifuge (VWR International. Darmstadt, Germany). Following, the supernatant was discarded, and the pellet obtained was redispersed in diH₂O. The washing process was repeated until reaching a neutral pH. Afterwards, the dispersion was dried in an oven at 50°C. Finally, dry COOH-MWNTs were weighed and dispersed in sterile diH₂O at desired concentration.

2.1.3 Amine MWNTs functionalisation

Aminated MWNTs were synthesised from carboxylated MWNTs in a reaction that consisted in mixing 100mg of COOH-MWNTs with 4mL of oxalyl chloride (Sigma-Aldrich. Dorset, UK) at 60°C under constant reflux in nitrogen atmosphere for 24h. The resulting dispersion was vacuum-evaporated and mixed with a solution containing 120mg of n-Boc-1,2-diaminoethane diluted in 5mL of dry tetrahydrofuran (Sigma-Aldrich. Dorset, UK). The reaction continued under the

conditions before explained for 48h. Once cooled down to RT, the product was washed 5 times with methanol and vacuum-dried. In order to cleave the Boc group, 3mg of the obtained compound were dispersed in 2mL of hydrochloric acid in dioxane solution (Sigma-Aldrich. Dorset, UK) for 5h at constant stirring. The final product was washed 5 times with 10mL of diethyl ether and vacuum-dried.

All MWNTs final dispersions were prepared at concentrations of 1 mg/mL and stored at 4°C. To ensure fully dispersion, functionalised MWNTs were sonicated for 5min before all experiments.

2.2 Multi-walled carbon nanotubes characterisation

2.2.1 Size distribution

Pristine and functionalised MWNTs dispersions at concentrations of 50 µg/mL (micrograms) in diH₂O were dropped onto 200 mesh Formvar coated copper grids (Agar Scientific. Essex, UK) and left to attach and dry overnight. Then, transmission electron microscopy (TEM) microphotographs were obtained using a LaB 6 filament JEOL operating an accelerating voltage of 80 kiloelectronvolts. To determine the length of MWNTs, images were processed and analysed using FIJI (ImageJ) software version 2.0.0 (National Institutes of Health. Bethesda, MD, USA).

2.2.2 Zeta potential

Superficial charge was analysed with a Zetasizer Nano-25 (Malvern Instruments Ltd. Malvern, UK). Samples of MWNTs were diluted to concentrations of 50 µg/mL in diH₂O. 1mL of MWNTs dispersion was added into a disposable capillary cell and the zeta potential was measured using a standard operating protocol (SOP) created for such study: absorption 0.5, 20°C, with water as dispersant. After instrument calibration, four independent experiments were performed, each one including 3 cycles of 50 readings.

2.2.3 Raman spectroscopy

Aqueous-dispersed pristine and functionalised MWNTs were dropped onto microscope glass slides (VWR International. Lutterworth, UK) and dried at 50°C overnight. The thin films of MWNTs obtained were analysed using an inVia Qontor confocal Raman microscope running the WiRE 3.1 software (Renishaw plc. Wotton-under-Edge, UK). Spectra were recorded using a green laser (532nm) and 50X magnification objective in a range from 0 to 3,000 cm⁻¹.

2.2.4 Thermogravimetric analysis and Fourier-transform infrared spectroscopy

Both FT-IR and TGA analysis were performed using PerkinElmer hyphenated technologies, coupling a Frontier spectrometer with a TGA 8000 (Waltham, MA,

USA). Herein, samples of 5mL of pristine and functionalised MWNTs at concentration of 1 mg/mL were placed in glass vials and dried overnight at 50°C. The next day, the nanomaterials were scraped off the vial and approximately 2mg were placed on a ceramic pan. Samples were weighed, zeroed, and temperature was set to rise 10°C per min. TGA measurements were recorded using the Pyris software in a range from 25 °C to 1,000 °C. Afterwards, the evolved gas from the samples was automatically transferred for time resolved FT-IR spectroscopy analysis. Spectra was obtained with the use of the Spectrum Timebase software.

2.3 Therapeutic efficacy assessment

2.3.1 Cell culture

MCF7 human breast ductal adenocarcinoma cells were obtained from Public Health England (Salisbury, UK) and cultured in complete medium (CM), comprised of minimum essential medium (MEM) (Gibco. Paisley, UK), supplemented with 10% heat inactivated fetal bovine serum (FBS) (v/v) (Gibco. Paisley, UK) and 1% penicillin/streptomycin (5,000 U/L) (v/v) (Gibco. Gaithersburg, USA). Cells were routinely maintained in 75cm² culture flasks at 37 °C and 5% CO₂ (v/v) in a humidified incubator (MCO-18AC-PE, Panasonic. Sakata, Japan), such conditions will be referred subsequently as “standard conditions”. Sub-cultured was performed when cells reached 80% confluence. The passage protocol consisted in removing and discarding the culture medium, followed by washing the cells monolayer with Dulbecco’s phosphate-buffered saline (PBS) (Gibco. Paisley, UK). The PBS was then discarded and 2mL of

trypsin-EDTA 0.05% (Gibco, Paisley, UK) were added; the culture was incubated for 2-4min at 37 °C. Once cells were round-shape, the flask was gently shaken to promote the cell detachment and suspension. Finally, the enzyme solution was neutralised by adding fresh medium. Cell suspension was diluted as necessary for experiments. In order to facilitate the description of methods in this chapter, MCF7 cells will be referred simply as “cells”. All culture methodologies were performed under sterile conditions, in biological safety cabinets (NO.NU-425-400E, Nuaire. Plymouth, UK).

2.3.2 Multicellular tumour spheroids

The generation of multicellular tumour spheroids (MCTS) was based on the liquid overlay technique. Thus, a suspension of 1% agar (w/v) (Sigma-Aldrich. Sintra, Portugal) in diH₂O was prepared and autoclaved. While it was liquid, 50µL per well were added to 96-well flat-bottom plates (Corning. UK). Once the agar cooled down and solidified, 8,000 cells in 100µL of CM were carefully plated on top of the agar per well and incubated under standard conditions. The MCTS formation was analysed daily using a transmitted light microscope (TML) (Primovert, Carl Zeiss. Suzhou, China). After 4 days, single MCTS were obtained in each well.

2.3.3 Cytotoxicity assessment

In order to determine the cytotoxicity of the nanomaterials, cells were seeded in 96-well plates at a density of 7,000 cells per well and incubated overnight under standard conditions. Then, the medium was removed and 100µL per well of functionalised MWNTs at concentrations ranging from 1 µg/mL to 300 µg/mL were added for 24 and 48h. Dimethyl sulfoxide (DMSO) 20% (v/v) (Sigma-Aldrich, Dorset, UK) was used as positive control with cytotoxic activity. The percentage of cell viability was evaluated using the modified lactate dehydrogenase (mLDH) assay.

Solutions of doxorubicin hydrochloride (Sigma-Aldrich, Dorset, UK) in diH₂O were prepared at concentrations of 1 mg/mL and stored at 4 °C. Similarly, CDDP (Cayman Chemical Company, Ann Arbor, MI, USA) was dissolved at equal concentration in PBS and stored at -20 °C. To ensure homogeneous solutions, preparations were vortexed and sonicated for 3min before use. Both drug stocks were sterilised using syringe filters with 0.22µm pore size (Millipore, Watford, UK). The efficacy of free DOX and CDDP was estimated in a similar procedure. The antineoplastic drugs were tested at concentrations in a range from 0.1µM to 1 millimolar (mM) for 24 and 48h. The mLDH assay was used to determine the percentage of cell inhibition.

2.3.4 Preparation of drug delivery systems

Previous assessments were refined to determine the concentrations to use in succeeding experiments. Thus, 40 and 50 $\mu\text{g/mL}$ of functionalised MWNTs and 12, 24 and 48 μM of anticancer drugs were established as optimal concentrations for the development of the nanocomplexes.

DDSs were prepared by mixing recently-sonicated functionalised MWNTs and DOX or CDDP at the above-mentioned concentrations in CM. Dispersions were then vortexed during 3min to homogenise the content. Nanocomplexes were always freshly prepared before all experiments.

2.3.5 Modified lactate dehydrogenase assay

After exposure time, media with treatments was replaced with 50 μL per well of a lysis solution containing 0.9% Triton X-100 (v/v) (Sigma-Aldrich. St. Louis, MO, USA) diluted in RPMI 1640, no phenol red medium (Gibco. Paisley, UK), and incubated for 1h at 37 °C. Then, the resulting lysates were collected in 1.5mL plastic tubes and centrifuged at 16,000 standard gravity (g), 20°C, during 5min. The nanomaterial was discarded by transferring 50 μL of the supernatant to new 96-well plates; following, 50 μL of Substrate Mix (Cyto Tox 96 Non-Radioactive Cytotoxicity Assay, Promega. Madison, WI, USA) were added. After an incubation period of 15min at RT, 50 μL of the Stop Solution provided in the kit were added to stop the colorimetric reaction. Finally, the absorbance of the

samples was recorded at 490nm using a Multi-mode Microplate Reader (FLUOstar Omega, BMG Labtech GmbH, Ortenberg, Germany).

The percentage of cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \left(\frac{\text{Average OD value}_{\text{Experimental group}}}{\text{Average OD value}_{\text{Untreated control group}}} \right) \times 100\%$$

2.3.6 Optical analysis of multicellular tumour spheroids

MCF7 MCTS were generated and microphotographs of each cellular structure were recorded using a TLM with 10X magnification. Analysis showed that, after 4 days of incubation, an average of 20µL of CM were evaporated. Thus, in order to avoid unnecessary disturbance to the MCTS, treatments were directly mixed with the CM in the plates for 24h and 48h. Desired treatment concentrations were calculated considering both volumes. DMSO was used as positive control. Concluded each time-point, MCTS were carefully collected, washed with PBS, and transferred to 24-well plates for imaging evaluation. Zen 2 (Blue Edition, Version 2, 2011. Carl Zeiss Microscopy GmbH, Jena, Germany) and FIJI software were used to process the obtained microphotographs.

The therapeutic efficacy of single treatments and nanocomplexes was determined based on the growth inhibition of MCTS. Hence, the circumference of MCTS was recorded at the experiment onset and compared to that measured

after 24h and 48h of treatment exposure. The growth ratio was calculated using the following equation:

$$\text{Growth ratio} = \frac{\text{Average circumference}_{\text{After treatment}}}{\text{Average circumference}_{\text{Before treatment}}}$$

All MCTS experiments were performed at least 3 independent times. Every individual assay considered at least 8 MCTS.

2.3.7 Annexin-V-FITC / Propidium iodide cell viability assay

MCTS were treated with functionalised MWNTs, single chemotherapeutics and nanocomplexes. DMSO 20% and staurosporine 1.5µM (Alfa Aesar. Heysham, UK) were used as positive controls to induce necrosis and apoptosis respectively. After 48h, the spheroids were collected in 15mL plastic tubes and washed twice with PBS. Then, 1mL of a mixture of StemPro Accutase (Gibco. Gaithersburg, USA) and trypsin at equal proportions was added. Samples were incubated for 10min at 37 °C, followed by gentle stirring to promote the MCTS disaggregation. The obtained cell suspension was centrifuged at 300 x g, washed with PBS and labelled using the Alexa Fluor 488 Annexin-V/Dead Cell Apoptosis Kit (Invitrogen. Oregon, USA). Following the supplier's protocol, a 100 µg/mL propidium iodide (PI) solution was prepared by diluting the provided stock in binding buffer. Afterwards, 1x10⁶ cells were resuspended in 100µL of binding buffer and mixed with 5µL of Annexin-V-FITC (fluorescein isothiocyanate) and 1µL of PI solution. After 15min of incubation at RT, additional 400µL of binding buffer were added,

followed by gently vortexing. Samples were then placed on ice and analysed within an hour using a BD LSRFortessa X-20 flow cytometer system running the software BD FACSDiva version 8.0.1 (Beckton, Dickinson and Company. New Jersey, NY, USA). The florescent excitation (ex) of FITC and PI were 488nm, while their emission (em) wavelengths were 538nm and 575nm respectively. Electronic compensation was set up based on unstained cells and cells labelled with the single stains.

At least three independent experiments were performed; each experiment considered at least 20 individual MCTS per sample. 10,000 events were analysed per sample. The obtained FCS files were analysed using the FlowJo (V.10.0.8, FlowJo, LLC. Ashland, OR, USA) software. Cells were classified based on their labelling as follows:

Viable cells: Annexin-V-FITC (-) / PI (-)

Early apoptosis: Annexin-V-FITC (+) / PI (-)

Late apoptosis/necrosis: Annexin-V-FITC (+) / PI (+) and Annexin-V-FITC (-) / PI (+)

2.3.8 Live/Dead viability assay

MCTS were generated and treated with functionalised MWNTs, free anticancer drugs and nanocomplexes. DMSO 20% was added as positive control for cell death induction. After 24h of incubation, MCTS were carefully collected, carefully washed with PBS, suspended in Live Cell Imaging Solution (Invitrogen. NY, USA), and transferred into 8-chamber culture slides (Falcon Fisher Scientific.

Loughborough, UK). Cytotoxicity and structural damage over MCTS were evaluated using the LIVE/DEAD Cell Imaging Kit (Invitrogen, Oregon, USA). The probe was prepared as follows: the two kit components were thawed and mixed as per the manufacturer protocol. The resulting solution was added to samples in equal proportion and incubated for 15min in the dark. Finally, MCTS were imaged using an inverted Zeiss LSM 780 multiphoton laser scanning confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) at (ex/em 488/515nm) and (ex/em 570/602nm). Maximum 3D projections were recorded merging the fluorescence obtained.

2.4 Mechanism of action of MWNTs + cisplatin

2.4.1 Cell transport

7,000 cells were seeded and left to attach overnight under standard conditions. Cultures were then exposed to treatments containing single COOH-MWNTs, free CDDP and combinations of these compounds for 3h and 6h at 4 °C. DMSO 20% (v/v) was used as positive control. To determine the percentage of cell viability, the mLDH assay was performed as described previously.

2.4.2 Cisplatin uptake

The content of cisplatin in treated cells was correlated with the concentration of Pt ions determined with the use of mass spectrometry techniques. Herein, 5×10^5

cells were plated in 6-well plates and grown overnight in standard conditions. Thereafter, cells were tested with single COOH-MWNTs, free CDDP and nanocomplexes. After one day of incubation, cells were pelleted, and acid digested in glass vials with the addition of aqua regia and incubation at 70 °C for 2h. Aqua regia contained a mixture of HNO₃ and hydrochloric acid (Honeywell, Dusseldorf, Germany) in 1:3 proportion respectively. After digestion, the samples were diluted to a final HNO₃ concentration of 2%, filtered using 0.22µm syringe filters, and stored at 4 °C for analysis. A calibration curve was prepared with serial dilutions of 1,000 ppm Pt standard solution (BDH Laboratory Supplies, Poole, UK) in 2% HNO₃. The concentrations used were the following: blank, 1 ppb, 10 ppb, 25 ppb, 50 ppb and 100 ppb. Iridium was used as internal standard (m/z 191).

Pt (m/z 195) was analysed using a NexION 300X ICP-MS (PerkinElmer, Waltham, MA, USA), incorporating a concentric nebuliser with a cyclonic spray chamber, and glass injector system. The sample uptake rate was 0.3 mL/min, nebuliser flow ~1 mL/min, auxiliary gas flow 1.2 mL/min, and plasma gas flow 18mL/min. Washes with 2% nitric acid were set between samples to eliminate any carry over. Results expressed the mean of three independent repetitions with 5 sets of 20 readings each.

2.4.3 Cell sensitisation to cisplatin

Cells were plated at density of 7,000 cells per well in 96-well plates and incubated overnight under standard conditions. Following this, medium was replaced with fresh one containing COOH-MWNTs at concentrations of 10 and 50 µg/mL. After 24h or 48h, the medium was discarded, cells were gently washed until complete removal of nanomaterial, and CDDP at 12µM, 24µM and 48µM was added for further 24h. In samples exposed to COOH-MWNTs alone, fresh media with same concentrations of nanomaterial was added. Finally, the mLDH assay was performed to determine the chemosensitisation to CDDP.

2.4.4 Cell cycle evaluation

2×10^5 cells per well were seeded in 12-well plates and left to attach overnight under standard conditions. The following day, cultures were synchronised by starvation: old medium was removed, cells were washed with PBS, and fresh media containing FBS 0.1% (v/v) was added for 24h. Then, low-FBS media was replaced with fresh one containing single COOH-MWNTs, free CDDP and COOH-MWNTs+CDDP and incubated for further 24h. Topotecan hydrochloride 3µM (Sigma-Aldrich. Shanghai, China) and etoposide 10µM (Sigma-Aldrich. Jerusalem, Israel) were included as positive controls. After exposure time concluded, the media was aspirated, cells were gently washed with PBS and detached with 200µL of trypsin. Afterwards, cells were collected in 15mL plastic tubes and centrifuged at 300 x g and 4 °C for 5min. The resulting cell pellet was washed with PBS and the centrifugation process was repeated. The next step

included the fixation of cells with ice-cold ethanol 70% (v/v), which was added dropwise with constant gently agitation; samples were stored at 4 °C overnight. Thereafter, cell suspensions were centrifuged and washed to remove completely the ethanol. Finally, cells were stained with 0.5mL of FxCycle PI/RNase Staining Solution (Invitrogen. Oregon, USA) and incubated at RT for 20min. The fluorescence intensity of samples was recorded by flow cytometry at an excitation of 488nm and emission of 585nm. Using the FACSDiva software, forward scatter and side scatter were set to identify the single cells and exclude cell doublets from the analysis. Cell cycle phases were evaluated using PI histograms with FlowJo software package.

The cell cycle assessment of MCTS followed a similar process. MCTS were generated and treated as explained in previous sections. After one day of treatment exposure, the 3D cultures were disaggregated and centrifuged at 300 x g. The obtained cell pellet was washed with PBS and centrifuged again. Then, the samples were fixed, labelled and analysed identically to the protocol of cell monolayers explained above.; Both cell suspensions and MCTS experiments were performed at least three independent times; 20,000 events per sample were examined in each study.

2.4.5 Reactive oxygen species assessment

Cells were plated at density of 2×10^5 cells per well in 12-well plates and incubated overnight under standard conditions. Following, single COOH-MWNTs at concentrations of 40 and 50 $\mu\text{g/mL}$, free CDDP at 24 μM and 48 μM and

nanocomplexes were added for 24h. Menadione 120 μ M (Sigma-Aldrich, Shanghai, China) was added during 1h as ROS inducer agent. Subsequently, cultures were washed, trypsinised, and centrifuged at 350 x g, 4°C for 5min. Following the manufacturer description, cells were then resuspended in RPMI 1640, no phenol red medium, containing 5 μ M of CellROX Deep Red Reagent (Invitrogen, Oregon, USA) and incubated for 30min at 37 °C. Lastly, samples were centrifuged to remove the unbound stain and cells were resuspended in 0.5mL of phenol red free medium. The fluorescence intensity of 10,000 events per sample was recorded using a flow cytometer at ex/em 644/665nm. Obtained histograms were analysed using the FlowJo software.

The generation of ROS in MCTS exposed to treatments was also assessed. Therefore, 3D cultures were exposed to 50 μ g/mL of COOH-MWNTs, 48 μ M of CDDP, complexes of these compounds and 150 μ M of menadione as positive control. Passed 24h of incubation, MCTS were collected and washed twice with PBS. As soon as the spheroids sedimented, the supernatant was replaced with RPMI 1640, no phenol red medium, containing 5 μ M of CellROX deep red reagent (ex/em 644/665nm) and incubated for 1h at 37 °C. Afterwards, the medium was discarded, MCTS were washed 3 times with PBS, then resuspended in 500 μ L of Live Cell Imaging Solution containing 1 drop of NucBlue Live Cell Stain Ready Probes Reagent (Invitrogen, Oregon, USA) (ex/em 360/460nm) and incubated for additional 15min at RT. Microphotographs were recorded using a laser scanning confocal microscope to detect ROS generation in red fluorescence,

whereas the nuclei were counterstain in blue. Both experiments were performed three independent times.

2.4.6 Reduced glutathione assessment

In these experiments, 2×10^5 cells per well were seeded in 12-well plates and left to attach overnight under standard conditions. Afterwards, old media was replaced with fresh one containing single COOH-MWNTs at concentrations of 40 and 50 $\mu\text{g/mL}$, free CDDP at 24 μM and 48 μM or nanocomplexes, and incubated for 24h. In this experiment, solutions of single menadione 100 μM and combined with N-acetyl-L-cysteine 1mM (Sigma-Aldrich. Shanghai, China) were added to cells for 1h as controls. Samples were then processed as per the manufacturer indications: after incubation period, media was discarded and cells were washed, detached, and centrifuged at 350 x g, 4 °C for 5min. Obtained pellets were resuspended in 250 μL of PBS mixed with ThiolTracker Violet dye solution (Invitrogen. Oregon, USA) to a final concentration of 10 μM , followed by a 30min incubation at 37 °C. Samples were then centrifuged to discard de unbound dye, resuspended in 500 μL of PBS, and analysed using a flow cytometer. 10,000 events were recorded at an ex/em 405/525nm; FCS files were analysed using the FlowJo software package. Experiments were performed at least three independent times.

2.4.7 Mitochondrial membrane potential evaluation

Changes in the mitochondria electrochemical gradient were evaluated using the MitoProbe TMRM kit (Invitrogen, Oregon, USA). In this experiment, 2×10^5 cells were grown in 12-well plates and treated with single COOH-MWNTs at 40 and 50 $\mu\text{g/mL}$, free CDDP at 24 μM and 48 μM and the DDSs. Carbonyl cyanide 3-chlorophenylhydrazone was provided in the kit as positive control; a concentration of 5mM was added to cells for 5min. Passed 24h of treatment exposure, medium was discarded, and the cells were trypsinised and pelleted at 350 x g, 4 °C for 5min. To label the cells, a solution of TMRM at 20nM was added, proceeded by 30min of incubation at 37 °C. Then, samples were centrifuged, the supernatant was removed, and pellets were resuspended in PBS. 10,000 events were recorded using a flow cytometry at ex/em 561/585nm. The FlowJo software was used to analyse the obtained histograms. Experiments were performed in independent triplicates.

2.4.8 DNA damage assessment

The terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay was performed to detect DNA fragmentation in tested MCTS. Following the generation of MCF7 spheroids, single COOH-MWNTs 50 $\mu\text{g/mL}$, free CDDP 48 μM and COOH-MWNTs+CDDP complexes were added for 24h. After incubation, MCTS were collected and fixed with 500 μL of 4%paraformaldehyde (v/v) (Alfa Aesar, Haverhill, MA, USA) in PBS overnight at 4 °C. In the next morning, the fixing solution was discarded and MCTS were washed twice with

PBS. In order to prevent cell damage and deformation, the multicellular structures were cyclically perfused with increasing concentrations of sucrose solutions (Sigma-Aldrich, Dorset, UK). Every cycle lasted 8h and the concentrations used were 10%, 20% and 30% (w/v). Following cryoprotection, the spheroids were embedded in Optimal Cutting Temperature compound mounting medium (VWR International, Leuven, Belgium) using disposable base moulds. This process was performed on pulverised dry ice for immediate sample freezing. As soon as the mounting medium was solid, samples were stored at -80 °C.

Samples were dissected using a Bright OT5030 cryostat microtome (Luton, UK) set at -20 °C. Obtained 10µm thick slices were thaw-mounted onto Superfrost Plus microscope slides (Thermo Scientific, Waltham, MA, USA), dried for 1h at 37 °C, and stored at -20 °C for analysis. Subsequently, slides were warmed to RT and gently washed with PBS. The samples were then fixed with 4% paraformaldehyde (v/v) during 25min at 4 °C and permeabilised by the addition of 0.2% Triton X-100 (v/v) for 5min at RT. The TUNEL assay was performed using the Promega DeadEnd Fluorometric TUNEL System (Madison, WI, USA). Attending the manufacturer instructions, slides were washed with PBS, placed on ice and covered for 8min with 100µL of the provided equilibration mixture, which contained 25mM Tris-HCl pH 6.6, 0.2mM DDT, 200mM potassium cacodylate, 0.25 mg/mL BSA and 2.5mM cobalt chloride. Afterwards, the mixture was replaced with 50µL of rTdT incubation buffer, consisting of 45µL of equilibration buffer, 5µL of fluorescein-tagged dUTP (ex/em 488/520nm) and 1µL of terminal deoxynucleotidyl transferase enzyme. Cover slips were gently placed

on top of the samples to ensure homogeneous reactions, followed by 1h of incubation at 37 °C in a humidified chamber. Such chamber was fully protected from light and humidity regularly checked to prevent samples to dry. To finish the reaction, the cover slips were removed, and slides were immersed in 2X saline sodium citrate buffer for 15min at RT. Once the incubation was completed, samples were washed with PBS three times for 5min each, proceeded by the addition of a drop of Vectashield Hardset with DAPI (ex/em 360/460nm) (Vector Laboratories. Burlingame, CA, USA), used as counterstain, and stored at 4 °C. Microphotographs were acquired and analysed the next day, using a Nikon A1R inverted confocal laser microscope system (Nikon Corp. Tokyo, Japan).

2.5 Statistical analysis

Statistical analysis was performed in all possible experiments. The statistical methods applied in this thesis were one-way analysis of variance (ANOVA) followed by Bonferroni comparisons using the software GraphPad Prism Version 8.4.3 (La Jolla, CA, USA). Results are presented as mean $\pm$ standard deviation. The significance levels are: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

CHAPTER 3

CHARACTERISATION OF MULTI-WALLED CARBON NANOTUBES

3.1 Introduction and summary

A high proportion of the research performed on NPs focus on their applications as DDS and interactions with cells rather than the analysis of physicochemical properties. More importantly, although the attention of nanotoxicology has increased in the last years, it is still common to get misleading conclusions often by disregarding the importance of nanomaterials characterisation (388).

The physicochemical characteristics are determinant factors that can dictate the safety and biocompatibility of any NP (372, 385). CNTs toxicity and poor dispersibility are greatly associated with features such as size, surface functionalisation and individualisation (383, 384). The evaluation of the inherent characteristics of the MWNTs used is of utmost importance in order to determine their integration in biological models.

Therefore, the aim of this chapter will be to have a clear understanding of the particular properties and assess the differences of as-synthesised pristine MWNTs, non-covalently functionalised F127-MWNTs and chemically surface-decorated COOH-MWNTs and NH_3^+ -MWNTs. The length, superficial charge, surface, purity and composition were evaluated using MWNTs dispersed in water or their resulting dried powder.

3.2 Results and discussion

3.2.1 MWNTs functionalisation and purification

CNTs tend to aggregate and bundle in most solvents and biological media, limiting their use. In this context, surface functionalisation offers a solution to integrate CNTs into biological applications by improving the nanomaterial dispersibility. There are several protocols to modify the CNTs surface including covalent and non-covalent functionalisations. Nonetheless, some of the most used methods are the adsorption of surfactants such as PEG and F127, oxidation with carboxylic derivatisation and 1,3-dipolar cycloaddition reactions for the production of ammonium-functionalised CNTs (229, 257).

Surface chemistry will not only modify their dispersibility, but also improves the CNTs biocompatibility, cellular interactions, and several *in vivo* features like biodistribution, degradation, elimination and even the ability to avoid the RES (260). Furthermore, functionalisation opens the possibility of further attachments

such as drugs, biomolecules and antibodies, conferring applications in controlled targeting, sensing and labelling, among others (328).

Besides functionalisation, the synthesis of CNTs is inevitably accompanied with amorphous carbon and metal catalyst impurities (237). Several studies have pointed the importance of CNTs purification to reduce the cytotoxicity (251, 389). For example, Pulskamp et al. suggested a concentration-dependant production of intracellular ROS with the presence of metal impurities (390). In contrast, purified CNTs showed few to none acute toxicity (390). In another study, Aldieri et al. compared the toxicity of MWNTs with and without Fe impurities on murine alveolar macrophages (391). The results demonstrated the association of Fe impurities with MWNTs cytotoxicity and genotoxicity, while the absence of the metal showed no adverse effects (391). The acid treatment and ultrasonication are not only effective to functionalise and improve the dispersibility of MWNTs, but are also effective methods of purification and length reduction (250, 392, 393).

As mentioned previously, the present study includes the development, evaluation and application of two MWNTs + anticancer drug combinations: MWNTs+DOX and MWNTs+CDDP. Even though both complexes were prepared by simple combination, it was expected that the interactions between the components were different. DOX has been described to conjugate with the external surface of CNTs through hydrophobic interactions and π - π stacking due to the aromatic nature of its structure (394). Ali-Boucetta et al. reported for the first time a non-covalent

MWNTs-DOX supramolecular complex (347). In their study, the interaction of the drug and nanomaterial was evaluated by analysing the emission spectrum. They observed that the combination of constant DOX concentrations with increasing MWNTs quantities quenched the drug fluorescence intensity. In conclusion, the π - π stacking resulting from the interaction of the planar hydroxyanthraquinone rings and the MWNTs structure led to conformational changes that diminished the DOX fluorescence signal (347). Furthermore, in another investigation, the strength of the interactions between SWNTs and DOX was found to be proportional to the nanotube diameter (395). SWNTs with larger diameter were associated with slower drug release rates.

The effect of the nanotube diameter was corroborated by Wang and Xu, mentioning also the relevance of the pH in the complexation of the anthracycline and CNTs (396). While DOX readily conjugates with SWNTs at pH higher than 7, lower pH triggers the drug release. The protonation of the DOX molecule in acidic solution enhances the solubility (396). This is important when considering the physiological pH and target of the DDS. On the other hand, the mixture of CNTs dispersions with platinum drugs such as CDDP produce complexes via hydrophilic interactions, hydrogen bonding Van der Waal forces and electrostatic interactions (397).

CNTs and CDDP complexation has been mainly reported via covalent functionalisations or encapsulation within the nanotubes. In an early study, longboat delivery systems were developed consisting in CDDP attached to

SWNTs (398). Researchers claimed to entrap 65 Pt (IV) cores per SWNT by covalently linking a Pt prodrug through axial COOH moieties to non-covalently derivatised SWNTs (398).

As mentioned above, other research groups have described protocols to load CDDP inside CNTs. Badea et al. proposed a method in which CDDP was dissolved in dimethylformamide and mixed with carboxylated MWNTs (399). The mixture was sonicated for 1h and stirred for further 2 days. Similar methodologies to entrap CDDP in CNTs have been developed by different research groups. Common procedures include the mixture of CDDP with non-covalently and covalently functionalised CNTs in aqueous (400), ethyl acetate (401), chloroform (402) or DMSO dispersions (403). Preparations were sonicated and stirred during prolonged periods of time followed by filtrations and extensive washes to remove any remaining drug attached to the outer surface of the CNTs.

Additionally, molecular dynamic simulations have provided important information about the interactions involved. Mejri et al. concluded that the entrapment of CDDP in CNTs is not only favoured due to strong Van der Waal interactions, but also hydrophobic interactions keep the drug confined, avoiding the need of capping the CNTs ends (404). Another publication mentioned the importance of considering the functionalisation of CNTs surface to understand the interaction of molecules and formation of complexes (405). Using density functional theory approaches, researchers determined that the inclusion of CDDP into oxidised CNTs produce stable systems (405). Also, computational structure modelling

showed hydrogen bonds between NH_3 moieties of the drug, while the hydroxyl and carboxyl groups of the derivatised nanomaterial stabilised the complex (405).

To our knowledge, the study here presented is the first to propose a simple, fast and reliable method to non-covalently conjugate functionalised MWNTs with CDDP without the use of organic solvents and avoiding filtration and long reaction periods.

3.2.2 Size distribution assessment

NPs dispersions are not homogeneous. Therefore, the determination of particle size and size distribution are valuable measurements of all nanomaterials including CNTs. Herein, non-covalently and chemically functionalised MWNTs were prepared following the procedures described previously. Then, the structure and length of MWNTs were analysed using a TEM; a collection of images are depicted in figure 3.1.

The obtained photomicrographs depicted clear differences among the different MWNTs. Pristine MWNTs dispersed in dH_2O (figure 3.1 A1-A2) were observed as long bundled nanotubes. The lack of functionalisation caused their evident entanglement, making the size assessment and use in biological studies almost impossible.

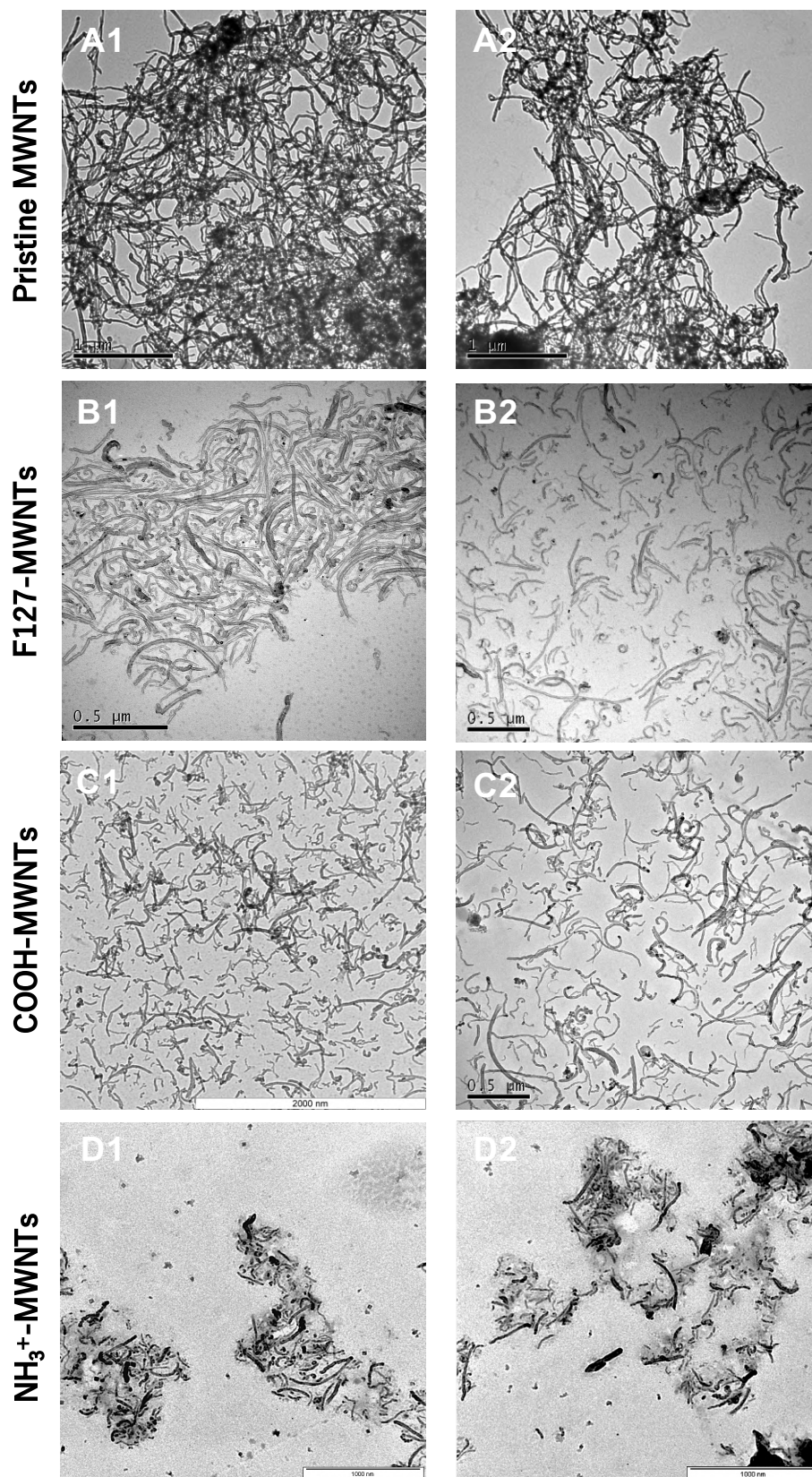


Figure 3.1 | TEM photomicrographs of non-functionalised, non-covalently functionalised and covalently functionalised multi-walled carbon nanotubes. (A1-A2) pristine MWNTs dispersed in diH₂O, (B1-B2) F127-MWNTs, (C1-C2) COOH-MWNTs and (D1-D2) NH₃⁺-MWNTs.

In contrast, all functionalisation procedures successfully increased the dispersibility of MWNTs. Although a few clusters were detected, F127-MWNTs microphotographs (figure 3.1 B1-B2) showed an improvement on the individualisation of nanotubes. However, the non-covalent derivatisation only involves the MWNTs coating with the polymer; thus, neither structural alterations nor size decrease were expected.

NH_3^+ -MWNTs demonstrated alike levels of individualisation and dispersion in diH_2O (figure 3.1 D1-D2); however, much shorter. Also, some agglomeration and artefacts were observed. Finally, COOH-MWNTs samples demonstrated to be the most homogeneously dispersed and length wise with practically absent nanotube bundles (figure 3.1 C1-C2).

It is important to note that the non-covalent derivatisation only involves the MWNTs coating with the polymer; thus, neither structural alterations nor size decrease were expected. On the other hand, as mentioned above, the acid treatment included in the chemical functionalisation is an efficient method to reduce the MWNTs length. Furthermore, TEM microphotographs of the promising COOH-MWNTs+CDDP and NH_3^+ -MWNTs+CDDP complexes, were acquired and analysed (figure 3.2).

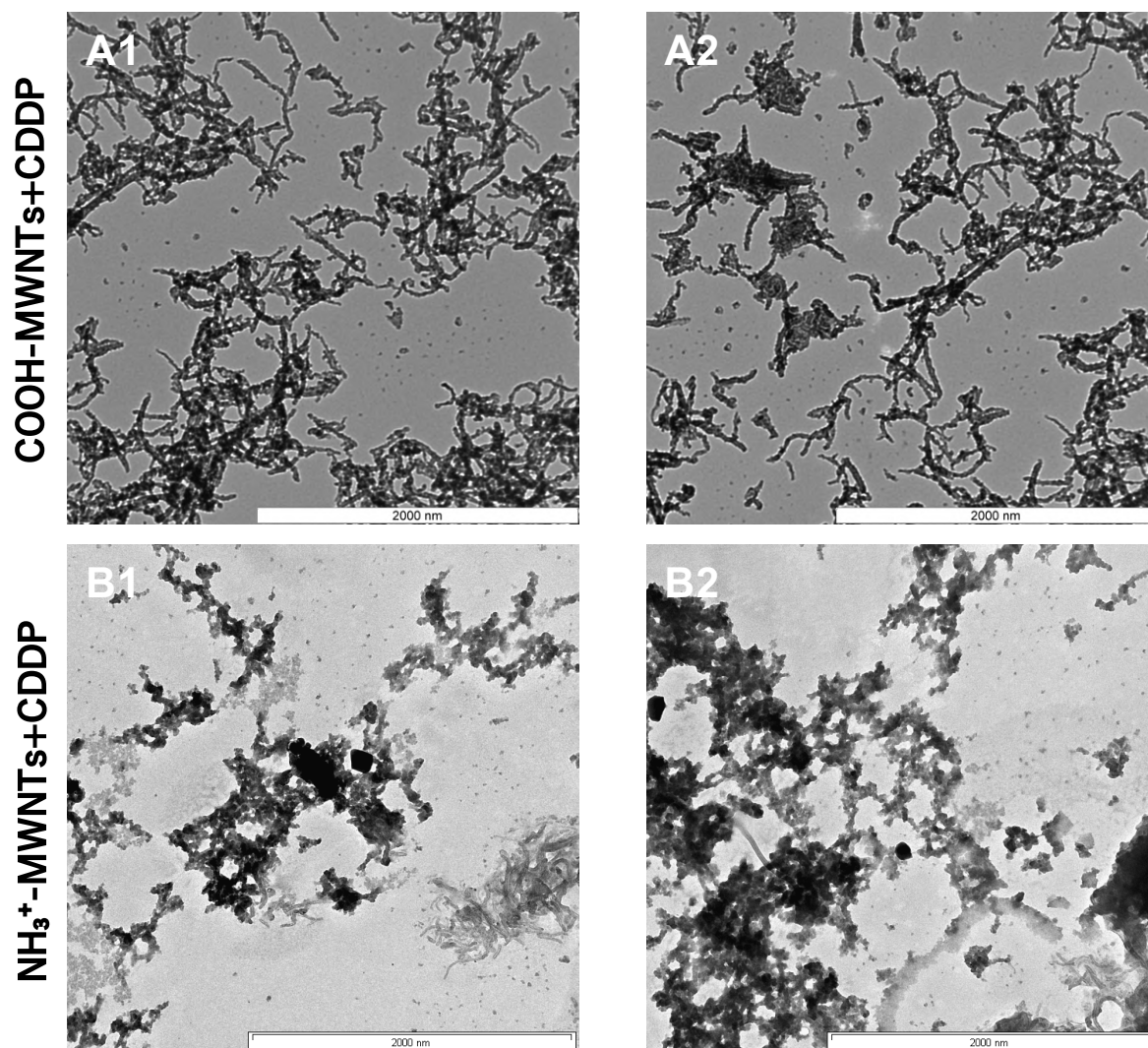


Figure 3.2 | TEM photomicrographs of multi-walled carbon nanotubes + cisplatin. (A1-A2) COOH-MWNTs+CDDP, (B1-B2) NH_3^+ -MWNTs+CDDP.

The complexed MWNTs depicted evident differences compared to those without the drug. Structural changes were observed in both evaluated combinations. COOH-MWNTs+CDDP showed small aggregates attached to the surface of the nanotubes modifying their appearance to granular nanotubes (figure 3.2 A1-A2). In the case of NH_3^+ -MWNTs+CDDP, the aggregates attached to the nanomaterial were bigger, producing dark spots that could be due to the bundle of the complex (figure 3.2 B1-B2). The overall structural transformation seemed more

homogeneous in the carboxylated MWNTs than the aminated. In the latter ones, some MWNTs were spotted without apparent modifications. As mentioned before, photographs of both nanoconstructs demonstrated an increased aggregation. Nonetheless, MWNTs+CDDP preparations did not show visible alterations on the dispersibility and stability.

Previous investigations by Bhirde et al. showed structural changes when CNTs were used to encapsulate CDDP (406, 407). The analysis of COOH-SWNTs-CDDP, COOH-SWNTs-CDDP attached to epidermal growth factor and PEG-SWNTs-CDDP using Z-contrast scanning transmission electron microscopy detected bright dots corresponding to Pt atoms and clusters. Besides, aggregates on the sidewalls were observed (406-408). In another study, SEM pictures exhibited COOH-MWNTs encapsulating CDDP with granular structure comparable to the nanoformulation we developed (399).

Even though data in some those publications are resulted from other assays than ours, microphotographs of their CNTs-CDDP complexes presented similar morphology changes. The preparation procedures of the CNTs complexes could explain the extent of surface aggregation. Their DDSs were thoroughly washed to remove any drug adsorbed to the CNTs surface, while ours did not go through any process after the nanomaterial-drug combination. Moreover, using molecular dynamic simulations, Konczak et. tested the stability of CNTs encapsulating CDDP and capped with functionalised AuNPs (409). Simulating two identical CNTs in crowded dispersion conditions, results showed that their interaction

aligned their orientation in parallel, caused their collision, weakened the capping and released the CDDP (409). These findings could be one of the possible explanations to the CNTs bundle. We suspect that the complexation of CDDP to MWNTs modifies the superficial charge of CDDP, which changes the interactions of the nanomaterial present in the dispersion.

Recorded images of the different MWNTs were used to determine the average particle size and size distribution. Figure 3.3 shows the size distribution of 1,000 individual MWNTs analysed and comparison of the different MWNTs types.

After analysing 1,000 individual MWNTs, results confirmed the effects of functionalisation type. Non-chemically coated F127-MWNTs demonstrated to be the longest MWNTs of all tested, with a mean length of 363 ± 187 nm (fig. 3.3A). On the other hand, chemically derivatised MWNTs measured less the half of the coated MWNTs. The mean particle size of NH_3^+ -MWNTs was 161 ± 89 nm (fig. 3.3D), practically the same of COOH-MWNTs which was the shortest with 158 ± 82 nm (fig. 3.3B). Measurements correspond to the fact that aminated MWNTs were synthesised from carboxylated MWNTs. Therefore, lengths of both MWNTs were expected to be analogous.

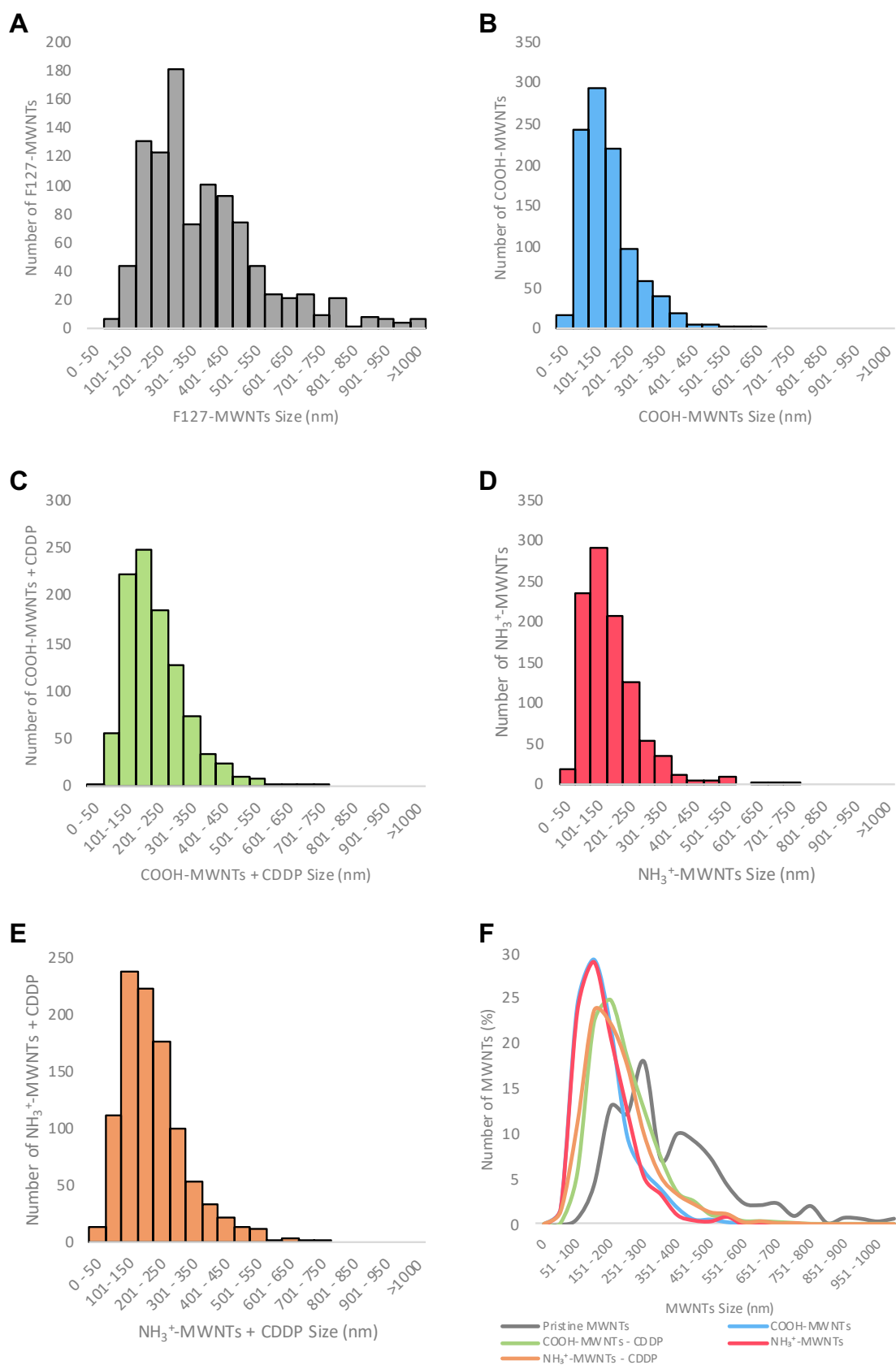


Figure 3.3 | Size distribution of sonicated MWNTs. (A) F127-MWNTs, (B) COOH-MWNTs, (C) COOH-MWNTs+CDDP, (D) NH_3^+ -MWNTs, (E) NH_3^+ -MWNTs+CDDP, (F) histogram comparison of MWNTs size.

Nanocomplexes described slightly longer particle sizes than the nanomaterial without the drug. The NH_3^+ -MWNTs-CDDP were marginally shorter than COOH-MWNTs-CDDP (fig. 3.3E and 3.3C correspondingly). The mean length of the aminated was $201 \pm 102 \text{ nm}$ while the latter was $214 \pm 97 \text{ nm}$. Figure 3.3F compares the particle size histogram of all samples.

Researchers have found a direct relationship between the length of CNTs and their biotoxicity. Macrophages play a particularly important role in the phagocytosis and clearance of CNTs. The efficiency of these bioprocesses is intimately related to the size of the nanomaterial. Investigations have demonstrated that CNTs longer than the diameter of the macrophages lead to frustrated phagocytosis (384, 410). The disruption of this process is partially responsible of the cytokines release and inflammation as well as fibrosis response to CNTs (377, 411). Sweeney et al. evaluated the impact of the MWNTs length on macrophages (412). Studies indicated that $20 \mu\text{m}$ long nanotubes were significantly cytotoxic; they increased the ROS generation and inflammatory mediators in human alveolar macrophages (412). Interestingly, the bioreactivity of short MWNTs of $0.6 \mu\text{m}$ was markedly lower or totally absent (412).

Moreover, Boyles et al. obtained alike findings by testing long ($>20 \mu\text{m}$) and short ($<20 \mu\text{m}$) MWNTs in three different macrophage models (413). The long MWNTs frustrated the phagocytosis, triggering inflammatory and fibrotic responses. In

contrast, short MWNTs not only demonstrated less to none cellular toxicity, but also the metal content and purity showed little influence (413). These results may suggest a high relevance of the nanomaterial's length over toxic responses.

3.2.3 Superficial charge evaluation

Superficial electrical charge, also defined as zeta potential (ZP), is the potential difference between the fluidic layer surrounding the NPs' surface and the medium of dispersion (414). Even though the superficial charge is not an absolute measurement of NPs stability, it is a key indicator of dispersion stability and cell interaction.

Typically, nanodispersions with ZP values higher than +30mV or lower than -30mV have been defined ideal due to the repulsion of the NPs. ZP magnitudes in the range of 20mV to 40mV (positive or negative) are considered good stability; enough to avoid NP aggregation. Lower values than those may, but not necessarily, produce aggregation, flocculation or coagulation (415, 416).

F127-MWNTs, COOH-MWNTs, NH_3^+ -MWNTs, and CDDP as well as the promising COOH-MWNTs-CDDP and NH_3^+ -MWNTs-CDDP nanoformulations were assessed to determine the superficial charge. Following methodologies explained beforehand, results are depicted in figure 3.4.

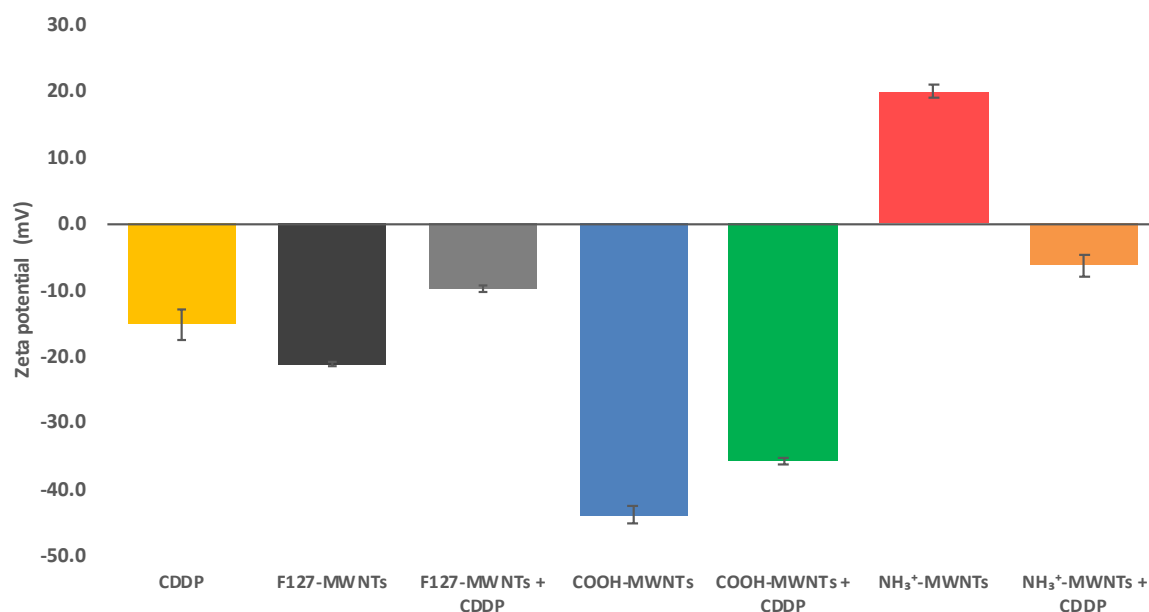


Figure 3.4 | Superficial charge of MWNTs nanoformulations. Determination of zeta potential of free CDDP, pluronic coated MWNTs, carboxylated MWNTs, aminated MWNTs and MWNTs + drug complexes.

As expected, the chemical nature of the performed functionalisations defined the superficial charge of tested MWNTs. The carboxyl group of COOH-MWNTs conferred an overall negative surface charge of $-43 \pm 1.3 \text{ mV}$ whereas the moieties attached to NH_3^+ -MWNTs produced a positive value of $19 \pm 0.9 \text{ mV}$. Besides, polymer-coated F127-MWNTs also showed a negative value of $-21 \pm 0.3 \text{ mV}$. Evaluation of F127 in solution resulted in a lower charge of $-10 \pm 2 \text{ mV}$, confirming that the ZP is closely related to the type of nanomaterial.

Our obtained ZP results agree with earlier studies. Superficial charge of oxidised MWNTs has been reported in a range of -60 to -30 mV whereas wrapped MWNTs dispersion showed values of -40 mV to 10 mV (278, 417). The aminated nanomaterial has also been reported with comparable positive values (418, 419). Nevertheless, it is important to mention that the equations used by the instrument

to determine the ZP assumes the presence of spherical-shape particles in the sample. Since MWNTs are non-spherical nanoparticles, studies have suggested to be aware of the challenges with this measurement (420, 421) and possible solutions (422).

Interesting is the fact that the presence of CDDP modifies the ZP of all analysed MWNTs. While the charge of the free drug in solution was -15.2 ± 2.4 mV, when it is complexed, it shifted towards neutrality positive and negative MWNTs. Negatively charged nanoformulations showed less evident changes than the positive ones. The combination of CDDP with F127-MWNTs quenched the ZP to -9 ± 0.4 mV while COOH-MWNTs-CDDP showed a minor effect with a decrease to -35 ± 0.5 mV. However, a marked change was observed in NH_3^+ -MWNTs-CDDP combination. The conjugation of CDDP dropped the ZP of the amine MWNTs to -6.3 ± 1.6 mV. We suspect that the platinum drug interacts directly with the chemical groups added on the MWNTs derivatisations, altering not only the structure, but also the overall physicochemical properties.

Previous studies have concluded that pH and the quantity of surfactant added to nanomaterial have important influence on the ZP and dispersion. While small percentages of surfactant tend to cause aggregation, high proportions increase the ZP value. Furthermore, the addition of other compounds to the nanodispersions can lead to drastic shifts of ZP (423). About the addition of compounds to NPs, Trummer et al. developed carboxylic acid and PEG bearing chitosan polymeric nanocarriers coordinated with CDDP (424). In the research,

they observed a ZP increase in the NPs when the CDDP was loaded, proposing that deprotonated acidic groups coordinated with CDDP, diminishing the negative charge values (424). Moreover, the loading of other drugs into MWNTs such as gemcitabine increased the ZP observed in MWNTs alone from -48mV to -10mV (425). Likewise, researchers concluded that the chemical structure of the drug interacted with the coating of the nanotubes (425). Also, grafted COOH-MWNTs with pyrazole derivatives showed similar trends of ZP shifting (426), depicting the interaction and adsorption of chemically different molecules with CNTs and the effect over the ZP.

Surface charge is known to be important on the opsonisation of NPs in blood. A reduced binding with plasma proteins and prolonged circulation have been associated with the use of hydrophilic polymers to coat NPs and a negative ZP (427). On the other hand, positively charged NPs have increased attraction to cells, which possess overall negative charge (428).

3.2.4 Raman spectroscopy

Raman spectroscopy is a technique used to detect the chemical composition of numerous materials in different states. It is based on the analysis of the vibration, rotation and other low-frequency modes of molecules in a system (429). The technique is widely used for the study of carbon-based materials due to its versatility and sensitivity to analyse all types of carbon allotropes (430).

The functionalisation of MWNTs' surface was expected to lead to important changes. Therefore, an assessment of MWNTs, free drug and combinations using Raman spectroscopy techniques were performed. Resulting spectra were compared (figure 3.5), paying special attention to the characteristic G and D bands. The G band ($1,580\text{--}1,600\text{cm}^{-1}$), also known as graphitic band, is originated from the vibration of the graphene sheets constructing to CNTs (275), while the D band ($1,350\text{cm}^{-1}$) results from sp^3 -hybridised carbon material related with disorder and defects in the aromatic structure (271).

All carbonaceous samples depicted clear G and D bands; however, differences between samples were observed. In general, derivatised MWNTs showed higher intensity D bands, suggesting the introduction of wall defects product of the functionalisation (431). Furthermore, shifting of the G bands was observed, which is related to the modification of the nanotube chemistry by the addition of chemical moieties (392). Increase in the G' bands ($2,700\text{cm}^{-1}$) on derivatised MWNTs is due to the interaction of the different graphene layers in the nanotubes; it is proportional to the number of layer and is created by double resonance transitions (271).

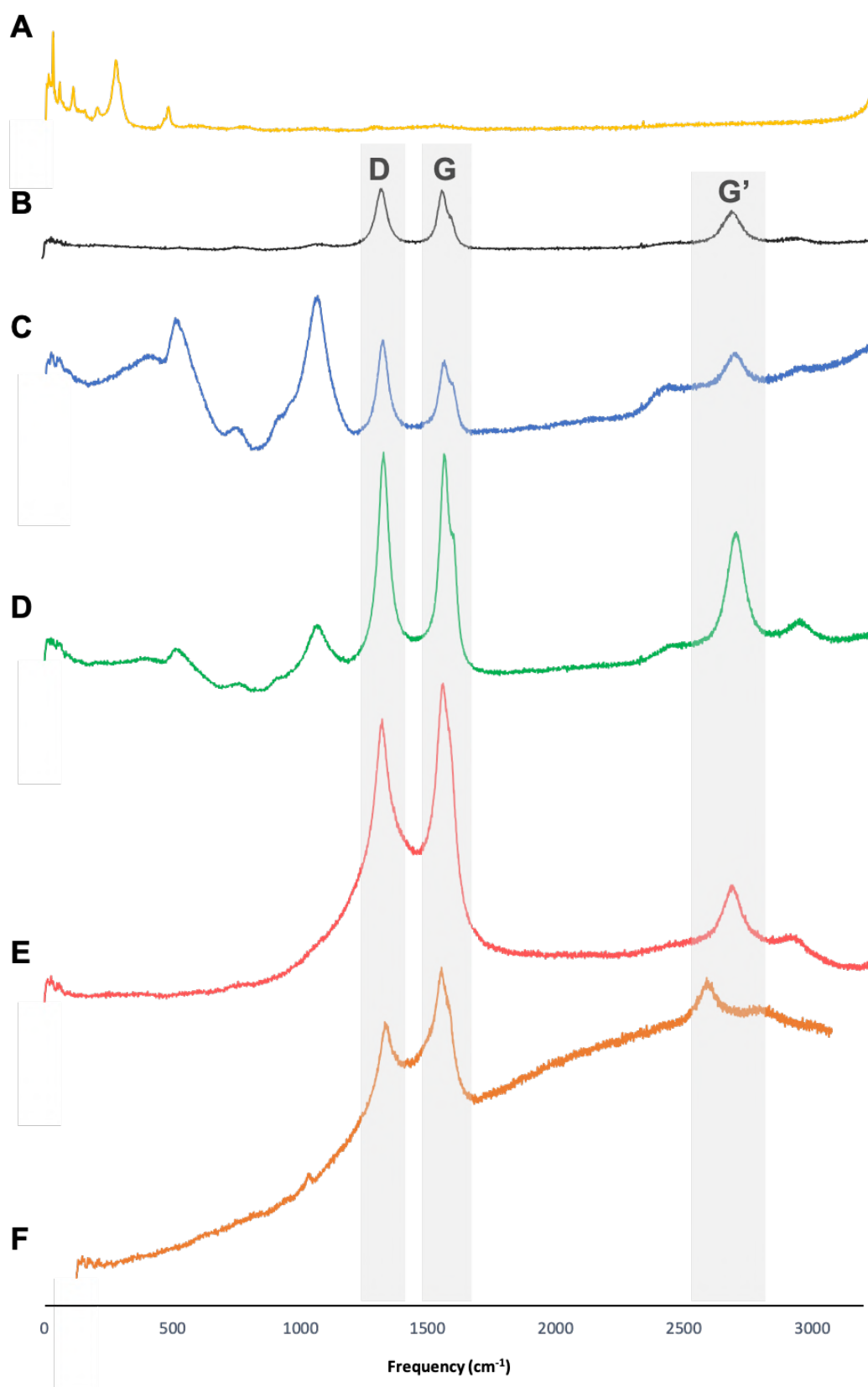


Figure 3.5 | Raman spectra of cisplatin, MWNTs, and nanoformulations. (A) CDDP, (B) Pristine MWNTs, (C) COOH-MWNTs, (D) COOH-MWNTs+CDDP, (E) NH_3^+ -MWNTs, (F) NH_3^+ -MWNTs+CDDP.

The D band ratios with respect to the G band (I_D/I_G) were also calculated. Pristine MWNTs, NH_3^+ -MWNTs and NH_3^+ -MWNTs-CDDP produced very similar results: 0.88, 0.87 and 0.86 respectively. In contrast, the ratio of the carboxylated nanomaterial was 1.12 and that of COOH-MWNTs-CDDP was 1.01. The I_D/I_G ratio is a proportional indicator of defects present in the nanomaterial. A low ratio is linked with low-defect CNTs, whereas a high number can also refer to the presence of carbonaceous impurities (275). It also has been associated with an increase of sp^3 -hybridised carbons, resulting from the oxidation of MWNTs (392). Additionally, aminated and carboxylated MWNTs showed a small band at $2,950\text{cm}^{-1}$. Reports have mentioned that it is related to C-H stretching, also product of the oxidative process (392). The presence of D' peaks ($1,620\text{ cm}^{-1}$) was observed as well, which are also due to defects on the graphene layers (432).

Free CDDP spectra is of particular interest in the low range as most of the stretching vibrations take place below 550cm^{-1} . A discrete band was observed at 525 cm^{-1} , which has been attributed to Pt-N stretching. Moreover, Pt-Cl frequency was detected at 323cm^{-1} , whereas the Cl-Pt-Cl bending mode was present at 162cm^{-1} (433, 434). CDDP spectra was masked when complexed with MWNTs; this effect can be explained due to the high intensity absorption of organic groups (405) and the incorporation of the drug in the MWNTs (403, 435).

3.2.5 Fourier-Transform Infrared Spectroscopy

FTIR techniques were used to confirm the chemical composition and surface modifications of MWNTs. FTIR spectroscopy is highly used to identify and characterise a wide range of carbon-based materials. The method measures the light absorbed by the vibration of atomic bonds originated from the IR irradiation of the material (276).

Pristine MWNTs, chemically decorated MWNTs, free CDDP and nanocomplexes were scanned as described before. Figure 3.6 shows the obtained spectra and a comparison of the different samples.

Characteristic peaks were detected at $1,680\text{cm}^{-1}$ associated with C=C due to aromatic rings and defects; bands around $3,600\text{cm}^{-1}$ were related with -OH groups, whereas C=O moieties were observed at $1,550\text{cm}^{-1}$ – $1,350\text{cm}^{-1}$ originated from carboxylic acids (269, 392, 436).

The presence of peaks at $3,600\text{cm}^{-1}$ registered in the pristine MWNTs samples could be attributed to ambient moisture, while widening of the signal indicated the oxidation of the nanomaterial. Additionally, previous research has explained that peaks observed at $1,445\text{cm}^{-1}$ and $1,756\text{cm}^{-1}$ are specific for MWNTs (437). Amine MWNTs presented typical bands in the range of $3,500\text{cm}^{-1}$ to $3,600\text{cm}^{-1}$, indicating NH moieties; small bands at $1,650\text{cm}^{-1}$ are associated with NH_2 groups (418, 431).

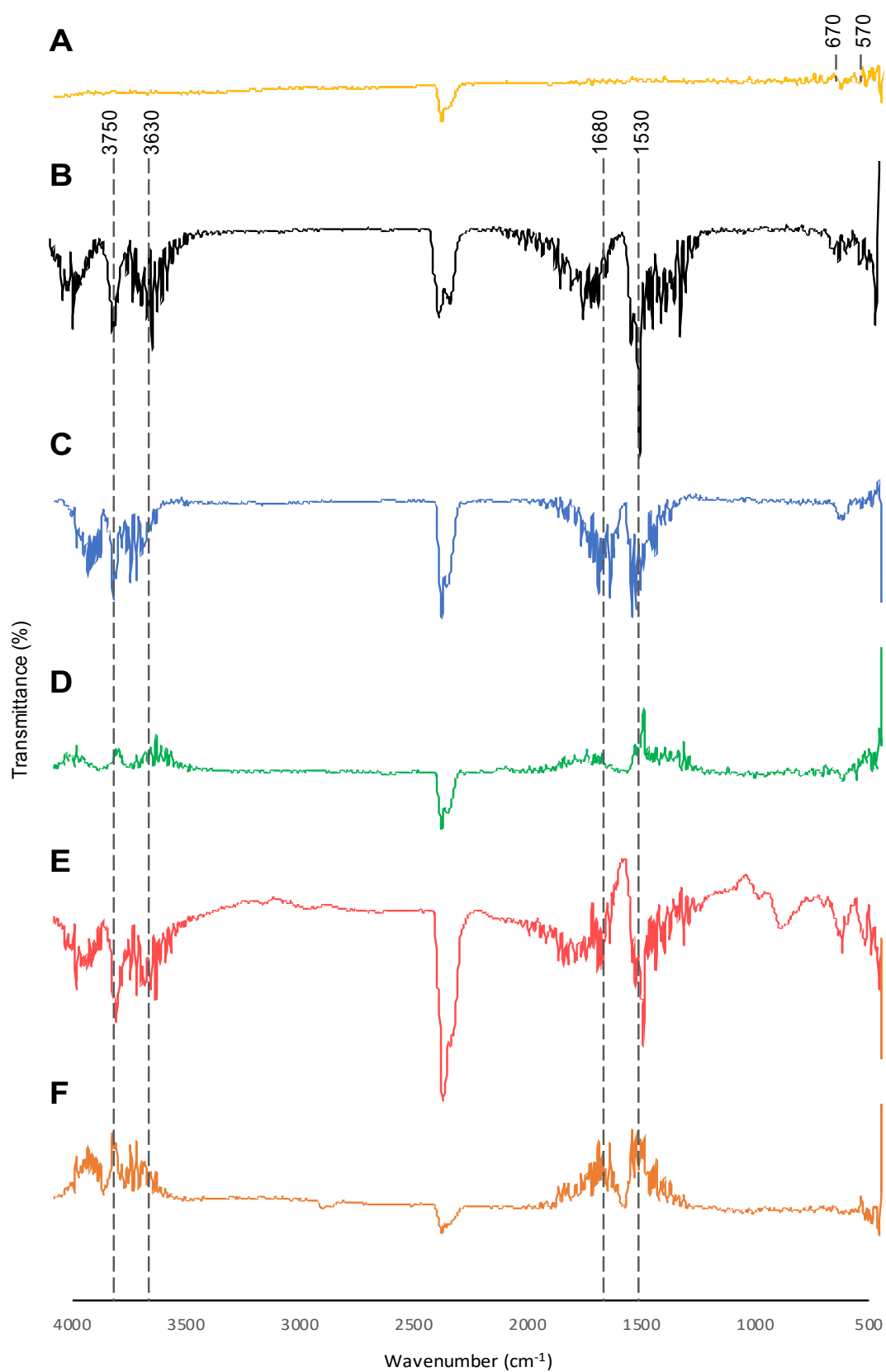


Figure 3.6 | Fourier-Transform Infrared Spectroscopy analysis of cisplatin, MWNTs, and nanoformulations. (A) CDDP, (B) Pristine MWNTs, (C) COOH-MWNTs, (D) COOH-MWNTs+CDDP, (E) NH_3^+ -MWNTs, (F) NH_3^+ -MWNTs+CDDP.

According to Bedea et al. the encapsulation of CDDP and MWNTs was detected at low frequencies (399). CDDP showed Pt-N and Pt-O chemical moieties at 670cm^{-1} and 570cm^{-1} respectively (399). Nonetheless, as observed in other investigations, characteristic stretching modes of the drug were hindered by the complexation with MWNTs, making them virtually indistinguishable (405). The detection of oxygen- and nitrogen-containing chemical groups confirmed that moieties were successfully introduced onto the MWNTs sidewalls, proving the efficacy of functionalisation methods.

3.2.6 Thermogravimetric analysis

TGA was the last of the assemble of measurements performed to determine the physicochemical properties of MWNTs. It is destructive method used to determine the thermal and oxidative stability as well as composition of materials by registering the weight change under constant increasing temperature (438).

In the particular case of CNTs, TGA is used to evaluate purity, composition and as indicator of surface modification of the nanomaterial by measuring the oxidation point, decomposition of carbon and residual mass (438, 439). Burning fractions may differ depending on the presence of synthesis catalysts, CNTs defects, and metal contaminants (275).

TGA techniques were performed to evaluate pristine MWNTs, covalently derivatised MWNTs, free CDDP and MWNTs+CDDP combinations. Spectra obtained are illustrated in figure 3.7.

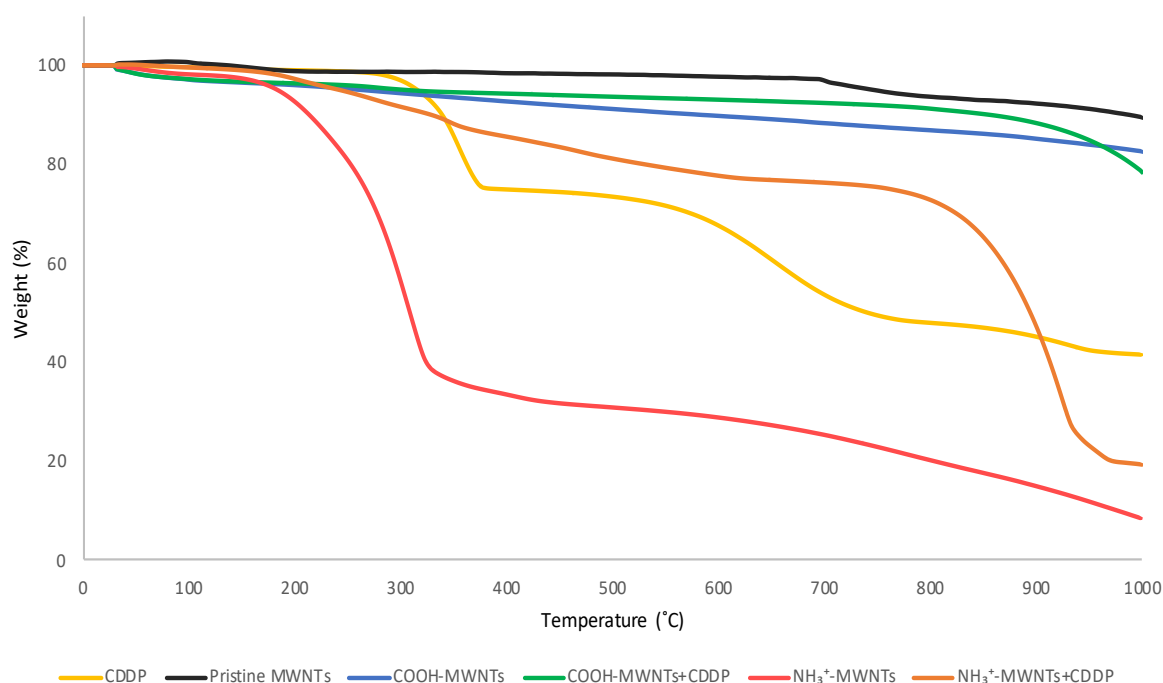


Figure 3.7 | Thermogravimetric analysis of MWNTs nanoformulations. The graph illustrates the percentage of mass loss in relation the temperature increase of free CDDP (**yellow**), pristine MWNTs (**black**), COOH-MWNTs (**blue**), COOH-MWNTs+CDDP (**green**), NH₃⁺-MWNTs (**red**) and NH₃⁺-MWNTs+CDDP (**orange**).

In general, samples demonstrated good thermal stability. Particularly, the initial stage of pristine MWNTs depicted a slight increase of weight due to humidity present in the sample. The little percentage of mass lost under 500 °C demonstrated the low content of amorphous carbon in the sample (268, 440). The initiation temperature, which marked the temperature of the carbon oxidation, started at 700 °C. The study ended with 89.5% of residual mass.

COOH-MWNTs and COOH-MWNTs+CDDP followed similar patterns. Both had sustained weight reductions throughout the experiment. COOH-MWNTs illustrated a constant mass decrease that began at 98 °C due to water evaporation and the loss of carboxylic, aldehyde and hydroxyl moieties as reported elsewhere (403, 441, 442). In contrast, the carboxylated MWNTs combined with CDDP showed a lower degradation rate, but a marked weight loss at 900 °C. As a result, both samples showed alike residual masses; COOH-MWNTs presented 82% while the nanocomplex resulted in 78%.

Regarding NH_3^+ -MWNTs, results presented various stages. During the first region, the sample presented approximately 5% of mass loss. As observed in other samples, the weight decrease was due to the volatilisation of moisture. Then, the sample rapidly reached the oxidation temperature at 180 °C, decomposing 62% of the nanomaterial. The weight loss was associated with the burning of amino, carboxylic and hydroxyl groups attached to the surface of nanotubes (419, 443, 444). Subsequently, the oxidation of the carbonaceous material was observed at 330 °C following a long weight decline. NH_3^+ -MWNTs demonstrated the lowest residual mass with 8.2%.

The complexation of aminated MWNTs with CDDP produced a completely different spectrum as the observed with the MWNTs alone. In this case, an important mass decrease was observed once 185 °C were reached. As explained above, the loss is related to the burning of moieties attached to the nanotube's backbone. Then, a major drop was recorded at 800 °C due to the oxidation of

MWNTs. Opposite to the residual mass obtained with NH_3^+ -MWNTs, this combination depicted 19% due to the Pt present in the sample.

Free CDDP showed a minimal initial weight loss, followed by a significant drop of 22% at 290 °C. That temperature has been described as the melting point of the drug (445, 446). The stage with the most material degradation initiated at 380 °C, combusting the amine and chloride groups (447). A gradual weight decline was recorded up to a total residue mass of 41.5% due to remaining Pt.

The different thermal decomposition patterns between single MWNTs, the free drug and complexes may lie in the interaction between the two molecules. Such interactions modified not only the oxidation rate of the samples, but also the mass loss. Furthermore, organic molecules from the drug attached or entrapped in the MWNTs promote different combustion patterns. Similar observations have been described in the analysis of other nanoconstructs (431, 446).

In conclusion, great emphasis was placed on improving the characteristics of pristine MWNTs and stressed the importance of thorough evaluation of the nanomaterials' properties. Thus, MWNTs were coated with F127 and covalently functionalised via acid treatment and 1,3-dipolar cycloaddition methods. Physicochemical characterisation assessment corroborated not only the successful carboxylation and amination of MWNTs, but also their good dispersion and individualisation in aqueous preparations. Studies confirmed as well the highly purity and thermostability of the nanomaterials developed. The described

characteristics, safe length, according to previous reports, and stability make the developed MWNTs biocompatible. Therefore, the next chapters will present and discuss the cytotoxic evaluation of the nanomaterials as well as the efficacy assessment of the nanocomplexes in cancer models.

CHAPTER 4

THERAPEUTIC EFFICACY ASSESSMENT OF MWNTS + ANTICANCER DRUGS IN 2D CELL CULTURES

4.1 Introduction and summary

New classes of DDS must be thoroughly evaluated to determine their therapeutic potential. Such analysis of optimisation of efficacy requires the use of testing models. In this context, biological models are experimental systems that recreate the human tissue function and spectrum of pathogenesis for meaningful scientific research (448, 449).

For the purpose of this thesis, pharmacological models provide quantitative tools to measure and understand the variables of pathophysiological processes and interactions with drugs (450). Likewise, cancer modelling represents features observed in human cancers. Ideally, cancer models are robust and dynamic systems, governed by complex genetic, metabolic and stress responses (451). These systems include cell lines, organisms and genetically engineered mice (452).

Used since the early 1900s, routinely cell cultures involve the maintenance and growing of cells in monolayer. Conventional cell cultures present challenges such as alterations of the cell morphology and pathway signalling that may diminish the reproducibility of promising therapeutic agents in clinal assays (453). Moreover, these culture systems are not completely equivalent to the real tissue; they present environmental and metabolic disadvantages that may result in stress and genotypic and phenotypic modifications (454, 455). Nevertheless, the use of 2D cultures is widely accepted and routinely performed in several scientific fields due to their cost-effectivity and feasibility to screen compounds as well as helpful on the efforts to reduce animal experimentation (455). Nowadays, they are extensively used and associated with virtually all current assays.

2D cultures are crucial in the drug discovery process, and assay such as potency (456), safety and toxicology determination (457), metabolic stability (458), cell proliferation and cytotoxicity (459), as well as pharmacokinetics and pharmacodynamics model simulators (460, 461) among others.

In recent years, the growing number of NPs introduced into nanomedicine and to the environment as waste has increased the concern of developing appropriate models for the exhaustive study of their toxicological and therapeutic profiles. This chapter focuses on the interactions of functionalised MWNTs with a 2D breast cancer model, particularly, on the cytotoxicity assessment of the nanomaterials and therapeutic efficacy of the MWNTs + anticancer drugs

combinations. The selected model was the human mammary tumour MCF7 cell line; an immortalised epithelial luminal line. Comparative analysis will be presented to determine the biocompatibility of MWNTs and the most promising nanocomplexes.

4.2 Results and discussion

4.2.1 Cytotoxicity assessment of functionalised MWNTs

The effect of MWNTs on cells viability is an important parameter to assess in the development of DDSs. Therefore, the cytotoxicity of F127-coated MWNTs and chemically derivatised MWNTs was assessed, followed by the therapeutic efficacy of free DOX and CDDP and finishing with MWNTs + drugs combinations. All assays were performed using the mLDH colorimetric assay.

The lactate dehydrogenase (LDH) is a cytoplasmic enzyme ubiquitous in all cells, whom leakage into extracellular media can be associated with cell membrane damage and therefore, irreversible cell death. The probe is based on the conversion of pyruvate to lactate and the inherent reduction of NAD⁺ to NADH; the reduced enzyme reacts with tetrazolium salts, producing coloured formazan crystals, quantizable at 490nm (462, 463). The assay is extensively accepted in cytotoxic studies and has been described as a fast, simple and consistent probe (464).

Nonetheless, studies have reported the interference of carbon-based nanomaterials with formazan-based cell viability assays, resulting in inaccurate results (465-468). Forest et al. studied the interferences occurring between MWNTs and the LDH probe, concluding that the effect was due to the natural absorbance of the nanomaterial and the adsorption of the enzyme on the surface of the MWNTs (469). Thus, in order to solve the problem, Ali-Boucetta et al. developed a modified LDH assay (470). The modification consisted in removing the nanomaterial from the sample and quantifying the intracellular LDH levels in intact cells instead of the leakage (470). Several studies have referred to this methodology as the most reliable to determine the cell viability in samples tested with carbonaceous NPs (471-474). Thus, it is the reason the mLDH was used in this work.

The cytotoxic effect of the F127-MWNTs, COOH-MWNTs, and NH_3^+ -MWNTs was determined using the mLDH colorimetric method. For these experiments, 2D MCF7 cultures were used as testing model. The concentration ranges of derivatised MWNTs were selected based on previous studies performed in our laboratory and related literature. All samples were assessed after 24h and 48h.

The first nanomaterial to be analysed was F127-MWNTs. Cells were incubated with the following concentrations of MWNTs: 7.8, 15.6, 31.2, 62.5, and 125 $\mu\text{g/mL}$. Results obtained were analysed and graphed in figure 4.1.

Overall, resulting graphs showed a concentration–response trend over time. The lowest concentration did not show apparent changes compared to untreated control. However, viability inhibition was detected from 15.6 $\mu\text{g/mL}$ and higher concentrations.

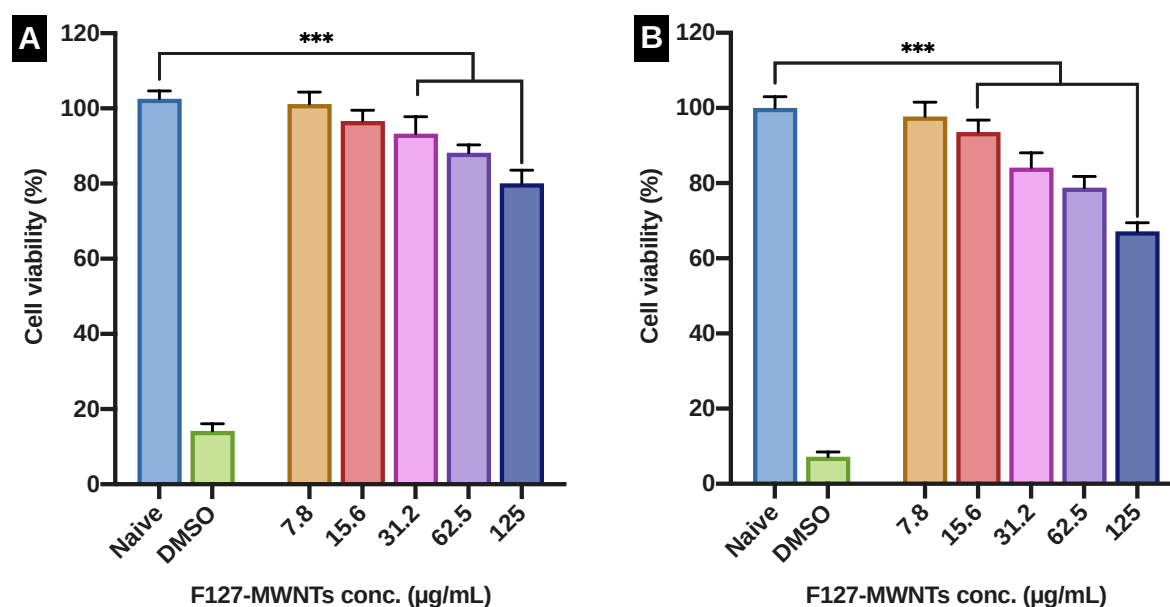


Figure 4.1 | Cytotoxicity of Pluronic F127-coated MWNTs in MCF7 cells. Cells were treated with increasing concentrations of F127-MWNTs for (A) 24h and (B) 48h. DMSO was used as positive control of cell death induction. Percentages represent the mean cell viability of 3 individual replicates ($n=3$) $\pm$ SD. Statistical significance compared to untreated control: *** $p<0.0001$.

Focusing at the 24h time-point (figure 4.1A), 15.6 $\mu\text{g/mL}$ depicted a slight cell death of $3.3\pm 2.8\%$. Nonetheless, it was on the 31.2 to 125 $\mu\text{g/mL}$ range that statistically significant cell death was observed ($p<0.001$), reaching a maximum of $20\pm 3.5\%$ inhibition at the highest concentration. As for the results obtained after 48h (figure 4.1B), a consistent drop in the cell viability as concentrations raised was observed. All treatments from 15.6 $\mu\text{g/mL}$ showed to be statistically

significant ($p < 0.001$); going from $6.4 \pm 3.1\%$ at $15.6 \mu\text{g/mL}$ to $32.7 \pm 2.2\%$ at $125 \mu\text{g/mL}$.

It is relevant to mention that, throughout the experiment, the highest concentration showed cytotoxicity values that exceeded 20% of cell viability. Moreover, $62.5 \mu\text{g/mL}$ caused similar effects after 48h. This is an important factor to consider in order to avoid the use of unsafe concentrations in further experiments.

The second nanomaterial to be evaluated was COOH-MWNTs. In order to determine the cytotoxicity, 2D cultures were treated with 5, 50, 150 and $300 \mu\text{g/mL}$ of carboxyl-MWNTs. After 24h and 48h the cell viability percentage was determined and illustrated in figure 4.2.

Herein, no clear trend over the time was observed. After one day of incubation (figure 4.2A), 5 and $50 \mu\text{g/mL}$ produced minimal cell inhibition with $4.2 \pm 3.6\%$ and $2.9 \pm 3.3\%$ decrease. Then, more concentrated samples showed increased cytotoxicities. $150 \mu\text{g/mL}$ demonstrated to be the most cytotoxic treatment at this time-point, lowering $14.4 \pm 4.2\%$ the cell survival; only 2% higher than that caused by $300 \mu\text{g/mL}$. These two concentrations showed to be statistically significant compared to naïve samples ($p < 0.001$).

On the second day post-treatment (figure 4.2B), only $50 \mu\text{g/mL}$ did not show changes on the cell viability. Besides, even though 5 and $150 \mu\text{g/mL}$ showed

statistically significant cell inhibition, it was discrete, with $5.2 \pm 4\%$ and $4.9 \pm 2.4\%$ respectively. As expected, 300 $\mu\text{g/mL}$ demonstrated the highest effect, causing $16.4 \pm 2.7\%$ of cell death.

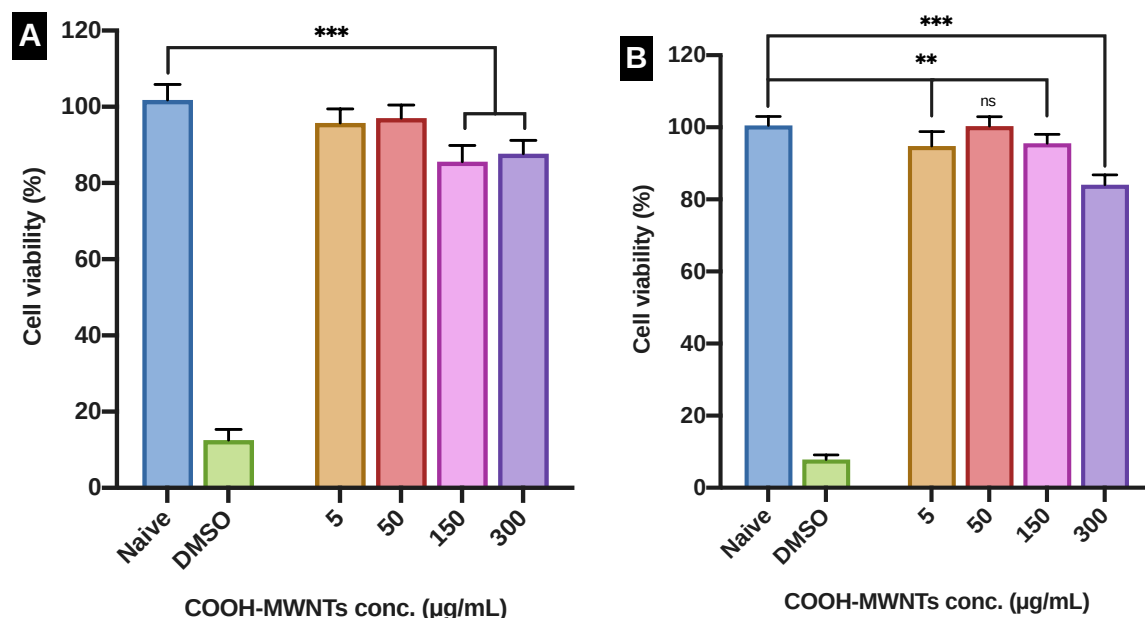


Figure 4.2 | Cell viability of MCF7 cells treated with carboxyl-functionalised MWNTs. Cells were treated with increasing concentrations of COOH-MWNTs for (A) 24h and (B) 48h. DMSO was used as positive control. Percentages represent the mean cell viability of 3 individual replicates ($n=3$) $\pm$ SD. ns=no statistical significance. Statistical significance compared to untreated control: ** $p<0.01$, *** $p<0.001$.

More importantly, the results obtained in this experiment indicated that the cytotoxicity of COOH-MWNTs was within the 20% range of cell inhibition. Suggesting the safety of the used concentrations.

Lastly, the cytotoxicity profile of NH_3^+ -MWNTs was determined by adding the nanoconstructs in concentrations ranging from 10 to 120 $\mu\text{g/mL}$ in 2D cultures for one and two days. The resulting analysis is depicted in figure 4.3.

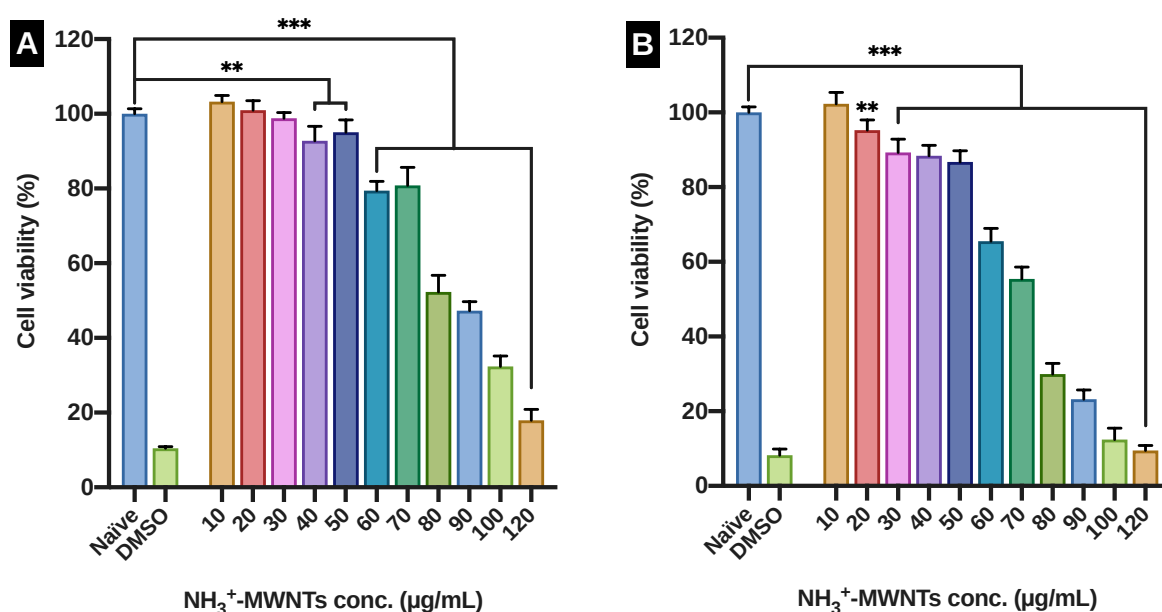


Figure 4.3 | Cell viability of MCF7 cells treated with amine-functionalised MWNTs. Cells were treated with increasing concentrations of NH_3^+ -MWNTs for (A) 24h and (B) 48h. DMSO was used as positive control. Percentages represent the mean cell viability of 3 individual replicates ($n=3$) $\pm$ SD. Statistical significance compared to untreated control: ** $p < 0.01$, *** $p < 0.001$.

A sustained concentration-dependant decline of the cell viability was observed at both time-points. Detailing firstly the results obtained at 24h (figure 4.3A), treatments from 30 $\mu\text{g/mL}$ and lower did not cause noticeable changes on the cell percentage. However, the addition of 40 and 50 $\mu\text{g/mL}$ produced a slight cytotoxic effect ($7.2 \pm 3.8\%$ and $5 \pm 3.2\%$ respectively) that steadily increased with subsequent concentrations, reaching a maximum inhibition of $82 \pm 2.9\%$ at the 120 $\mu\text{g/mL}$ treatment. Secondly, after two days of incubation (figure 4.3B), the range of 10 to 50 $\mu\text{g/mL}$ described a small but maintained inhibition, being no greater than 14%. Nonetheless, 60 $\mu\text{g/mL}$ induced a fall on the viability percentage to $65.5 \pm 2.4\%$. Another major drop was observed at 80 $\mu\text{g/mL}$, diminishing the

viability to $29.9 \pm 2.8\%$. The cytotoxicity peaked at the highest concentration, inhibiting more than 90% of the cells.

The increasing applications of CNTs in the biomedical field have increased the need to unveil the properties and cytotoxicity of the nanomaterial. Various reports have assessed the safety of CNTs in different cellular models. For example, a previous study compared the cytotoxic impact of F127-MWNTs and NH_3^+ -MWNTs; two of the MWNTs tested in this chapter. The analysis concluded that both functionalisations produced good dispersibility. However, the chemically-derivatised MWNTs depicted the greatest cellular uptake of the two. Besides, the coated MWNTs exerted higher cytotoxic responses, attributed to the toxicity of the polymer (470). Another study communicated the NH_3^+ -MWNTs low toxicity even compared to other NPs (363). Similarly, evaluating NH_3^+ -MWNTs and COOH -MWNTs in three different culture models, no significant loss of cell viability was detected (475).

Moreover, previous analysis performed in A549 cells determined that COOH -MWNTs started to show minimal cytotoxicity at $50 \mu\text{g/mL}$, increasing in a concentration-dependant manner at higher concentrations. Nevertheless, COOH -MWNTs were the least toxic nanomaterial compared to pristine and OH -MWNTs (386). Additionally, pristine MWNTs and pluronic-coated COOH -MWNTs were tested in macrophage cultures, resulting in small toxicity from both types of nanotubes (476). Interestingly, intracellular accumulation of COOH -MWNTs were

much higher than the pristine ones, leading authors to suggest the internalisation via receptors (476).

Vittorio et al. provided information about F127-MWNTs and oxidised-MWNTs (477). In vitro analysis concluded that highly pure MWNTs barely affected the cell viability even during prolonged incubation periods (477). On the other hand, 97% purity nanotubes showed significant proliferation inhibition. Confirming the association of MWNTs' purity with the cytotoxicity (477). These findings are consistent our results.

Considering the results of the three types of MWNTs, NH_3^+ -MWNTs demonstrated to cause greater cytotoxicity. The effect could be due to the positive surface charge, granting higher affinity for the cells. Nevertheless, F127-MWNTs showed higher cytotoxicity at low concentrations. In contrast, COOH-MWNTs were the safest nanomaterial of all, even at high concentrations.

Although current literature is inconclusive, previous studies have reported higher cytotoxicities of amine CNTs compared to other types of functionalisation. For example, Li et al., analysed the fibrogenic potential of various functionalised CNTs in different cell lines, concluding that the superficial charge is determinant (478). Particularly, neutral and weak cationic CNTs showed differences in cellular uptake, lysosomal damage, cathepsin B release, and inflammasome activation as compared to anionic CNTs (478). Additionally, the positive charge produces a

moderately hydrophobic surface, compromising the stability of the nanomaterial in aqueous medium (478).

Acute and subchronic toxicity of functionalised SWNTs and MWNTs conducted in male albino mice concluded that the former nanomaterial was slightly more biocompatible than the latter (479). Furthermore, carboxylated CNTs did not show significant systemic toxicity nor histopathological changes, whereas the administration of aminated CNTs was accompanied by an increase in mortality, abnormal behaviour, and moderate affections in liver, lung, and spleen. Such observations were associated with increased ROS levels, lipid peroxidation, and apoptosis induction detected in a time-dependant manner (479). Researchers hypothesised the higher toxicity could be linked with the physical stability of the nanomaterial, particularly, to higher sedimentation rates (479).

In another example, Balas et al. loaded carboplatin into oxidised and aminated MWNTs and evaluated the cytotoxic effect in MDA-MB-231 cells (480). In their results, aminated MWNTs showed a concentration-dependant cytotoxicity as opposed to oxidised MWNTs, which did not show changes compared the untreated control (480). The cytotoxicity was related to an increase in superoxide anion production and decrease of GSH levels, leading to cell death (480).

It is important to consider that apart of functionalisation type, physical characteristics of nanomaterials such as length and purity are determinant factors of cytotoxicity.

4.2.2 Therapeutic evaluation of anticancer drugs

Once the cytotoxicity profile of the three derivatised MWNTs was established, DOX and CDDP, two extensively used anticancer drugs were selected to be complexed with the MWNTs. Therefore, to determine the ideal therapeutic concentrations, the free drugs were tested in the above-mentioned breast cancer model.

Two structurally different anticancer drugs provide the possibility of different interactions and complexation with MWNTs. Having an aromatic structure, DOX has been reported to adsorb to CNTs predominantly by hydrophobic interactions and π - π stacking (329, 347, 481). On the other hand, CDDP has been reported to bind through weaker interactions such as Van der Waals, hydrogen bonds and electrostatic interactions (404, 405). Therefore, the importance of testing and comparing alternative compounds. Furthermore, conjugating the two drugs with three differently functionalised MWNTs present more possibilities to find good therapeutic prospects. The anthracycline was the first drug evaluated. 2D cultures were treated with 3, 6, 12, 24, 48, and 96 μ M for 24h and 48h. After each time-point the mLDH was performed and results were analysed and graphed in figure 4.4.

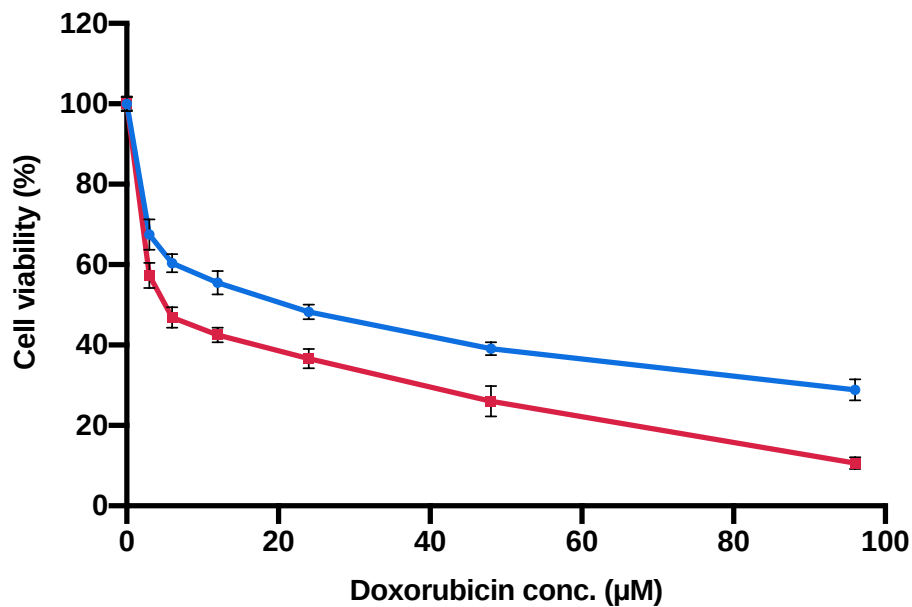


Figure 4.4 | Therapeutic assessment of doxorubicin in 2D cell cultures. MCF7 cells were treated with increasing concentrations of DOX for 24h (● blue) and 48h (■ red). Percentages represent the mean cell viability of 3 individual replicates ($n=3$) $\pm$ SD.

Both 24h (blue line) and 48h (red line) results presented concentration–dependant decreases on the cell viability percentage. Moreover, the patterns followed over the two time-points were parallel. From 3µM to 48µM, the difference of cell death between the two days was 10-14%. The exception was 96µM which caused a cell survival of $28.8 \pm 2.6\%$ at 24h and $10.6 \pm 1.4\%$ after 48h; a difference of 18.2%.

We then continued with the analysis of CDDP; the other antineoplastic drug nominated for this research. Likewise, concentrations of 3, 6, 12, 24, 48, and 96µM of the platinum drug were added and analysed with the same colorimetric method after one and two days. Figure 4.5 depicts the results obtained.

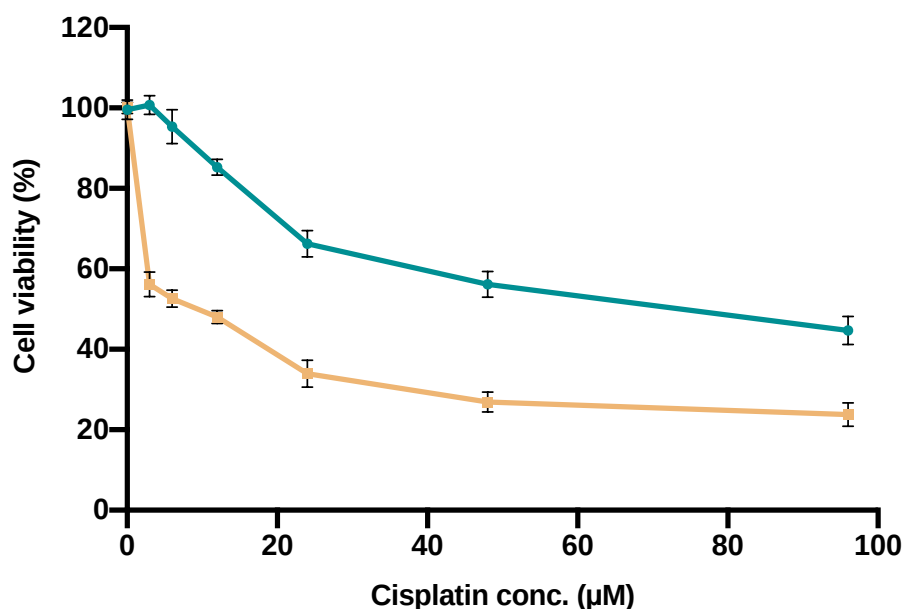


Figure 4.5 | Therapeutic assessment of cisplatin in 2D cell cultures. MCF7 cells were treated with increasing concentrations of CDDP for 24h (● green) and 48h (■ yellow). Percentages represent the mean cell viability of 3 individual replicates ($n=3$) $\pm$ SD.

As estimated, concentration–dependant inhibition profiles were obtained over the whole time of the experiment. Comparing the two time-points, CDDP demonstrated a stronger cytotoxic effect at 48h, especially at low concentrations. At 24h, 3μM had a minor increase in the viability percentage compared to the untreated control. However, this result could be due to cell growth variations. Then, from 6μM, a sustained decrease on the cell survival was observed, starting in $95.3 \pm 4.2\%$ and finishing in $44 \pm 3.5\%$ viability, caused at 96μM.

On the 48h results, even the lowest concentration, 3μM, produced a marked drop of the living cells, killing $43.8 \pm 3\%$. The two following concentrations, 6 and 12μM, produced a lower impact with $47.3 \pm 2.1\%$ and $51.9 \pm 1.5\%$ respectively. Then, the

inhibiting fashion continued with $66\pm3.3\%$ on $24\mu\text{M}$ up to $76.3\pm2.9\%$ at the highest concentration.

The evaluation of both antineoplastic drugs demonstrated high activity against MCF7 cultures, corroborated by other analysis (482). Furthermore, as mentioned before, the two structurally different anticancer drugs bind to MWNTs through a variety of interactions. The interaction type is an important factor not only on the complex formation, but also on the release of the drugs. π - π stacking has been described to produce stronger interactions and thus, slow release, while CDDP interactions are prone to dissociation (394, 397). Therefore, based on the characteristics of MWNTs and drugs, we expected the formation of stable and effective nanocomplexes.

Once the cytotoxic profiles of the three functionalised MWNTs were assessed and the efficacy of the anticancer drugs was determined, we proceeded to prepare combinations among them. In order to develop highly effective and safe DDSs, the effect on cell viability of the nanomaterials and drugs was carefully analysed. Hence, considering concentrations that caused no more than 15% of cell inhibition, we selected 40 and 50 $\mu\text{g/mL}$ of the nanocarriers. Furthermore, designated concentrations of DOX and CDDP were 12, 24 and $48\mu\text{M}$. This range of concentrations would allow the assessment and modulation of cell growth inhibition.

4.2.3 Efficacy evaluation of MWNTs + Doxorubicin

Nanocomplexes were developed by combining F127-coated MWNTs and the two chemically derivatised MWNTs with DOX. Mixtures of different nanomaterial-drug ratios were prepared and evaluated in the 2D breast cancer model to determine the therapeutic efficacy and select promising DDSs.

Block copolymer Pluronic F127-coated MWNTs and DOX was the first combination tested. 40 and 50 $\mu\text{g/mL}$ of nanotubes were mixed with increasing concentrations of the drug and added to cell cultures as well F127-MWNTs and free DOX at same concentrations. After 24h and 48h, the cell viability was determined using a colorimetric assay. Results were graphed in figure 4.6, comparing the efficacy of the combinations to free DOX at equivalent concentrations.

Overall, 24h and 48h results depict a concentration-dependant response from single DOX and F127-MWNTs+DOX combinations. Firstly, results at 24 hours (figure 4.6A) showed a consistent cell viability decrease in free DOX treatments. Going from $71.2 \pm 3.2\%$ at the lowest concentration to $51 \pm 1.5\%$ at $48\mu\text{M}$. About combinations, F127-MWNTs $40\mu\text{g/mL}$ + DOX $12\mu\text{M}$ and F127-MWNTs $40\mu\text{g/mL}$ + DOX $48\mu\text{M}$ showed statistically significant decrease in cell survival of $67.6 \pm 2.2\%$ and 45.5 ± 2.5 respectively compared to DOX at same concentrations. As for combinations with F127-MWNTs at $50\mu\text{g/mL}$, only its mixture with DOX $12\mu\text{M}$ depicted significantly difference to free DOX, causing 10.3% more cell

death. F127-MWNTs 50 $\mu\text{g/mL}$ + DOX 24 μM and F127-MWNTs 50 $\mu\text{g/mL}$ + DOX 48 μM were marginally more efficient than free DOX at the same concentrations.

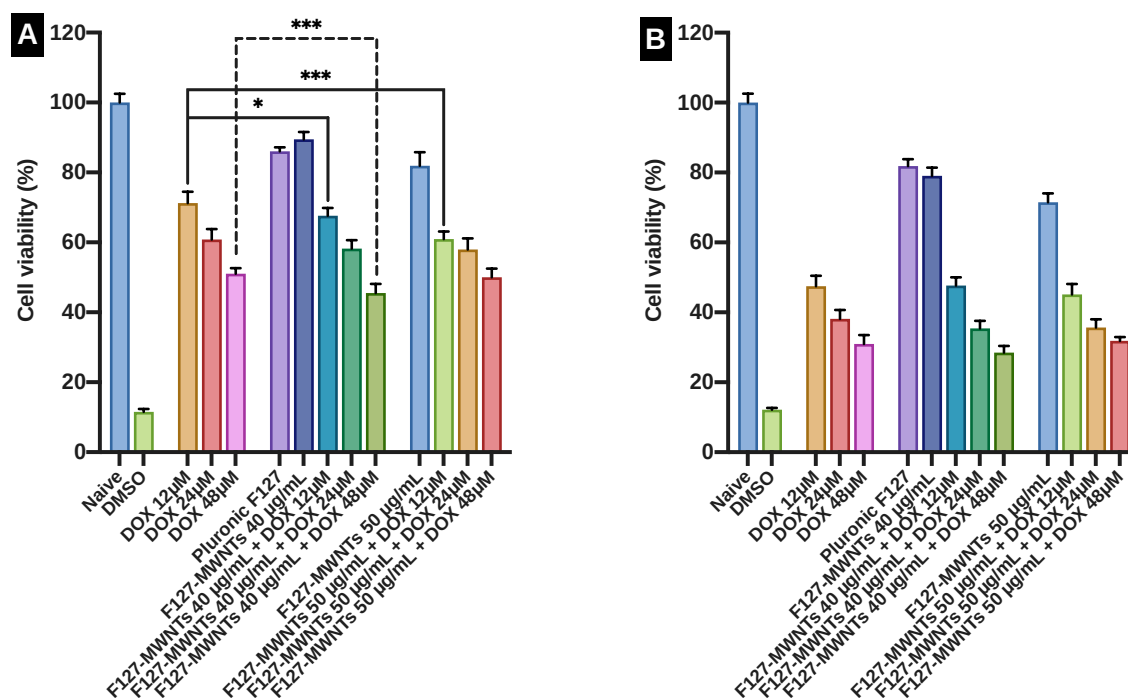


Figure 4.6 | Therapeutic efficacy of Pluronic F127-coated MWNTs and doxorubicin complexes. MCF7 cells were treated with increasing concentrations of F127-MWNTs, free DOX and combinations of these compounds for (A) 24h and (B) 48h. DMSO was used as positive control for cell death induction. Percentages represent the mean cell viability of 3 individual replicates ($n=3$) $\pm$ SD. Statistical significance compared to free DOX at equal concentrations: * $p<0.05$, *** $p<0.001$.

Treating cells with 40 $\mu\text{g/mL}$ of single F127-MWNTs, reduced $10.5\pm 2\%$ the population and 50 $\mu\text{g/mL}$ $18.7\pm 3.8\%$. It is important to note that the addition of Pluronic F127 in solution inhibited the growth of practically the same cell percentage of that observed by F127-MWNTs 50 $\mu\text{g/mL}$. Both treatments contained the same concentration of Pluronic, confirming that the cytotoxicity of the coated nanomaterial is mostly related to the copolymer.

Then, after 48h (figure 4.6B), all treatment groups followed clear concentration-dependant cell death trends. Free DOX had an important impact on the cell viability, diminishing 47.4 ± 2.9 the percentage at lowest concentration and $30.9 \pm 2.5\%$ at the highest one. On the combinations side, even though all combinatory treatments depicted good efficacy, none of them resulted significantly different of the effect calculated by the free drug. Finally, Pluronic F127 in solution produced $81.8 \pm 1.9\%$ cell survival; slightly higher viability percentage than $40 \mu\text{g/mL}$ and considerably higher than $50 \mu\text{g/mL}$, which caused approximately 20% and 30% cell reduction respectively.

Possibly the most relevant study was carried by Ali-Boucetta et al. They developed a non-covalent supramolecular complex comprising F127-MWNTs and DOX via π - π stacking, enhancing significantly the anticancer activity (347). Moreover, other investigations using polymer-coated MWNTs such as PEI-HA-MWNTs were used to load DOX (355). Using fluorescent imaging analysis, the conjugated DOX showed 7-fold higher cell internalisation compared to the free drug, though protein-mediated uptake (355).

Experimentation continued with COOH-MWNTs+DOX complexes. Mixtures were constituted at same concentrations and fashion above-explained. The efficacy assessment performed on 2D MCF7 cultures is illustrated in figure 4.7.

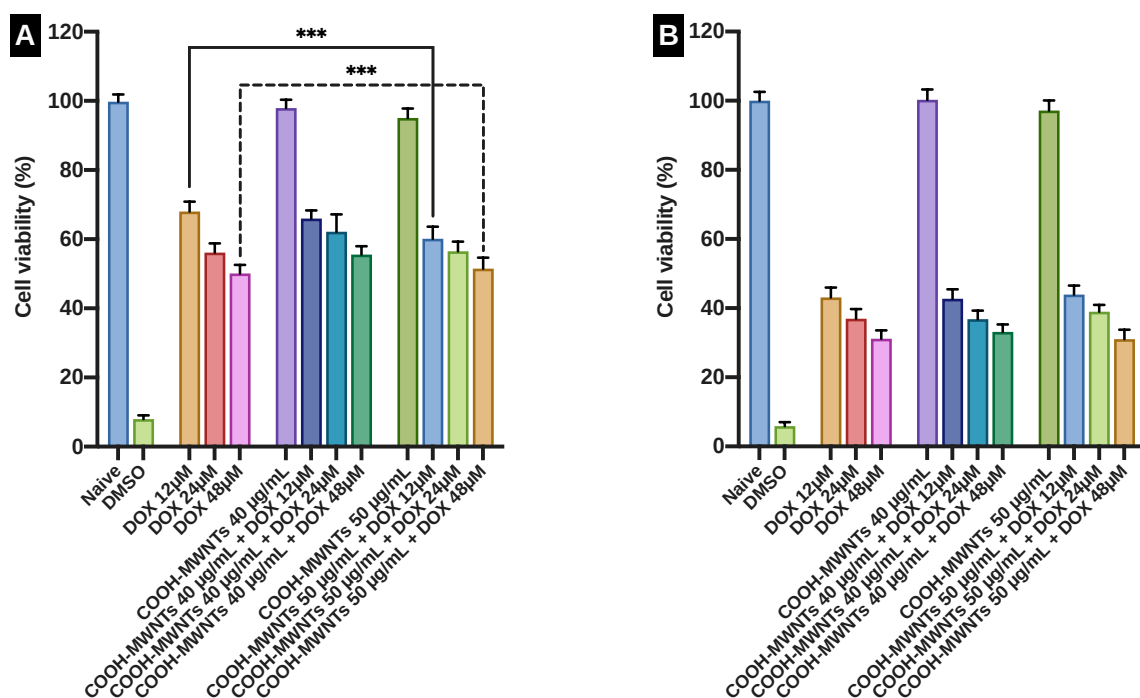


Figure 4.7 | Therapeutic efficacy of carboxyl-functionalised MWNTs and doxorubicin complexes. MCF7 cells were treated with increasing concentrations of COOH-MWNTs, free DOX and combinations of these compounds for (A) 24h and (B) 48h. DMSO was used as positive control for cell death induction. Percentages represent the mean cell viability of 3 individual replicates ($n=3$) $\pm$ SD. Statistical significance compared to free DOX at equal concentrations: *** $p<0.001$.

Both time-points showed concentration-dependant trends in all DOX-containing treatments. Besides, the two concentrations of single COOH-MWNTs tested demonstrated minimal effect on the cell viability.

Results obtained at 24h (figure 4.7A) showed that the three increasing concentrations of free DOX reduced $68 \pm 2.8\%$, $56.1 \pm 2.6\%$ and $50 \pm 2.4\%$ the cell viability respectively. On the other hand, the three combinations of COOH-MWNTs $40 \mu\text{g/mL}$ + DOX did not result in any statistically difference compared to the ones obtained with free DOX at equivalent concentrations; in fact, COOH-MWNTs $40 \mu\text{g/mL}$ + DOX $24\mu\text{M}$ and COOH-MWNTs $40 \mu\text{g/mL}$ + DOX $48\mu\text{M}$

depicted higher cell survival percentages. At higher nanocarrier concentration, only the COOH-MWNTs 50 $\mu\text{g/mL}$ + DOX 12 μM combination described significantly efficacy enhancement compared to 12 μM of free DOX, killing 7.8% more cells.

Then, after 48h (figure 4.7B), even though free DOX and COOH-MWNTs+DOX showed high therapeutic efficacy, when compared at equal concentrations, neither of them showed statistically significant difference. To illustrate, the cell death difference between the free drug and nanocomplexes was never greater than 2%.

In addition to the evaluations performed to combinations based on F127-coated MWNTs and carboxylated MWNTs, NH_3^+ -MWNTs were also complexed with DOX. Assays were performed using identical concentrations and evaluated at same time-points. Resulting calculations are represented in figure 4.8.

Generally speaking, free DOX as well as combinatory treatments exhibited concentration-dependant behaviours. Besides, single NH_3^+ -MWNTs showed significant decrease of the cell viability compared to the untreated control. Same way, all NH_3^+ -MWNTs+DOX combinations proved to be significantly more effective than the drug alone.

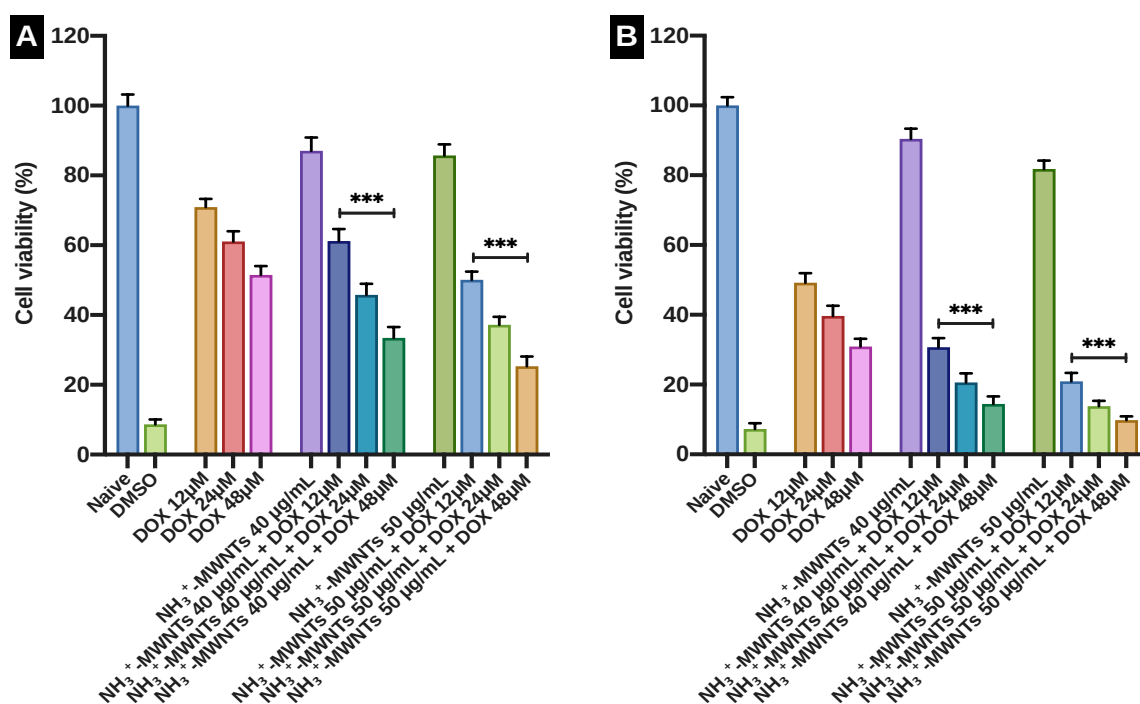


Figure 4.8 | Therapeutic efficacy of amine-functionalised MWNTs and doxorubicin complexes. MCF7 cells were treated with increasing concentrations of NH₃⁺-MWNTs, free DOX and combinations of these compounds for (A) 24h and (B) 48h. DMSO was used as positive control for cell death induction. Percentages represent the mean cell viability of 3 individual replicates (n=3) ± SD. Statistical significance compared to free DOX at equal concentrations: ***p<0.001.

Detailing the results observed at 24h (figure 4.8A), 40 μg/mL of single NH₃⁺-MWNTs induced 12.9±3.7% cell death, while 50 μg/mL was marginally more cytotoxic, producing 14.2±3.1%. Upon adding free DOX, viability percentages were further reduced approximately 10% on each concentration. Cell death went from 29±2.3% to 48.5±2.5%. Considering that all nanocomplexes depicted higher cytotoxicity values than free DOX, it is important to mention that combinations based on 50 μg/mL NH₃⁺-MWNTs were notably the most effective treatments. While NH₃⁺-MWNTs 40 μg/mL + DOX 48μM caused 33.4±3.1% cell death, NH₃⁺-MWNTs 50 μg/mL + DOX 48μM reduced the viability to 25.2±2.7%.

After 48h (figure 4.8B), 40 $\mu\text{g/mL}$ of single nanomaterial displayed a cytotoxicity of $9.5\pm 2.8\%$, whereas the higher concentration had two-fold the effect. As observed previously, the presence of NH_3^+ -MWNTs enhanced the DOX efficacy. Comparing treatments at $12\mu\text{M}$, free DOX showed a cell survival of $49.1\pm 2.7\%$; NH_3^+ -MWNTs 40 $\mu\text{g/mL}$ + DOX $12\mu\text{M}$ presented $30.6\pm 2.5\%$ and NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + DOX $12\mu\text{M}$ had $20.9\pm 2.3\%$ inhibition. Similarly, the most concentrated free DOX treatment resulted in a cell population decrease to $30.9\pm 2.2\%$; NH_3^+ -MWNTs 40 $\mu\text{g/mL}$ + DOX $48\mu\text{M}$ depicted $14.4\pm 2.1\%$ and NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + DOX $48\mu\text{M}$ showed $9.8\pm 1\%$ viability; being the latter, the greatest cell death observed.

DDSs composed of CNTs and DOX have been reported previously. For example, Li et al. developed a nanocarrier integrating MWNTs, FeNPs, DOX and folate for active targeting and using irradiation to activate the release of the drug (483). Furthermore, Liu et al. loaded DOX into PEG-SWNTs by mixing the components for 18h, followed by repeated filtration and washing processes (481). Similarly, another methodology describes the loading of the drug into polymer-coated MWNTs during 48h of incubation accompanied a similar procedure of excess drug elimination (334). DOX has been also covalently attached to CNTs via hydrazone (484) and carbodiimide bonds (485), among others.

Although some of the MWNTs+DOX combinations presented here showed slight in vitro therapeutic advantage compared to free DOX, there are other relevant issues to consider. For example, an important factor is the toxicity reduction as

described by Liu et al (481). In the study, DOX was encapsulated into PEG-SWNTs, proving not only an enhancement of the cytotoxic efficacy, but also a clear toxicity reduction was observed in in vivo studies (481).

In this context, a good example is Doxil, the first-approved nanosystem for the treatment of various human cancers. Although the liposome-encapsulated DOX demonstrated enhanced anticancer activity in preclinical studies (220), a significant increase of the therapeutic efficacy in patients is inconclusive (223). Nevertheless, a marked improvement of the drug's pharmacokinetic parameters (222) and the significant diminish of cardiomyopathy development have been reported (221). Even though current carbon-based DDSs are still in preclinical evaluations, nanosystems under clinical studies and already commercialised have paved the path for a promising future in biomedicine.

Formerly explained, we aimed to develop DDSs based on different functionalised MWNTs conjugated with anticancer compounds and evaluate their therapeutic performance. Therefore, once the in vitro efficacy of MWNTs+DOX was assessed, studies continued with CDDP complexation.

4.2.4 Efficacy evaluation of MWNTs + Cisplatin

The objective was to assess the therapeutic activity of MWNTs+CDDP nanocomplexes, compare results and select the most promising ones. Thus, identical conditions and concentrations to preceding experiments of both

functionalised nanomaterial and CDDP were examined in the 2D breast cancer model.

The studies started using F127-MWNTs physically combined with CDDP. After 24h and 48h incubating cells with treatments, the mLDH assay was performed to determine the final viability percentages, which are depicted in figure 4.9.

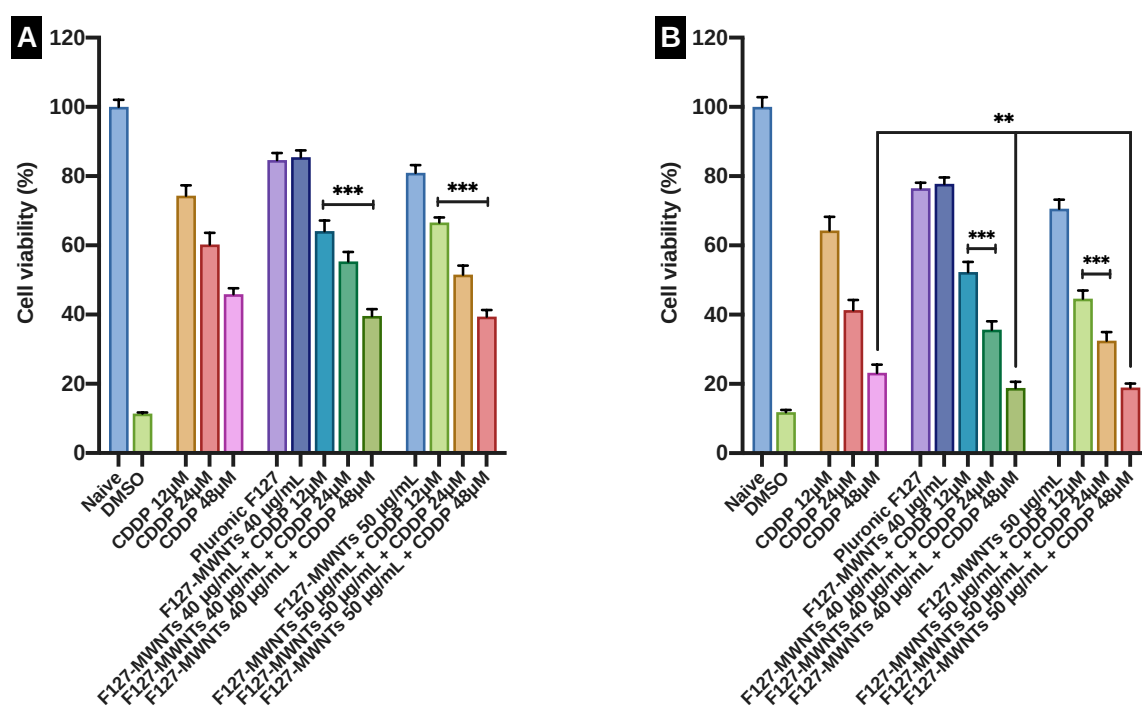


Figure 4.9 | Therapeutic efficacy of Pluronic F127-coated MWNTs and cisplatin complexes. MCF7 cells were treated with increasing concentrations of F127-MWNTs, free CDDP and combinations of these compounds for (A) 24h and (B) 48h. DMSO was used as positive control for cell death induction. Percentages represent the mean cell viability of 3 individual replicates (n=3) $\pm$ SD. Statistical significance compared to free CDDP at equal concentrations: **p<0.01, ***p<0.001.

Overall, a clear concentration-dependant cytotoxic trend was detected in all the treatments in which CDDP was present. Nevertheless, all F127-MWNTs+CDDP combinations depicted significantly higher efficacies than free CDDP at

proportionate concentrations. The addition of single F127-MWNTs resulted again in higher cytotoxicity compared to the untreated control.

Particularly at 24h (figure 4.9A), 40 $\mu\text{g/mL}$ of single F127-MWNTs caused a viability of $85.4 \pm 1.9\%$; almost the same percentage caused by Pluronic alone ($84.6 \pm 2\%$), whereas, 50 $\mu\text{g/mL}$ showed 3.7% further reduction. Free CDDP showed a maximum growth inhibition of $54.1 \pm 1.7\%$. However, F127-MWNTs+CDDP enhanced that effect, killing $64.2 \pm 3\%$ and $66.7 \pm 1.4\%$ of the cell population at F127-MWNTs 40 $\mu\text{g/mL}$ + CDDP 48 μM and F127-MWNTs 50 $\mu\text{g/mL}$ + CDDP 48 μM treatments respectively. Interestingly, these two concentrations depicted a narrow difference, which may suggest the efficacy enhancement is related to the presence of MWNTs rather than the concentration.

Incubating F127-MWNTs for 48h (figure 4.9B) demonstrated cytotoxicities that surpassed 20% of cell death. After analysing 40 and 50 $\mu\text{g/mL}$ of the nanomaterial, $22.2 \pm 1.8\%$ and $29.3 \pm 2.5\%$ of viability reduction was observed respectively. At same time-point, free CDDP ended up triggering increasing cell death rates, ranging from $35.6 \pm 3.9\%$ at the lowest concentration to $76.7 \pm 2.3\%$ at the highest. As for the F127-MWNTs+CDDP combinations, F127-MWNTs 50 $\mu\text{g/mL}$ + CDDP 12 μM showed to be more effective than F127-MWNTs 40 $\mu\text{g/mL}$ + CDDP 12 μM , since the first one caused 7.7% more cell death. Similarly, comparing F127-MWNTs 40 $\mu\text{g/mL}$ + CDDP 48 μM and F127-MWNTs 50 $\mu\text{g/mL}$ + CDDP 48 μM , the latter treatment caused 3.18% more cytotoxicity. However, the rest of the combinations showed to cause the same proportion of cell

inhibition. Important to note is that all combinations proved to be statistically significant when compared to equivalent concentrations of free CDDP.

With high expectations, evaluation continued with COOH-MWNTs complexed with CDDP. Consequently, compounds were combined and tested in the same fashion as previously explained. Single nanomaterial and the free drug were analysed as well. Figure 4.10 illustrates the cell viability calculated after performing the mLDH assay at 24h and 48h post-treatment.

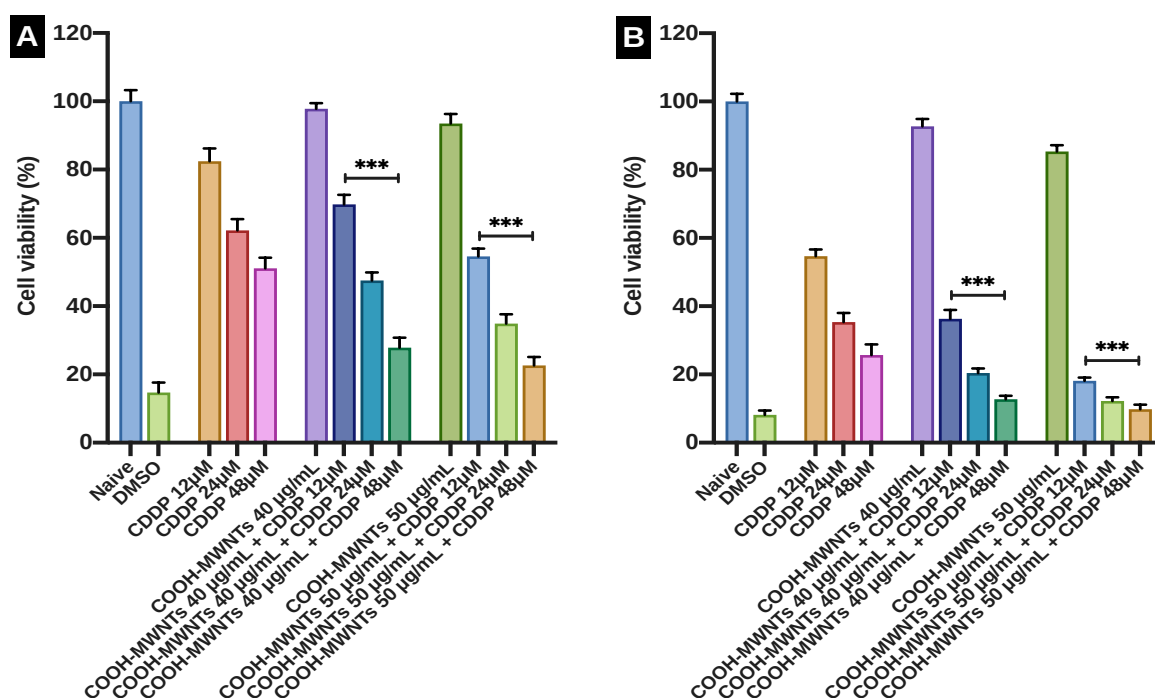


Figure 4.10 | Therapeutic efficacy of carboxyl-functionalised MWNTs and cisplatin complexes. MCF7 cells were treated with increasing concentrations of COOH-MWNTs, free CDDP and combinations of these compounds for (A) 24h and (B) 48h. DMSO was used as positive control for cell death induction. Percentages represent the mean cell viability of 3 individual replicates (n=3) $\pm$ SD. Statistical significance compared to free CDDP at equal concentrations: ***p<0.001.

It was evident that CDDP-containing treatments described consistent concentration-dependant cell inhibition trends. More importantly, all combinatory treatments showed to be statistically significant more effective than free CDDP at equal concentrations. This demonstrates a strong therapeutic activity enhancement in the presence of COOH-MWNTs. Besides, the nanomaterial alone exhibited a low cytotoxicity. Examined concentrations caused less than 7.5% of cell death, with the exception of 50 $\mu\text{g/mL}$ at 48h, which increased to $14.6\pm1.8\%$.

Focusing on the 24h findings (figure 4.10A), minimal cytotoxicities were detected in the presence of single COOH-MWNTs, being $6.5\pm2.6\%$ the greatest. In contrast, drug-containing treatments showed important inhibition levels; however, different efficacies were detected. The free platinum drug caused the lowest therapeutic effect, followed by nanocomplexes based on 40 $\mu\text{g/mL}$ and finally, combinations based on 50 $\mu\text{g/mL}$ depicted the highest efficacy. For instance, 12 μM of free CDDP resulted in 82.4 ± 3.7 cell survival, while COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 12 μM increased that inhibition 1.7 times. More importantly, COOH-MWNTs 50 $\mu\text{g/mL}$ + CDDP 12 μM showed 2.5 times higher effect of that seen in free CDDP 12 μM . Furthermore, free CDDP at 48 μM inhibited half of the cell population. Interestingly, this effect is comparable to COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 24 μM ($52.2\pm2.3\%$) and COOH-MWNTs 50 $\mu\text{g/mL}$ + CDDP 12 μM ($45.4\pm2.2\%$), using half and a quarter of the drug concentration respectively.

In 48h assays (figure 4.10B), single COOH-MWNTs showed statistically significant cytotoxicity compared to the untreated control. However, the concentrations used did not surpassed 15% cell inhibition. At this time-point, nanocomplexes using COOH-MWNTs 50 $\mu\text{g/mL}$ confirmed to cause the greatest therapeutic efficacy of all tested compounds. Free CDDP at lowest concentration cut the cell number close to half (45.3 ± 1.9). Nonetheless, combinatory treatments containing the same concentration of the anticancer drug showed evident higher death rates. COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 12 μM caused a cell viability decrease of $63.6 \pm 2.6\%$, while COOH-MWNTs 50 $\mu\text{g/mL}$ + CDDP 12 μM depicted a remarkable inhibition of $81.8 \pm 2.6\%$. This tendency continued with the rest of the treatments. Outstandingly, though the effect of free CDDP 48 μM was notable ($74.3 \pm 3.1\%$), COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 48 μM triggered the death of $87.2 \pm 0.9\%$ of the plated cells. Nevertheless, similarly to the effect observed at 24h, COOH-MWNTs 50 $\mu\text{g/mL}$ + CDDP 48 μM showed an astounding inhibition percentage of $90.2 \pm 1.3\%$. To picture the enhanced effect of the combinatory treatments, COOH-MWNTs 50 $\mu\text{g/mL}$ combined with 12 μM , the lowest CDDP concentration used, resulted in more than the double cytotoxic efficacy than that produced by free CDDP with 4 times higher the concentration.

The last system tested was the NH_3^+ -MWNTs+CDDP nanocomplex. Therefore, MCF7 2D cultures were treated with either the nanomaterial, free drug, or combinations of these compounds. Resulting measurements from mLDH assay were graphed in figure 4.11.

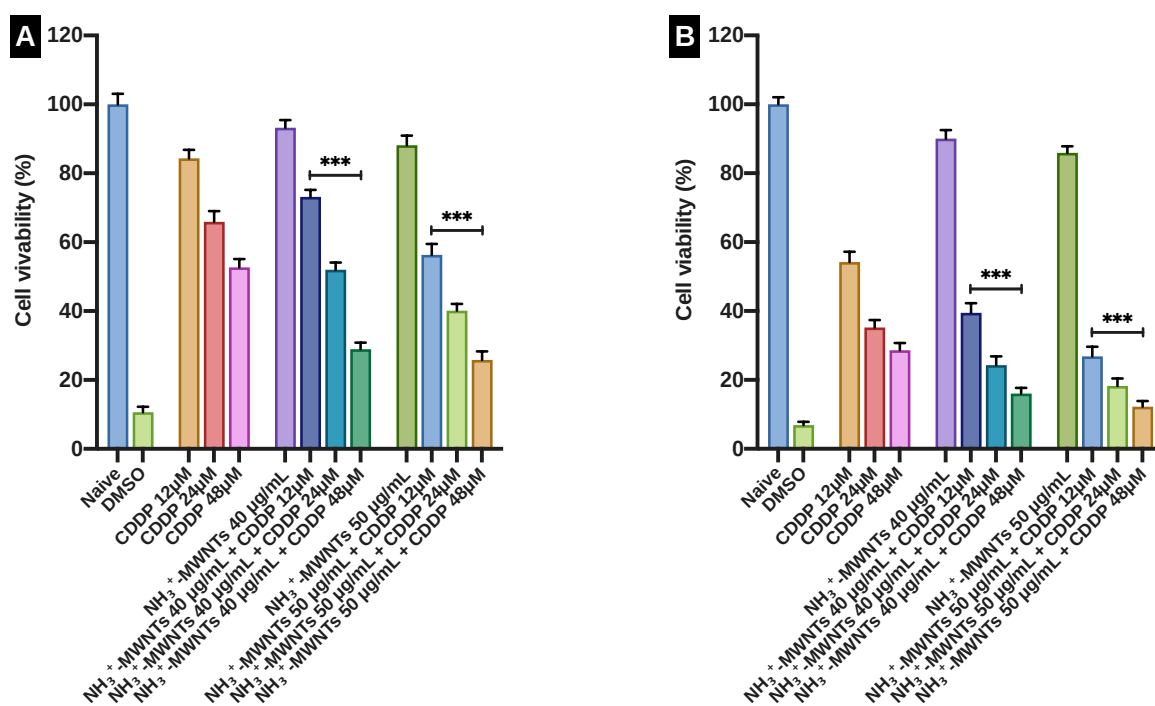


Figure 4.11 | Therapeutic efficacy of amine-functionalised MWNTs and cisplatin complexes. MCF7 cells were treated with increasing concentrations of NH₃⁺-MWNTs, free CDDP and combinations of these compounds for (A) 24h and (B) 48h. DMSO was used as positive control for cell death induction. Percentages represent the mean cell viability of 3 individual replicates (n=3) ± SD. Statistical significance compared to free CDDP at equal concentrations: ***p<0.001.

Obtained values were similar to those observed in COOH-MWNTs+CDDP previous experiments. All compounds followed gradual falls of cell viability. More importantly, all combinatory treatments were significantly more effective than free CDDP at identical concentrations. The nanomaterial alone showed significant cytotoxicity compared to the untreated control. However, none of the tested concentrations exceeded 15% of cell death.

The most important fact to note is the enhanced efficacy that combinatory treatments depicted. For example, on the first day of treatment (figure 4.11A), NH₃⁺-MWNTs 40 μg/mL + CDDP 24μM caused a cell viability decrease by half. However, the same effect was observed with free CDDP at double the

concentration. Remarkably, a similar effect was observed when NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + CDDP 12 μM combination was added to cells, containing the lowest drug concentration. Besides, comparing nanocomplexes, the cell inhibition percentage that NH_3^+ -MWNTs 40 $\mu\text{g/mL}$ + CDDP 24 μM caused was very similar to the obtained with NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + CDDP 12 μM , using only half the CDDP concentration. The most concentrated combination, NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + CDDP 48 μM , reduced the cell population by almost 75%.

Results at 48h (figure 4.11B) described a similar trend to the previous day. The highest concentration of free CDDP caused slightly more than 70% of cell death. However, NH_3^+ -MWNTs 40 $\mu\text{g/mL}$ + CDDP 24 μM and NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + CDDP 12 μM showed alike inhibition rates. Both treatments containing considerably less CDDP concentrations. NH_3^+ -MWNTs 40 $\mu\text{g/mL}$ + 48 μM CDDP diminished $84 \pm 1.6\%$ of the total cells. Likewise, NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + CDDP 24 μM decreased the cell viability only 2% less. The maximum cell death was reached with NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + CDDP 48 μM , reducing $88 \pm 1.6\%$ the cell population.

Even though the complexation of MWNTs with both drugs enhanced their efficacy, combinations with CDDP demonstrated especially strong therapeutic improvement. These findings may suggest that their administration in further studies can potentially diminish side effects associated with CDDP due to possible dose reductions.

Published research on nanoconstructs has focused on the encapsulation of CDDP into CNTs and covalent binding rather than the adsorption. Reports have described extensive loading processes, concentrating on options to retain the drug inside the nanomaterial as well as trigger its liberation (399, 400, 402). Everything considered, we aimed for simple, fast, and reliable complexation methods over the complexity of other protocols.

The weak interactions between CNTs and CDDP are likely to disrupt and dissociate. Computational analysis described that the CNTs+CDDP complexation process is energetically favoured (404) and stabilised by interactions between the ammonia molecules of CDDP and hydrophilic moieties of derivatised CNTs (405). More importantly, evaluations using lipid membranes to mimic the CNTs-CDDP and cell interactions showed that the drug is readily released near the cell membrane as the electrostatic interactions between them increase (404).

Furthermore, PEG- and albumin-dispersed carbon nanofibers and CNTs were found to sensitise several cancer cell lines to CDDP and carboplatin (486, 487). While the cell proliferation was diminished, researchers concluded that not only carbon-based nanomaterials possess anticancer activity, but also potentiate the effect of conventional chemotherapeutics. Such effect has been reported with other NPs like SWNTs and graphene oxide and drugs such as paclitaxel (488).

On the other hand, examining the interaction between oxidised MWNTs and DOX, researchers found that the nanomaterial has high capacity to bind the drug. However, the release process is not only slow, but also pH-dependant. Studies showed that DOX attached to CNTs predominantly at pH higher than 7 (489). In contrast, it hardly detached at pH 7.4 and 5.5, whereas it is readily released at lower pH values (489). Also, an inverse proportion between the CNTs' diameters and the speed release of the drug has been explained; larger diameters slowed the drug release (395, 396).

Pistone et al. developed and assessed nanoconstructs of oxidised MWNTs and PEG-MWNTs with DOX (334). Their studies corroborated the relation of acidic pH on the DOX detachment from MWNTs and proved that the conjugations were temperature-sensitive at more than 40 °C (334). More importantly, investigators concluded that drug loading onto MWNTs and release was a function of the functionalisation type and degree. This may explain the variation of therapeutic efficacies observed on the different nanocomplexes presented herein. Also, it supports the application of CNTs in different therapies such as hyperthermia and the possibility to tune the drug loading and release rate.

A possible explanation of the therapeutic efficacy enhancement could be the increase of intracellular uptake of the drug. Previously, labelled proteins adsorbed to oxidised SWNTs demonstrated to be rapidly internalised into the cell (490). Nanotubes were found inside endosomes and, once released into cytoplasm, attached proteins integrated in biological functions (490).

MWNTs can attach a high number of drug molecules and transport them inside the cell via the nanoneedle mechanism. We hypothesise that the non-energy dependant mechanism of internalisation may facilitate the drug uptake. Inside the cell, the weak interactions of the nanosystem are disrupted due to external forces, releasing the drug. This process may lead to increased intracellular drug concentration and enhanced cytotoxicity.

In conclusion, stable and reliable MWNTs + anticancer drugs systems were developed. Detailed assessments of the components included in all nanocomplexes were performed on a 2D breast cancer model, establishing the cytotoxicity and efficacy profiles. All resulting DDSs proved comparable or greater therapeutic efficacy than the free drugs. Particularly, MWNTs+CDDP combinations demonstrated impressive efficacy enhancement. Limited cytotoxicity was associated with the nanocarrier in tested nanocomplexes. Nevertheless, in order to confirm the observed effects, analysis in complex experimental systems would be convenient. Therefore, the next chapter will evaluate and discuss the therapeutic efficacy of selected nanocomplexes in 3D breast cancer models.

CHAPTER 5

THERAPEUTIC EFFICACY ASSESSMENT OF MWNTS

+ ANTICANCER DRUGS IN 3D MULTICELLULAR SPHEROIDS

5.1 Introduction and summary

The increasing need of physiologically relevant biological models has made researchers to develop tools that can accurately mimic cellular mechanisms and in vivo tissues. In this search, 3D multicellular spheroids, have attracted enormous attention due to their advantages over conventional monolayer cultures.

The interest in 3D cultures has exponentially increased since the first publication in 1968 (491). Opposite to 2D cultures, where cells are adhered to a plastic surface and directly exposed to media, multidimensional cultures possess intricate cell arrangements embedded in extracellular matrix. While preserving in vivo phenotypic, genotypic and metabolic characteristics, cells integrate complex

dynamic interactions (492, 493). Therefore, spheroids are considered the bridge between in vitro and in vivo models (494).

The simulated microenvironment of 3D models have showed advances in the study of biological processes such as cell morphology, proliferation, differentiation, migration and invasion, metabolism and gene expression (492, 495). Some important advantages of three-dimensional systems are the possibility to design and incorporate different cell types into specific models, spatial and temporal cellular development and accurate in vivo simulations, which may result in the reduction of animal experimentation (454, 496, 497).

Nowadays, spheroids have vast scientific applications, being cancer research the field that most frequently uses them. Multicellular tumour spheroids (MCTS) are the most attractive 3D platform for cancer models. These biological systems replicate important features of solid tumours like structural organisation (necrotic, quiescent and proliferative zones), radio- and chemotherapy limited penetration as well as nutrients, gases and pH gradients (498-500).

MCTS are regularly used in imaging techniques (501, 502), radiotherapy and hyperthermia (503, 504), antigen expression and immunotherapy (505, 506), and drug discovery (507-509). Particularly in nanomedicine, MCTS are suitable to evaluate the biosafety and efficacy of DDSs by means of properties optimisation, cellular uptake and mapping, drug delivery and NP-mediated imaging, among others (510-512). Often, DDSs that displayed favourable results in 2D systems,

fail to reproduce those responses in more complex systems; thus, MCTS represent a valuable option of assessment (513).

For the purposes of this chapter, breast cancer MCTS, also known as mammospheres, were developed using the liquid overlay method, originally developed by Ivascu and Kubbies (514). Thereafter, the three-dimensional model was used to evaluate the therapeutic activity of chemically functionalised MWNTs + anticancer drug combinations, measuring spheroids' growth ratio and cytotoxicity using various techniques.

In the former chapter, 2D cell cultures were used to assess the cytotoxicity of F127-MWNTs, COOH-MWNTs and NH_3^+ -MWNTs. Results showed that the F127-coated MWNTs were less biocompatible than their covalent-functionalised counterparts, which might limit their use as DDS. In contrast, COOH-MWNTs and NH_3^+ -MWNTs + drugs complexes showed promising therapeutic efficacy. Therefore, F12-MWNTs and its combinations were discarded, continuing the experiments with carboxylated and aminated MWNTs in the 3D platform. Comparative analysis of biocompatibility and therapeutic efficacy of treatments will be presented.

5.2 Results and discussion

5.2.1 Therapeutic efficacy assessment of MWNTs + doxorubicin

Further studies were needed to validate the anticancer effects of nanocomplexes observed in 2D cell cultures. Therefore, an economical and reliable method to generate MCF7 cell-derived MCTS was established. The technique proved consistency on the formation of compact, uniform-sized spheroids. In previous evaluations, MCF7 MCTS demonstrated advanced-stage cancer phenotype, including reduced ER and E-cadherin expression as well as superior cell migration, angiogenic potential and drug resistance (515); characteristics associated with poor prognosis and metastasis. Thus, MCTS are excellent platforms for drug testing that may predict in vivo outcome.

Herein, the anticancer activity of the free drugs, and nanocomplexes as well as the cytotoxicity of the nanomaterials were tested in the 3D cell system. To determine the efficacy of treatments, the circumference, shape, circularity and integrity of MCTS were visually evaluated from microphotographs. Hence, the growth inhibition 24h and 48 after the onset of the experiment, was calculated. Besides, careful analysis of structural damage was performed. The most effective treatments were those that produced greater growth inhibition.

Optical microscopy is one of the most commonly used end-point methods to evaluate and validate therapeutic compounds (511). MCTS are regularly used to

assess the efficacy of anticancer therapies, associating size variations to determine the growth inhibition and structural injury (516, 517).

The first experiment performed included individually generated MCF7 MCTS treated with free DOX, single COOH-MWNTs, and combinations of these compounds. In order to compare the efficacy of compounds between 2D and 3D cell models, same concentrations as in last chapter were tested. Briefly, DOX was added at 12 μ M, 24 μ M and 48 μ M, while the concentrations of nanotubes used were 40 and 50 μ g/mL.

Microphotographs of MCTS obtained at 24h and 48h post treatment addition are illustrated in figure 5.1. Images depict morphological changes of untreated spheroids, those tested with the highest concentrations of free DOX, COOH-MWNTs, and the combinations COOH-MWNTs 40 μ g/mL + DOX 48 μ M and COOH-MWNTs 50 μ g/mL + DOX 48 μ M to demonstrate the effect of each treatment.

The technique presented herein to develop MCF7 MCTS produced well-defined, compact spherical structures (figure 5.1 A1-A2). Our 3D model comply with the spheroid classification of 3D breast cancer cultures based on morphological characteristics of more than 40 different experimental methodologies performed by Froehlich et al (518). Additionally, spheroids formed with the same number of MCF7 cells and incubation periods as ours have been reported with analogous measurements and characteristics as those illustrated here (519).

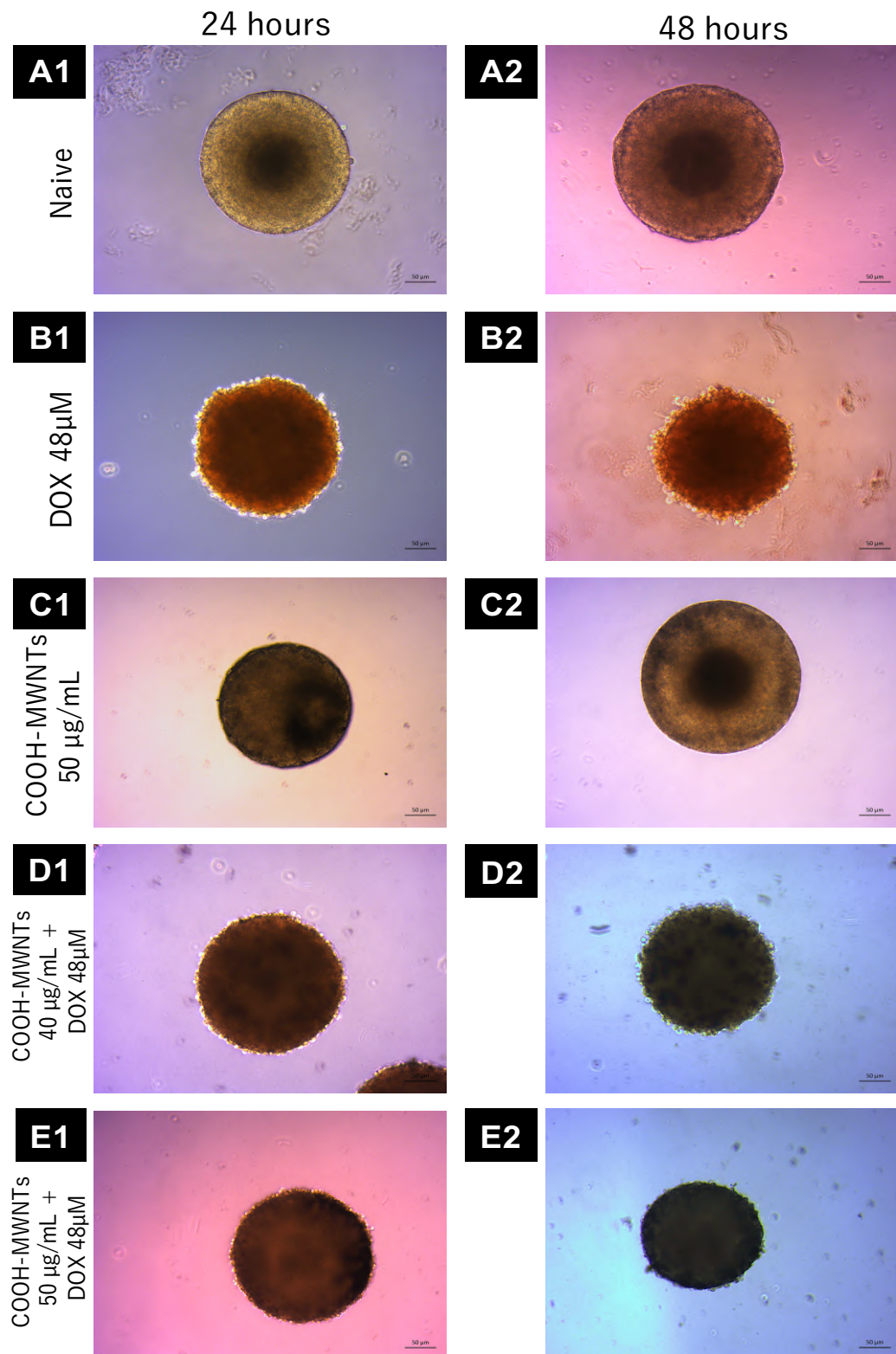


Figure 5.1 | Microphotographs of MCTS treated with carboxyl-functionalised MWNTs + doxorubicin complexes. Images of MCF7 MCTS depicting: (A) untreated control, (B) free DOX, (C) single COOH-MWNTs, (D) COOH-MWNTs 40μg/mL + DOX 48μM complex, and (E) COOH-MWNTs 50 μg/mL + DOX 48μM complex for (1) 24h and (2) 48h post-treatment.

The addition of single COOH-MWNTs showed no sign of rim damage or effects over the growth (figure 5.1 C1-C2). A time-dependant growth rate was observed in both analyses. In contrast, free DOX caused a clear damage to MCTS' rim and growth inhibition with greater effects at longer exposure periods (figure 5.1 B1-B2). Spheroids were coloured in orange tones, product of the interaction with the drug. Likewise, nanocomplexes showed similar effects on the rim and size, being more noticeable after 48h (figure 5.1 D1-E2). COOH-MWNTs+DOX combinations showed comparable size reductions to free DOX; however, the latter seemed to cause greater cellular damage.

Microphotographs were then used to determine the therapeutic efficacy of the treatments. Thus, the growth ratio was calculated recording the circumference of each MCTS immediately before the addition of treatments and after each time-point. Measurements were graphed in figure 5.2, showing the effect of treatments at 24 and 48h.

While overall inhibition patterns are similar to those observed in the 2D cultures, these showed to be less effective. Herein, free DOX demonstrated to be equally or more effective than the nanocomplexes. Moreover, inhibition trends at both time-points were very similar, suggesting slow effects. Important as well is the fact that treatments showed to produce limited efficacy and differences between them. To exemplify, after 24h (figure 5.2A), free DOX 12 μ M was the only

treatment that showed significant inhibition compared to other DOX-containing treatments at equal concentrations.

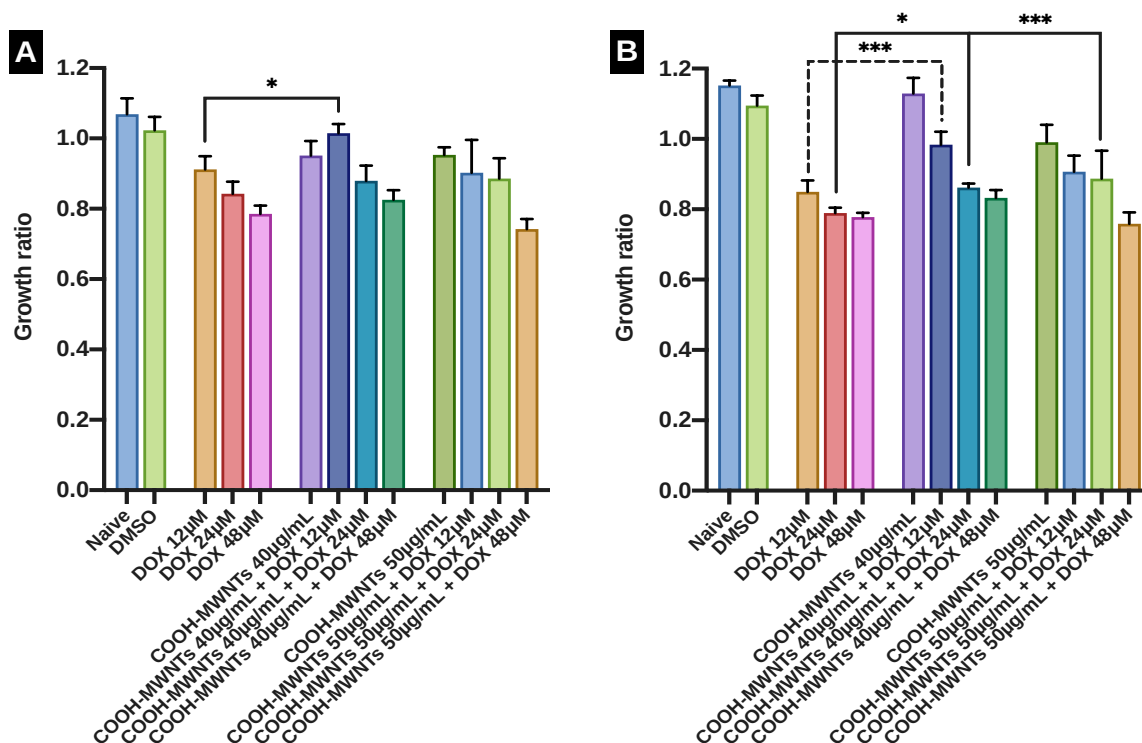


Figure 5.2 | Therapeutic efficacy of carboxyl-functionalised MWNTs + doxorubicin complexes in MCTS. Growth ratio of MCF7 MCTS treated with increasing concentrations of free DOX, single COOH-MWNTs and COOH-MWNTs+DOX for (A) 24h and (B) 48h. DMSO was used as positive control. Values represent the mean growth ratio of 3 individual experiments (n=3) $\pm$ SD. Each assessment considered at least 8 spheroids per treatment. Statistical significance compared to free DOX at equal concentrations: *p<0.05.

At this time-point, COOH-MWNTs 50 μ g/mL + DOX 48 μ M depicted the greatest growth reduction with 0.74 ± 0.02 ; a marginal decrease compared to free DOX 48 μ M. As for single nanomaterial, both concentrations caused a marginal growth decrease; just 0.1 times less growth than the untreated control.

Similar results were obtained after 48h (figure 5.2B). Free DOX and combinations showed a concentration-dependant growth inhibition. Besides, ratio difference

among treatments was modest as well; for example, the highest effect observed of a DOX-containing treatment was only 0.14 greater than the least effective treatment. All free DOX concentrations showed to be more efficient than combinations at equal drug concentrations. COOH-MWNTs+DOX combinations based on 40 µg/mL were the treatments with the lowest efficacies observed. To illustrate this, COOH-MWNTs 40 µg/mL + DOX 48µM (0.83) showed almost the same ratio reduction as that observed with free DOX 12µM (0.84). Untreated MCTS and those treated with single COOH-MWNTs 40 µg/mL showed ratio increases of 1.15 and 1.12 respectively, while 50 µg/mL showed no noticeable change.

In previous sections, the free drug and nanocomplexes demonstrated the ability to cause structural and morphological changes in MCTS as well as to diminish the growth of 3D cell cultures. Nonetheless, in the search of determining the cell fate in such spheroids, the percentage cell viability and cell death process were studied using fluorescent techniques.

Cell death is the irreparable damage that leads to irreversible failure of vital mechanisms (520). According to the morphological process, it is classified into apoptosis, necrosis and autophagy (521). While the two first are the major forms, apoptosis is the most frequent death pathway (522). Also defined as programmed cell death, apoptosis is an organised, sequential process, characterised by chromatin condensation, blebbing, cell shrinkage, chromosomal degradation, nuclear fragmentation and cellular components resorption with limited

inflammation and extracellular leakage (523, 524). Regarding necrosis, it is a rapid, accidental event caused by an external trauma. Associated with a severe metabolic failure, necrosis is characterised by inflammation, vacuole formation in the cytoplasm, nuclear membrane disruption and cell lysis (523, 525, 526).

Cells undergoing apoptosis present several structural and biochemical features that can be measured, depending on the apoptosis trigger, cell type and particular step of the process at which the analysis is executed (527). In this context, flow cytometry techniques allow the performance of multiparametric assessments based on characteristic biomarkers of cell death.

One of the most important apoptosis markers is phosphatidylserine, which is translocated from the inner leaflet of the cell membrane to the cytoplasmic surface upon apoptosis onset (527). The exposure of the aminophospholipid can be accurately estimated using the flow cytometry-based Annexin-V assay. The probe contains the Annexin-V protein conjugated with FITC (or alternative dyes) that will fluoresce upon binding with phosphatidylserine, and the red-fluorescent dye PI. Excluded from membrane-intact cells, PI specifically intercalates between the DNA bases of permeable, necrotic cells. Both late apoptosis and early necrosis are Annexin-V/PI-double positive, making their discrimination difficult. In order to differentiate the stages, a detailed time-course analysis is necessary plus validating assays such as caspase assays (528).

Therefore, using the same concentrations already detailed, MCTS treated during 48h were disaggregated into a single-cell dispersion. Then, cells were labelled with Annexin-V-FITC/PI and analysed using a flow cytometer. Cells were classified based on their labelling: Annexin-V-FITC(-)/PI(-) were considered viable; Annexin-V-FITC(+)/PI(-) were determined undergoing early apoptosis, while Annexin-V-FITC(+)/PI(+) and Annexin-V-FITC(-)/PI(+) were categorised as late apoptosis/necrosis. At least 10,000 events per sample were quantified in each individual experiment (n=3) and illustrated in figure 5.3.

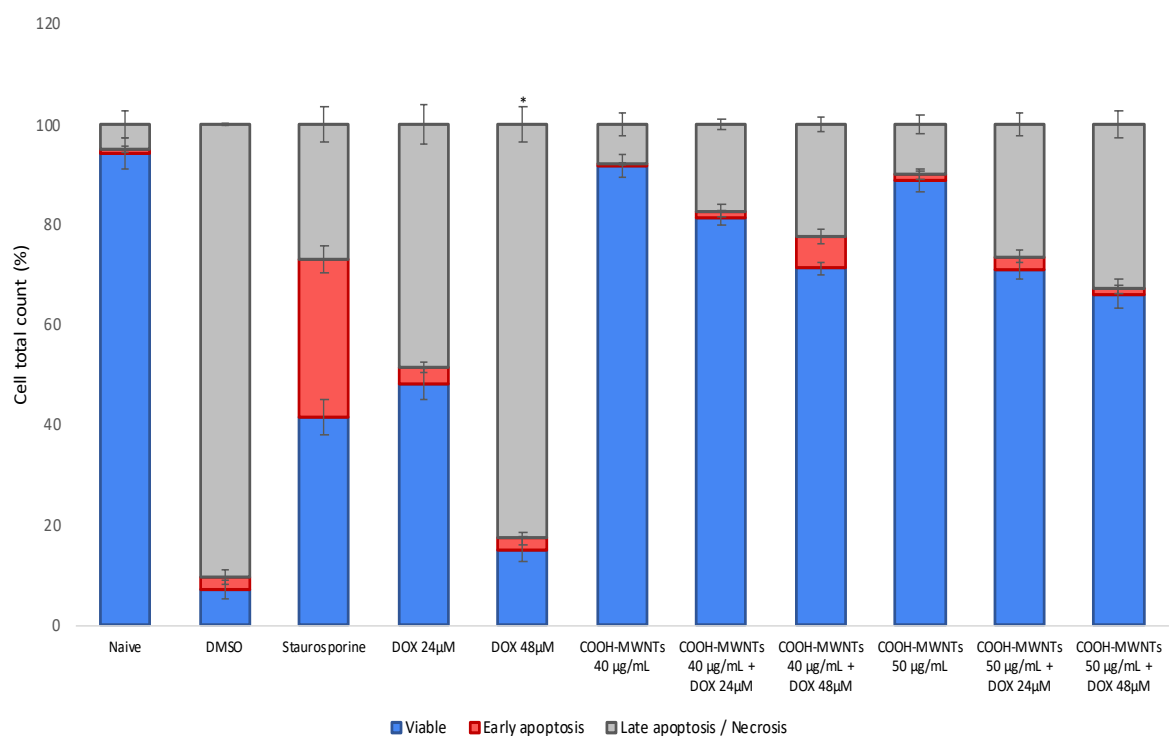


Figure 5.3 | Cell death assessment of carboxyl-functionalised MWNTs + doxorubicin complexes in Annexin-V-FITC/PI labelled MCTS. MCF7 spheroids treated with increasing concentrations of free DOX, single COOH-MWNTs and COOH-MWNTs+DOX were disaggregated and fluorescently stained. Staurosporine and DMSO were used as positive controls of early apoptosis and late apoptosis/necrosis respectively. Percentages of viable cells (**blue**), early apoptosis (**red**), and late apoptosis/necrosis (**grey**) are depicted in each bar. Values represent the mean of 10,000 events (n=3) $\pm$ SD. Statistical significance compared to free DOX at equal concentrations: *p<0.05.

The examination of cell death demonstrated that an elevated percentage of cells contained in untreated MCTS was viable; only $5\pm 2.5\%$ of them were in late apoptosis/necrosis stages. Likewise, single COOH-MWNTs 40 and 50 $\mu\text{g/mL}$ presented minor cytotoxicity rates with $7.9\pm 2.3\%$ and $10\pm 1.8\%$ respectively.

These proportions of necrotic cells were probably associated with the necrotic core present in MCTS. As mentioned previously, MCTS present three zones depending on the accessibility of oxygen and nutrients: necrotic, hypoxic or quiescent and proliferating. All zones have important differences worth studying; while the necrotic layers are product of starvation, the low availability of glucose and oxygen lead cells to produce growth factors that have been related with angiogenesis (529, 530). It has been described that heterogeneous cell organisation present in MCTS and solid tumours is partially responsible of the impaired activity of anticancer drugs. For instance, the lack of oxygen may diminish the efficacy of ROS-generating chemotherapeutics like DOX and CDDP (517).

Contrarily, all DOX-containing treatments showed to cause concentration-dependant cell death. Thus, confirming previous findings, both free DOX 24 μM and 48 μM showed the highest cytotoxicities among all treatments. Causing mostly late apoptosis/necrosis, their percentages were superior than any combination added. Free DOX 24 μM inhibited 51.8% of the total population, whereas DOX 48 μM reduced the viable cells 84.8%, of which $82.6\pm 3.5\%$ underwent late apoptosis/necrosis. On the nanocomplexes side, COOH-MWNTs

50 µg/mL + DOX 48µM depicted the cytotoxicity peak of all combinations, inhibiting a total of 34% of the cells. COOH-MWNTs 40 µg/mL + DOX 48µM showed to cause $6.3 \pm 1.3\%$ of early apoptosis, being the highest percentage of early apoptosis, which indicated that at 48h post-treatment the cells had gone either directly to necrosis or most probably had surpassed early apoptosis. Evidence suggest that apoptosis can switch to necrosis under certain conditions, for example, when cellular energy levels are rapidly depleted or under redox state imbalance (531).

Following the same protocol, experimentation continued with MCTS treated with free DOX, NH_3^+ -MWNTs, and complexes of both compounds. Microphotographs were obtained of at least 8 individual spheroids per sample to compare their physical characteristics and morphological changes (figure 5.4).

Upon analysis, untreated and single NH_3^+ -MWNTs 50 µg/mL samples showed increasing ratios (figure 5.4 A1-A2 and C1-C2), both depicting intact rims, and compact structures. On the other hand, the rest of the treatments that include DOX inhibited the spheroids' growth, showing a similar orange tone due to the colour of the drug (figure 5.4 B1-B2 and D1-E2). Furthermore, all these treatments depicted damaged structures with loose integrity and detached cells surrounding the main cellular body. The effect was evidently greater after two days of treatment, particularly in MCTS treated with the nanocomplexes.

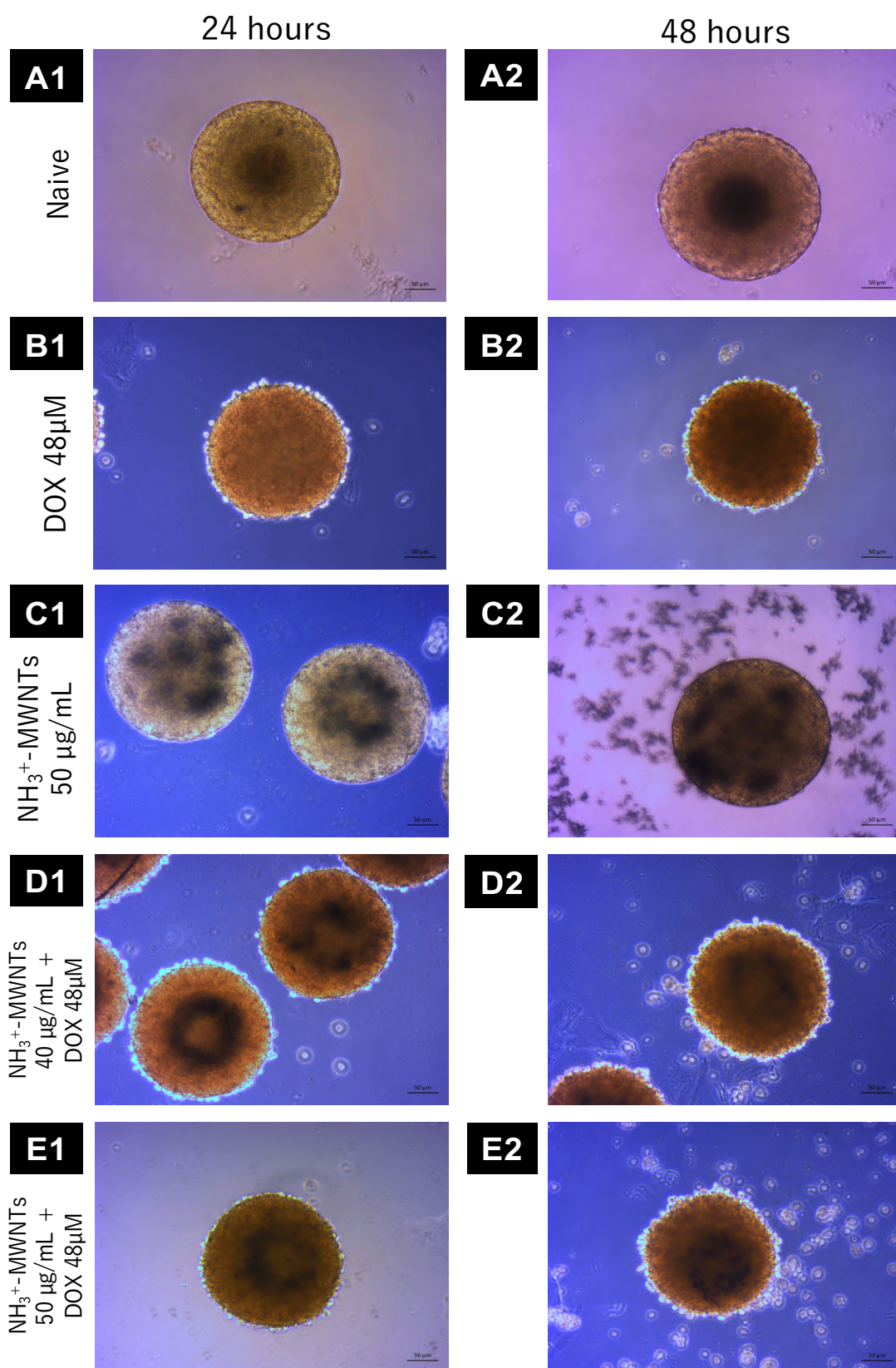


Figure 5.4 | Microphotographs of MCTS treated with amine-functionalised MWNTs + doxorubicin complexes. Images of MCF7 MCTS depicting: (A) untreated control, (B) free DOX, (C) single NH₃⁺-MWNTs, (D) NH₃⁺-MWNTs 40 μg/mL + DOX 48μM complex, and (E) NH₃⁺-MWNTs 50 μg/mL + DOX 48μM complex for (1) 24h and (2) 48h post-treatment.

Subsequently, circumference measurements were recorded previous to the addition of treatments and after 24h and 48h. Then, growth ratios of each group were calculated, graphed and compared in figure 5.5.

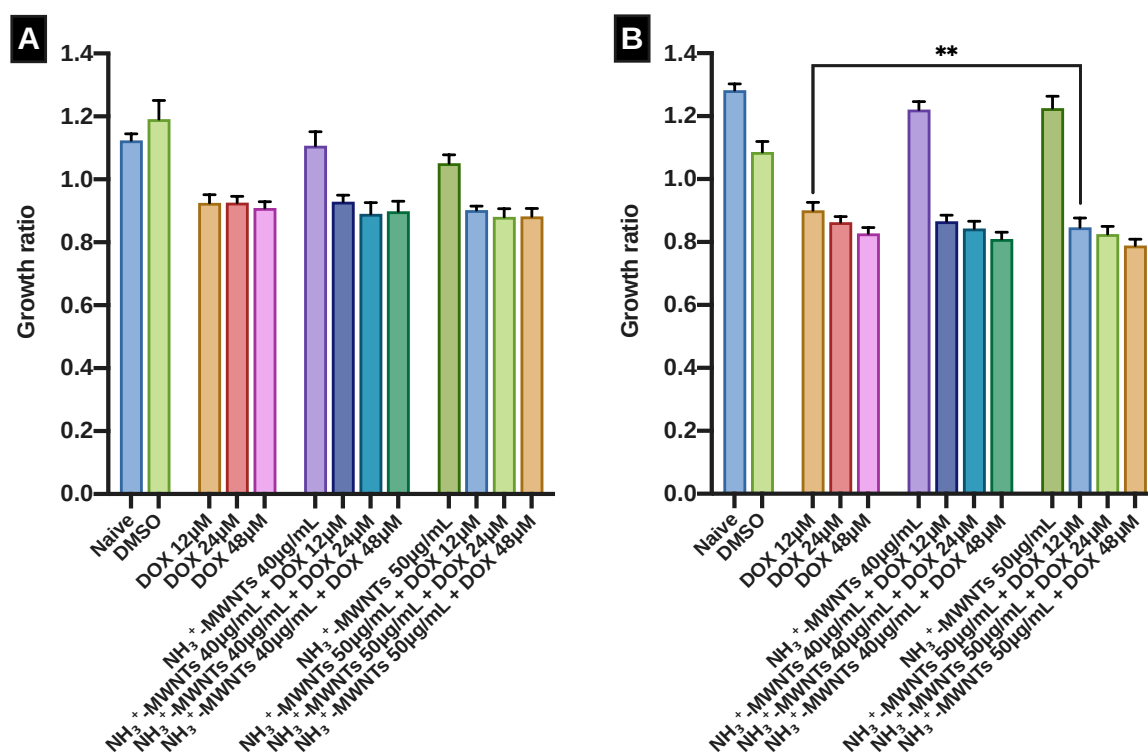


Figure 5.5 | Therapeutic efficacy of amine-functionalised MWNTs + doxorubicin complexes in MCTS. Growth ratio of MCF7 MCTS treated with increasing concentrations of free DOX, single NH₃⁺-MWNTs and NH₃⁺-MWNTs+DOX complexes for (A) 24h and (B) 48h. DMSO was used as positive control. Values represent the mean growth ratio of 3 individual experiments (n=3) ± SD. Each assessment considered at least 8 spheroids per treatment. Statistical significance compared to free DOX at equal concentrations: *p<0.05.

This experiment corroborated the observed in the microphotographs: only untreated MCTS and those treated with single NH₃⁺-MWNTs followed growing rates, while free DOX and combinatory treatments showed inhibitory effects. However, efficacies of DOX-containing treatments did not differ much as no observable statistically significant changes were detected at 24h (figure 5.5A).

For example, NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + DOX 24 μM produced the greatest growth inhibition (0.88 ± 0.02), whereas NH_3^+ -MWNTs 40 $\mu\text{g/mL}$ + DOX 12 μM showed the lowest (0.92 ± 0.02) with a proportion difference of only 0.04.

Free DOX treatments showed to be the weakest of these experiments with an inhibition peak at 48 μM of 0.9 ± 0.02 . Importantly, the effect was slightly greater to that produced by NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + DOX 12 μM , containing four times less drug concentration. Nanocomplexes based on NH_3^+ -MWNTs 40 and 50 $\mu\text{g/mL}$ described analogous growth ratios when compared at same drug concentration.

On the second time-point evaluation (figure 5.5B), all treatments containing DOX followed concentration-dependant growth inhibitions. Free DOX demonstrated the least efficient treatment, showing a maximum inhibition of 0.82 ± 0.01 at highest concentration. In contrast, NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + DOX 48 μM combination caused the greatest ratio reduction with 0.78 ± 0.02 . Nonetheless, only NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + DOX 12 μM showed to be significantly different to free DOX at equal concentration. NH_3^+ -MWNTs+DOX combinations based on 40 $\mu\text{g/mL}$ proved higher inhibitory activity than free DOX, but marginally lower than complexes of 50 $\mu\text{g/mL}$.

We then moved with the cytotoxicity assessment of the testing compounds. Figure 5.6 presents percentages of viability and death type of disaggregated cells from MCTS.

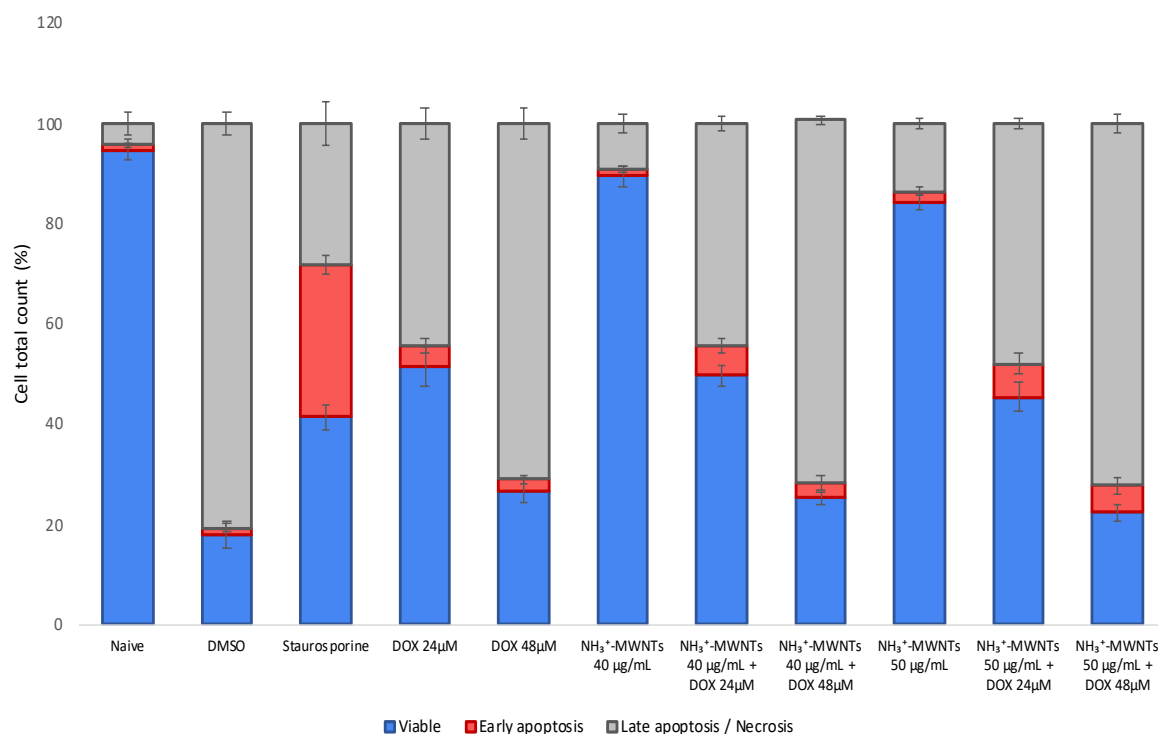


Figure 5.6 | Cell death assessment of amine-functionalised MWNTs + doxorubicin complexes in Annexin-V-FITC/PI labelled MCTS. MCF7 spheroids treated with increasing concentrations of free DOX, single NH₃⁺-MWNTs and NH₃⁺-MWNTs+DOX were disaggregated and fluorescently stained. Staurosporine and DMSO were used as positive controls of early apoptosis and late apoptosis/necrosis respectively. Percentages of viable cells (**blue**), early apoptosis (**red**), and late apoptosis/necrosis (**grey**) are depicted in each bar. Values represent the mean of 10,000 events (n=3) ± SD.

Fluorescence-based evaluation supported our previous findings on 2D cell cultures. First, even though the nanomaterial alone depicted a small degree of cytotoxicity, mainly necrosis, the effect did not surpass 16% of cell death.

Secondly, the concentration of the anthracycline drug was associated to the cell inhibition extent. Interestingly, all treatments at equivalent concentrations depicted alike results, particularly in the late apoptosis/necrosis proportions, which were predominant cell death type. To illustrate this, free DOX 48µM caused

71±3% of late apoptosis/necrosis; at same DOX concentration, NH₃⁺-MWNTs 40 µg/mL + DOX 48µM inhibited, via the same mechanism, 72.2±0.8% of the cell population. Finally, NH₃⁺-MWNTs 50 µg/mL + DOX 48µM demonstrated practically the same effect with 72.3±2%. Due to the modest difference between the treatments, no statistical significance was observed. About early apoptosis, differences depicted among treatments were minor as well. Percentages ranged from 2.1±0.6% obtained from MCTS treated with free DOX 48µM, to a maximum of 6.5±2.8%, result of the NH₃⁺-MWNTs 40 µg/mL + DOX 24µM treatment.

HA-functionalised amino MWNTs loaded with DOX displayed significant volume inhibition in MDA-MB-231 spheroids and managed to break the structures into pieces after 5 days of treatment (532). The construct increased the rate of apoptotic cells; however, free DOX doubled the percentage (532). Other CNTs-nanocomplexes depicted to cause cell death through necrosis, such as DOX-loaded folic-acid-conjugated MWNTs, which significantly inhibited the growth of HeLa cells via necrosis (533).

Even though the objective was to use equal concentrations as those tested before, the complexity of 3D systems may reduce the drug uptake and efficacy. A higher chemoresistance in MCTS compared to traditional cultures has been widely studied (534-536). For example, DOX IC₅₀ values were found to be approximately 50-fold higher in 3D MCF7 models compared to traditional cultures; concentrations were relative to the spheroids' size (519). Furthermore, studies have proved that disaggregated cells from MCTS are also more resistant

to drugs (537). In a relevant example, Nunes et al. demonstrated that disaggregated MCF7 spheroids and subsequently 2D-cultured cells were significantly more resistant than regular MCF7 cultures (538). Importantly, MCTS depicted even higher resistance to such monolayer derived-cultures. Other researchers found that MCF7 2D and 3D cultures presented similar sensitivity to DOX and paclitaxel (539). However, having generated different mammospheres and obtained loose MCF7 MCTS, authors explained that the spheroid's density is intimately related to the drug resistance and hypoxia development (539). It is important to state that the MCTS developed in this work are densely integrated.

Deficient drug penetration into MCTS and solid tumours is one of the main reasons of therapy failing and an urgent issue to tackle. Considering the resemble in extracellular microenvironment and diffusion impediment between the clinical and in vitro model, Wang et al assessed the diffusion of free DOX using fluorescence microscopy techniques (540). Previous results showed that during the first 4h, the drug can only reach superficial layers and approximately 70µm after 8h. Nonetheless, DOX-loaded chitosan NPs were detected in the centre of the spheroids at short time-periods (540). A similar analysis, which included the generation of MCF7 spheroids plating the same number of cells as ours, reported a penetration into the multicellular structure of 100µm in comparable time periods as ours (519). Since vasculature is not present in in vitro models, the increased internalisation of NPs may be associated with facilitated cell transport and receptor mediated uptake.

5.2.2 Therapeutic efficacy assessment of MWNTs + cisplatin

Following the therapeutic assessment of free DOX and combinations with functionalised MWNTs on MCTS was determined, we continued the investigation using CDDP as active compound. Hence, MCF7 MCTS were tested with free CDDP, COOH-MWNTs and complexes as DDSs. The concentrations used of the drug were 12 μ M, 24 μ M, and 48 μ M, while the nanomaterial was added at 40 and 50 μ g/mL; both compounds were also mixed at same concentrations.

Physical evaluation of treated spheroids was performed using microphotographs captured 24h and 48h after the onset of the experiment (figure 5.7). Herein, untreated controls depicted compact, well-defined cellular structures (figure 5.7 A1-A2). They showed a marked growth particularly after 48h. Equally, single COOH-MWNTs did not cause any apparent damage to the spheroids (figure 5.7 C1-C2). The rim seemed well preserved and cells compactly together.

The opposite effect was observed in CDDP-containing treatments, where growth inhibition and damage to MCTS were observed. In this context, free CDDP 48 μ M demonstrated to harm the cells of the outer layer at both time-points (figure 5.7 B1-B2). However, while 24h MCTS (figure 5.7B1) conserved defined shapes, 48h ones lost circularity, with evident damaged and smaller spheroids, suggesting a time-dependant effect. Nonetheless, the addition of COOH-MWNTS+CDDP combinations revealed higher damage than free CDDP (figure 5.7 D-E); they seemed to be able to penetrate deeper into the MCTS.

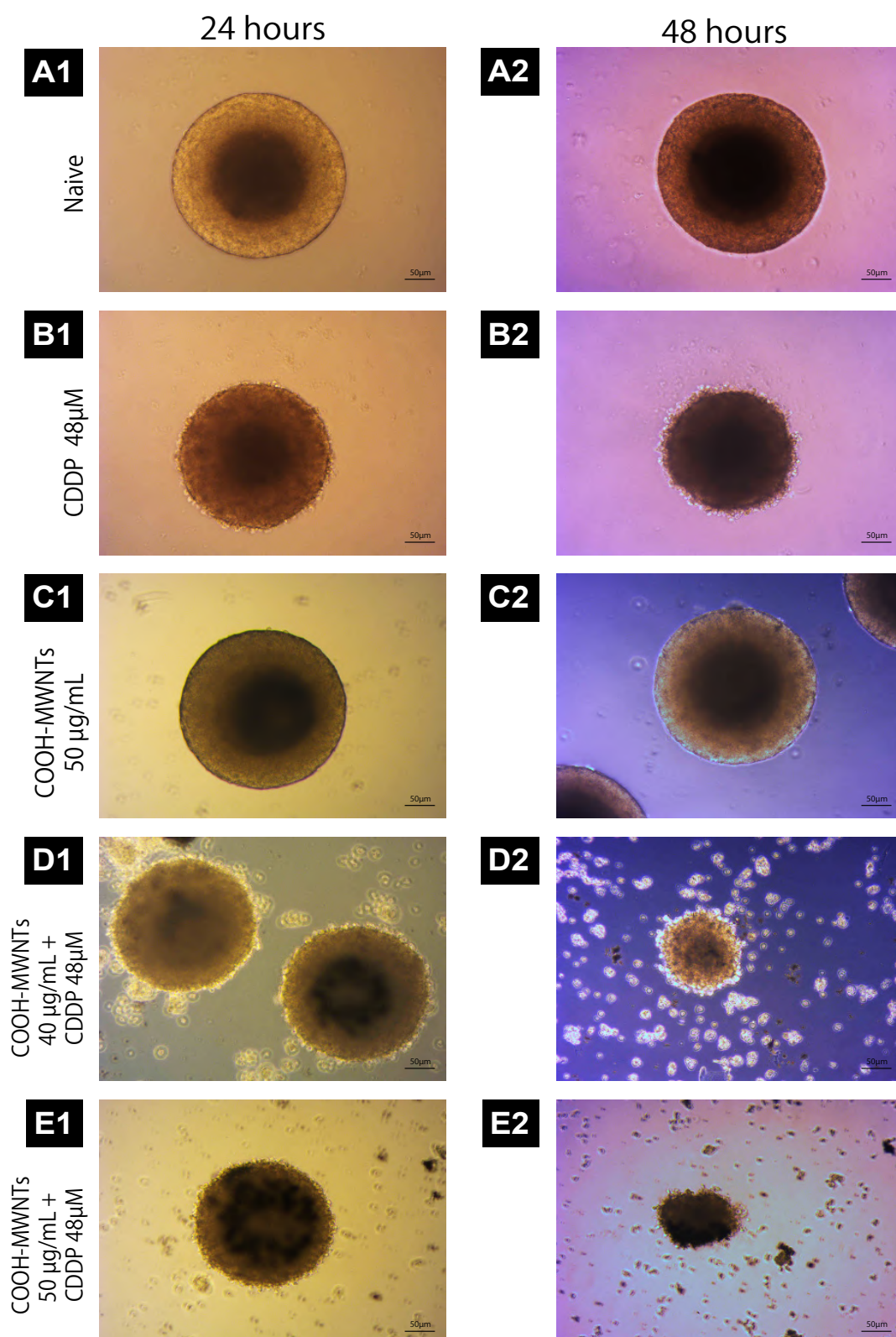


Figure 5.7 | Microphotographs of MCTS treated with carboxyl-functionalised MWNTs + cisplatin complexes. Images of MCF7 MCTS depicting: (A) untreated control, (B) free CDDP, (C) single COOH-MWNTs, (D) COOH-MWNTs 40 µg/mL + CDDP 48µM complex, and (E) COOH-MWNTs 50 µg/mL + CDDP 48µM complex for (1) 24h and (2) 48h post-treatment.

Furthermore, detached rounded cells, presumably dead, were found surrounding these MCTS. Then, most surprisingly, results obtained at 48h were exceptional. A major effect was observed in MCTS treated with COOH-MWNTs 40 µg/mL + CDDP 48µM and COOH-MWNTs 50 µg/mL + CDDP 48µM, disrupting the MCTS structures, detaching an important number of cells and reducing the size to a great extent (figure 5.7D2 and E2). Besides, the morphology and sphericity changed completely, resulting in cell aggregates. Some spheroids were fractured into big cellular aggregates and floating rounded cells, depicting an unparalleled effect.

The visual analysis was followed by growth inhibition evaluations, correlating the circumference of individually measured MCTS before the addition of the treatments and after 24h and 48h of testing (figure 5.8).

Overall, concentration-dependant trends were observed in all treatments containing CDDP. Besides, the growth inhibition was more pronounced after 48h, indicating a time-dependant effect. Relatable to the analysis of microphotographs, all complexed treatments depicted smaller ratios than free CDDP, demonstrating greater efficacy at identical concentrations. In terms of the nanomaterial, the concentrations used did not show to cause relevant effects. Though 50 µg/mL depicted a lower ratio than the untreated control, MCTS continued growing during the experiment, confirming biosafety to be used for DDS purposes. The effect of single MWNTs over the MCTS' growth presented in

this work agree with other reports. For example, the evaluation of oxidised MWNTs at equal concentrations as ours in colon adenocarcinoma spheroids showed an increase in the median volume (485). Similar studies demonstrated that 50 $\mu\text{g/mL}$ of CNTs produced a cytostatic effect on MCF7 MCTS, whereas higher concentration motivated their growth (541).

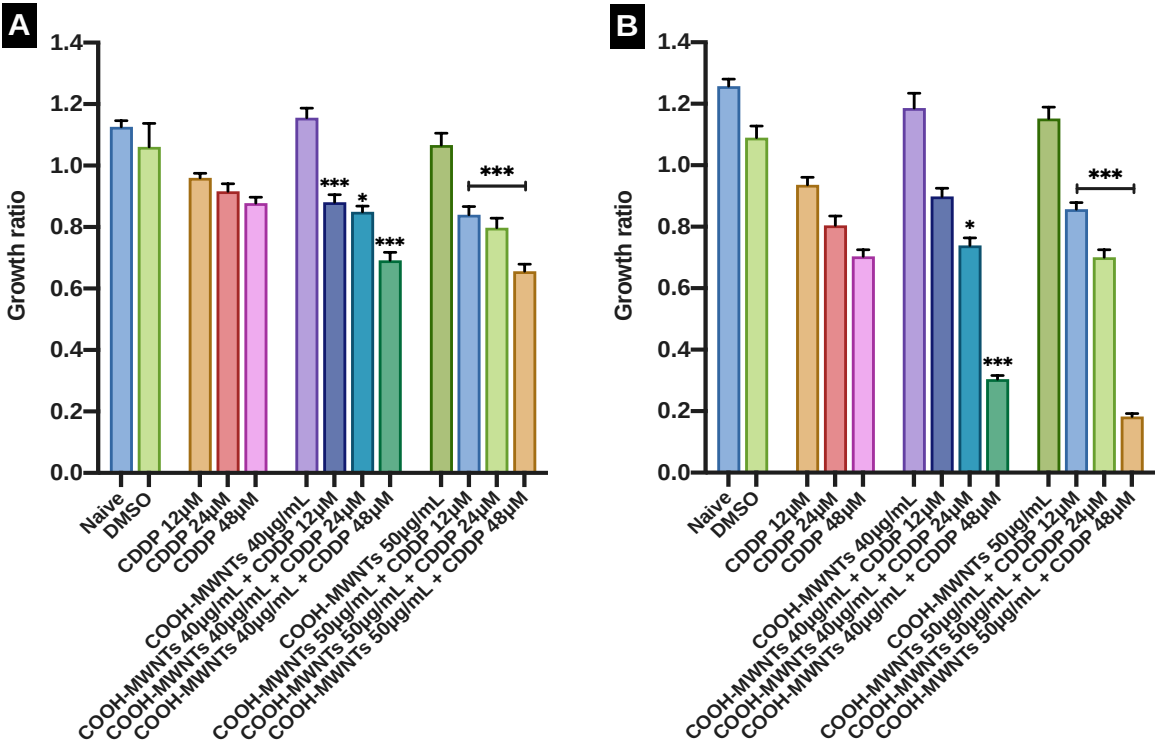


Figure 5.8 | Therapeutic efficacy of carboxyl-functionalised MWNTs + cisplatin complexes in MCTS. Growth ratio of MCF7 MCTS treated with increasing concentrations of free CDDP, single COOH-MWNTs and COOH-MWNTs+CDDP for (A) 24h and (B) 48h. DMSO was used as positive control. Values represent the mean growth ratio of 3 individual experiments ($n=3$) $\pm$ SD. Each assessment considered at least 8 spheroids per treatment. Statistical significance compared to free CDDP at equal concentrations: ** $p<0.01$, *** $p<0.001$.

Regarding 24h evaluations (figure 5.8A), although free CDDP showed a consistent growth decline, the effect was rather modest. To illustrate, the growth ratio difference between the lowest and highest concentrations of free drug was only 0.0824. Similar results were observed with COOH-MWNTs 40 $\mu\text{g/mL}$ +

CDDP 12 μ M and COOH-MWNTs 40 μ g/mL + CDDP 24 μ M, having a small difference of 0.0315. However, it is important to note that the size inhibition that COOH-MWNTs 40 μ g/mL + CDDP 12 μ M produced (0.88 ± 0.02) was practically the same as that obtained with free CDDP 48 μ M (0.87 ± 0.01). More significantly, COOH-MWNTs 40 μ g/mL + CDDP 48 μ M decreased the initial MCTS' average circumference more than 30%; being more the double effect of free CDDP at equal concentration. With respect to the combinations with COOH-MWNTs 50 μ g/mL, all demonstrated the best efficacies of all treatments. COOH-MWNTs 50 μ g/mL + CDDP 12 μ M was slightly more effective at inhibiting the MCTS' growth than the highest concentration of free CDDP, using 4 times less drug concentration. Even comparing between combinations, COOH-MWNTs 50 μ g/mL + CDDP 48 μ M showed higher efficacy than COOH-MWNTs 40 μ g/mL + CDDP 48 μ M.

The next section of the analysis was the results obtained at 48h (figure 5.8B). Generally speaking, although free CDDP demonstrated important decreases of MCTS' average size, it was the least efficacious treatment compared to the nanocomplexes. To exemplify, free CDDP 48 μ M, the most concentrated treatment, depicted a comparable growth ratio (0.7 ± 0.02) to those observed with COOH-MWNTs 40 μ g/mL + CDDP 24 μ M (0.73 ± 0.02) and COOH-MWNTs 50 μ g/mL + CDDP 24 μ M (0.7 ± 0.02). These combinations produced analogous results with half the concentration of drug.

COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 48 μM and COOH-MWNTs 50 $\mu\text{g/mL}$ + CDDP 48 μM showed remarkable growth ratios reductions. The former treatment demonstrated a reduction of about 70% of the original size, while the latter treatment diminished the initial measurements more than 80%. Based on the results, COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 48 μM was ~ 2.3 times more effective than free CDDP, whereas COOH-MWNTs 50 $\mu\text{g/mL}$ + CDDP 48 μM was ~ 3.8 times more.

The therapeutic evaluation continued with the analysis of cell death type. For this purpose, fluorescence-labelled cells were assayed to determine the percentages of viability, early apoptosis and late apoptosis/necrosis.

Results in figure 5.9 contrast the high percentage of viable cells present in single COOH-MWNTs-treated samples with the elevated necrotic cell populations in CDDP-treated samples. On the single COOH-MWNTs side, both concentrations had almost identical results; a large population of viable cells representing more than 94%, 4.5% of necrosis and less than 1% of early apoptosis. Since untreated controls showed virtually the same results, the percentage of necrosis could be attributed to the necrotic core developed in MCTS.

On the other hand, all CDDP-containing treatments, particularly the nanocomplexes, showed outstanding cell inhibitions, mainly thorough late apoptosis/necrosis. Corroborating past findings, free CDDP showed to be the least effective treatment compared to the combinations. The free drug at 24 μM

resulted in $65.1 \pm 3.7\%$ of cell viability and $33 \pm 3.5\%$ of late apoptosis/necrosis, whereas $48 \mu\text{M}$ reduced the cell population to half, causing $47.6 \pm 2.1\%$ of late apoptotic/necrotic cells. More importantly, among all nanocomplexes, COOH-MWNTs $40 \mu\text{g/mL}$ + CDDP $24 \mu\text{M}$ showed the lowest efficacy; nevertheless, it caused 9.9% more cell death than the most efficient free CDDP treatment, demonstrating superior cell inhibition.

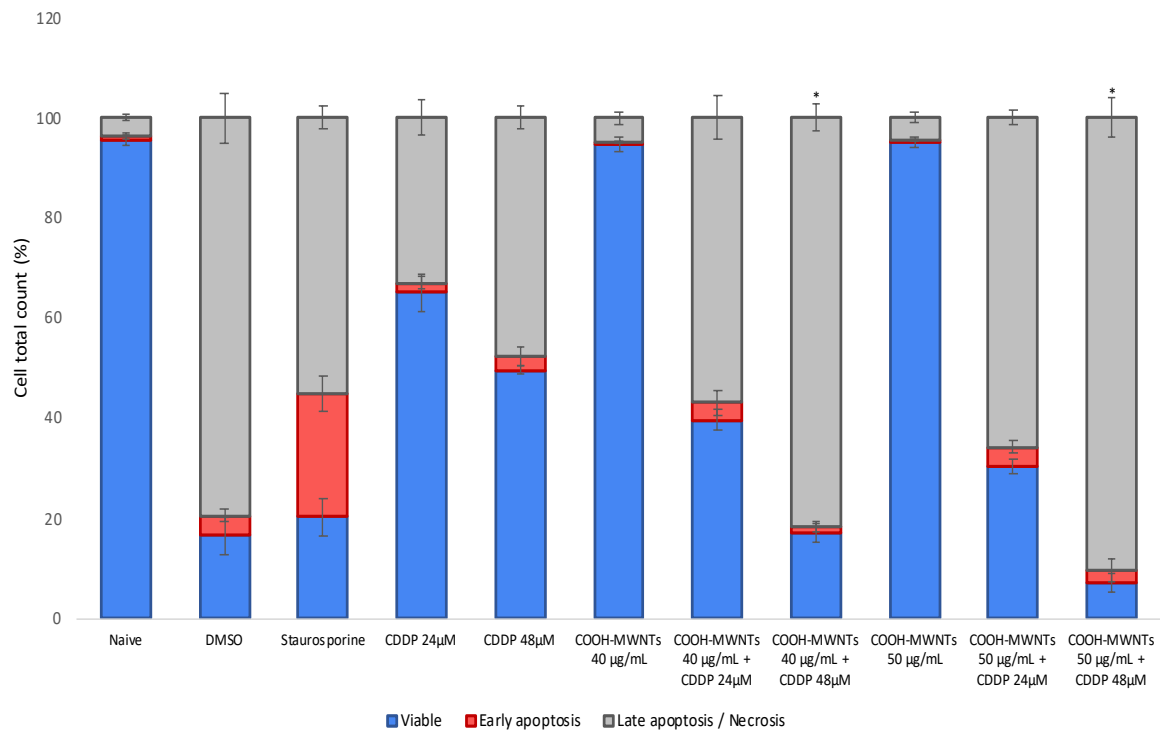


Figure 5.9 | Cell death assessment of carboxyl-functionalised MWNTs + cisplatin complexes in Annexin-V-FITC/PI labelled MCTS. MCF7 spheroids treated with increasing concentrations of free CDDP, single COOH-MWNTs and COOH-MWNTs+CDDP were disaggregated and fluorescently stained. Staurosporine and DMSO were used as positive controls of early apoptosis and late apoptosis/necrosis respectively. Percentages of viable cells (blue), early apoptosis (red), and late apoptosis/necrosis (grey) are depicted in each bar. Values represent the mean of 10,000 events ($n=3$) $\pm$ SD. Statistical significance compared to free CDDP at equal concentrations: * $p<0.05$.

Comparing combinations, COOH-MWNTs $40 \mu\text{g/mL}$ + CDDP $48 \mu\text{M}$ showed an impressive $81.5 \pm 2.8\%$ of late apoptotic/necrotic cells. However, COOH-MWNTs

50 µg/mL + CDDP 48µM managed to improve that result with an outstanding 90.4±4% of late apoptosis/necrosis and 2.5±2.2% of early apoptosis. These values depicted an efficacy enhancement of almost the double compared to free CDDP at equivalent concentration. Furthermore, these findings were in line to those described previously and confirmed to produce better results than the MWNTs+DOX combination.

The last combination to be analysed was the NH₃⁺-MWNTs+CDDP complex. Initially, microphotographs of MCTS treated with increasing concentrations of the free drug, nanomaterial and combinations were obtained. Then, a detailed evaluation of their physical characteristics was performed after 24h and 48h of incubation with treatments (figure 5.10).

Herein, the appearance of untreated MCTS and those tested with single NH₃⁺-MWNTs were in line with previous analysis. Spheroids depicted intact, compact structures with defined necrotic cores (figure 5.10 A1-A2 and C1-C2).

Of utmost importance is the fact that all MCTS treated with MWNTs presented dark spots distributed along the structures. We hypothesise that such observations are the internalisation of MWNTs into the multicellular assemblies.

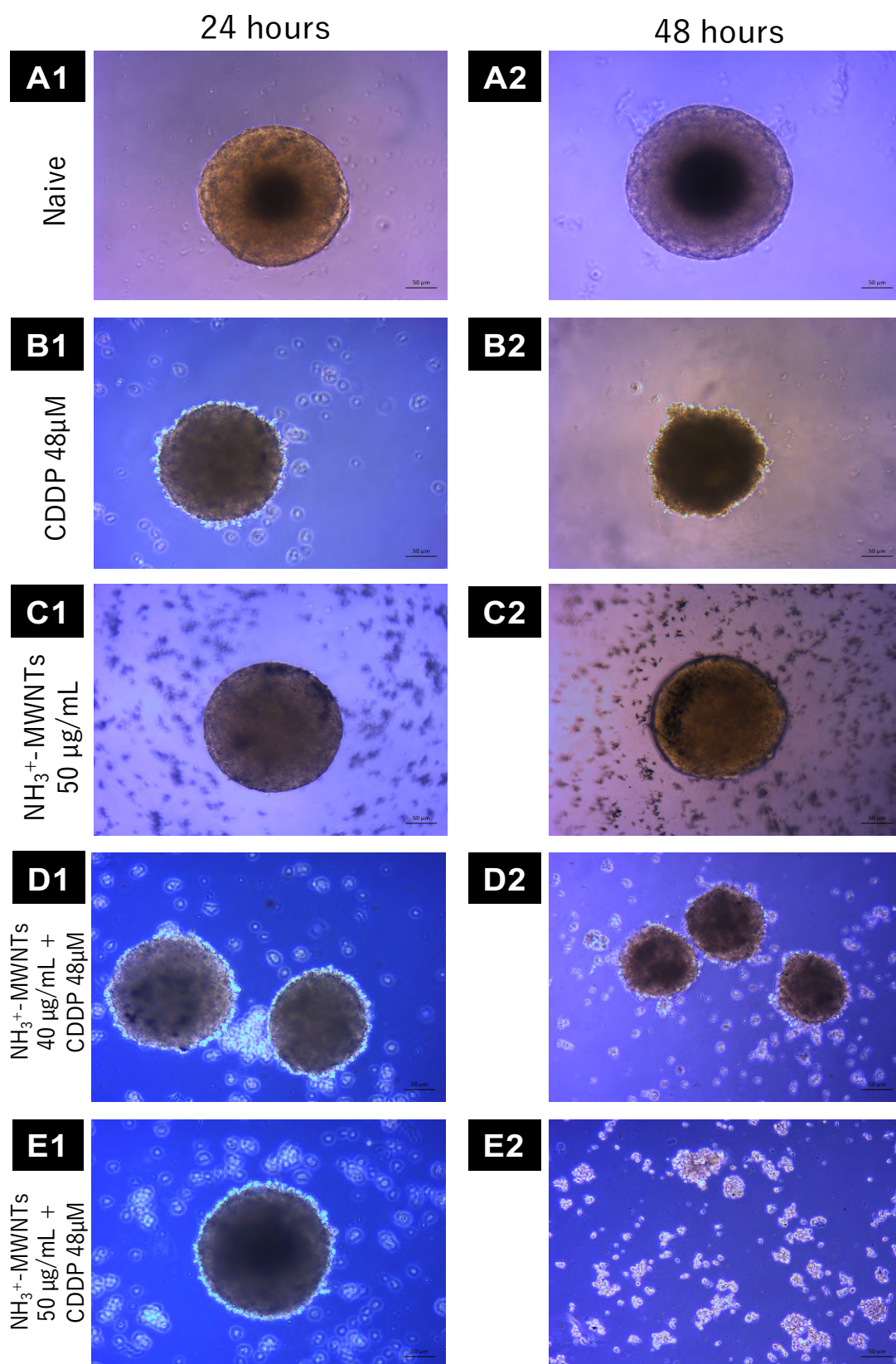


Figure 5.10 | Microphotographs of MCTS treated with amine-functionalised MWNTs + cisplatin complexes. Images of MCF7 MCTS depicting: (A) untreated control, (B) free CDDP, (C) single NH₃⁺-MWNTs, (D) NH₃⁺-MWNTs 40 μg/mL + CDDP 48μM complex, and (E) NH₃⁺-MWNTs 50 μg/mL + CDDP 48μM complex for (1) 24h and (2) 48h post-treatment.

The penetration of several NPs into MCTS have been studied, concluding that the size and superficial charge play important roles in the penetration of MCTS. While smaller particle sizes tend to be internalised by spheroids rapidly and homogeneously, larger NPs were observed in the external layers. Moreover, aminated NPs with overall positive charge, penetrated faster the MCTS than negatively-charged carboxylated NPs (542).

Our results agree with previous reports such as the study conducted by Jena et al. who concluded that CNTs are well internalised in MCF7-derived MCTS (513). Using advanced microscopy techniques, researchers determined that nanotubes promptly penetrate spheroids, observed as punctate spots distributed throughout the structure (513). Moreover, MCF7 cells cultured with CNTs successfully assembled MCTS; demonstrating that the presence of the nanomaterial does not affect the ability of cells to aggregated into 3D cultures (513).

The uptake and distribution of MWNTs in spheroids are key findings that most probably are connected with the degree of damage, structural disorganisation, cell death and overall therapeutic efficacy of nanocomplexes.

Opposite to previous well-preserved MCTS, all CDDP-containing treatments showed damage to some extent. First, the addition of free CDDP for 24h showed to cause rim damage, killing and detaching cells of the outer layer that could be observed in the media (figure 5.10B1). More importantly, the complexed

treatments showed to improve those results as damage reached deeper in the structure, causing the detachment of significantly more cells (figure 5.10D1, E1). Results led to the conclusion that nanocomplexes induce their effects faster and more profound than free CDDP.

Subsequently, microphotographs captured after 48h illustrated the increasing effect of free CDDP (figure 5.10B2). It caused higher structural injury with larger portions of rounded cells and improved growth inhibition. Despite these observations, combinatory treatments exerted exceptional effects. NH_3^+ -MWNTs 40 $\mu\text{g/mL}$ + CDDP 48 μM demonstrated a remarkable growth ratio reduction (figure 5.10D2), whereas NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + CDDP 48 μM outstandingly disintegrated the MCTS into small, loose cell aggregates (figure 5.10E2). The more concentrated combination affected spheroids to a point that their structure totally lost the initial morphology, detaching and dispersing the vast majority of composing cells.

Turning now to the growth inhibitory effect of treatments, microphotographs were used to determine the size ratio of MCTS 24h and 48h after assay onset. Results in figure 5.11 correlate to the observed in the MCTS pictures. Naïve and single NH_3^+ -MWNTs groups showed steady growth ratios over the two time-points; though, the nanomaterial reduced lightly the rate compared to the control. Also, all CDDP-including samples depicted consistent concentration-dependant ratio decreases. In addition, the NH_3^+ -MWNTs+CDDP combinations showed to be more efficient than free CDDP, although marginally in certain treatments.

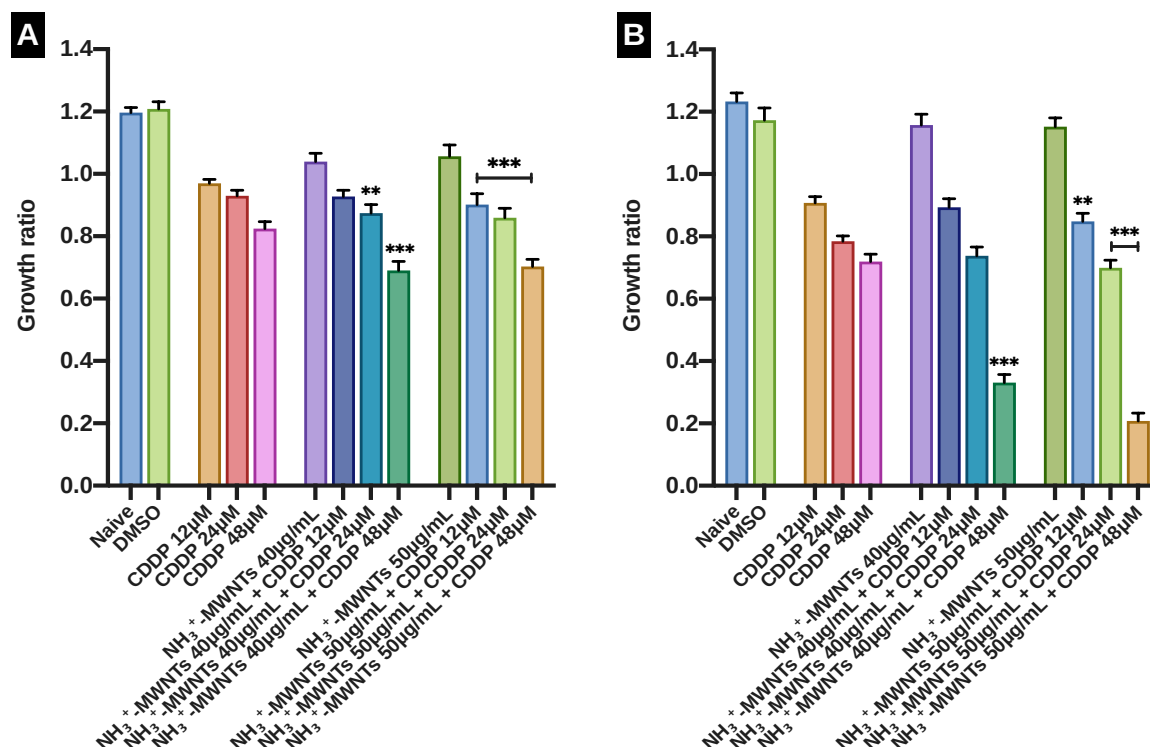


Figure 5.11 | Therapeutic efficacy of amine-functionalised MWNTs + cisplatin complexes in MCTS. Growth ratio of MCF7 MCTS treated with increasing concentrations of free CDDP, single NH₃⁺-MWNTs and NH₃⁺-MWNTs+CDDP complexes for (A) 24h and (B) 48h. DMSO was used as positive control. Values represent the mean growth ratio of 3 individual experiments (n=3) ± SD. Each assessment considered at least 8 spheroids per treatment. Statistical significance compared to free CDDP at equal concentrations: **p<0.01, ***p<0.001.

Focusing on results obtained at 24h (figure 5.11A), despite the fact that free CDDP effectively inhibited the growth ratio, reductions were discrete. Especially free CDDP 12μM and 24μM, which decreased the size ~3% and ~7% respectively. The highest concentration showed improved results, but it only reached an inhibition of 17.5±2.1%.

On the other hand, all nanocomplexes showed to enhance the CDDP effect. For instance, NH₃⁺-MWNTs 40 μg/mL + CDDP 24μM described alike results to free CDDP 48μM, whereas NH₃⁺-MWNTs 50 μg/mL + CDDP 12μM resulted in

significantly greater inhibition than the free drug 24 μ M; both combinations used half the concentration of the platinum drug to obtain the same effect. More importantly, NH₃⁺-MWNTs 40 and 50 μ g/mL + CDDP 48 μ M decreased ~30% the circumference of MCTS.

Moving to the analysis at 48h (figure 5.11B), as expected, an improved effect was observed in all treatments. The efficacy of the free drug improved, reaching a maximum growth ratio decrease of 28 $\pm$ 2.3%. NH₃⁺-MWNTs 40 μ g/mL + CDDP 24 μ M and NH₃⁺-MWNTs 50 μ g/mL + CDDP 24 μ M depicted similar inhibitions over the spheroids' growth with ~30% reduction. Nevertheless, those effects were comparable to free CDDP 48 μ M, using half the drug concentration. Finally, as microphotographs demonstrated, the best results were observed in NH₃⁺-MWNTs 40 and 50 μ g/mL + CDDP 48 μ M combinations. Notably, while the first nanocomplex resulted to diminish the MCTS' growth 66.9 $\pm$ 2.5%, the latter demonstrated an amazing inhibition of 79.2 $\pm$ 3%, being the greatest effect from all treatments evaluated. Remarkably, calculations indicated that these combinations were 2.17 and 3.7 times respectively more effective than free CDDP at equivalent concentration. Results not only confirmed the observations in MCTS microphotographs, but also validated the enhancing effect of MWNTs in the CDDP activity.

Lastly, in order to determine the viability of cells conforming the treated MCTS, the Annexin-V-FITC/PI assay was performed. Therefore, percentages of cell death were assessed and classified into proportions of viable, early apoptotic,

and late apoptotic/necrotic cells. Results in figure 5.12 indicated that single NH_3^+ -MWNTs and the untreated control showed very close results. 40 $\mu\text{g/mL}$ demonstrated to maintain $92.9 \pm 1.1\%$ of the cells healthy; only 2.7% less than the naïve group. Moreover, 50 $\mu\text{g/mL}$ of nanomaterial, with $89.1 \pm 0.8\%$ of living cells, was slightly more cytotoxic; however, no statistical difference was observed compared to untreated MCTS.

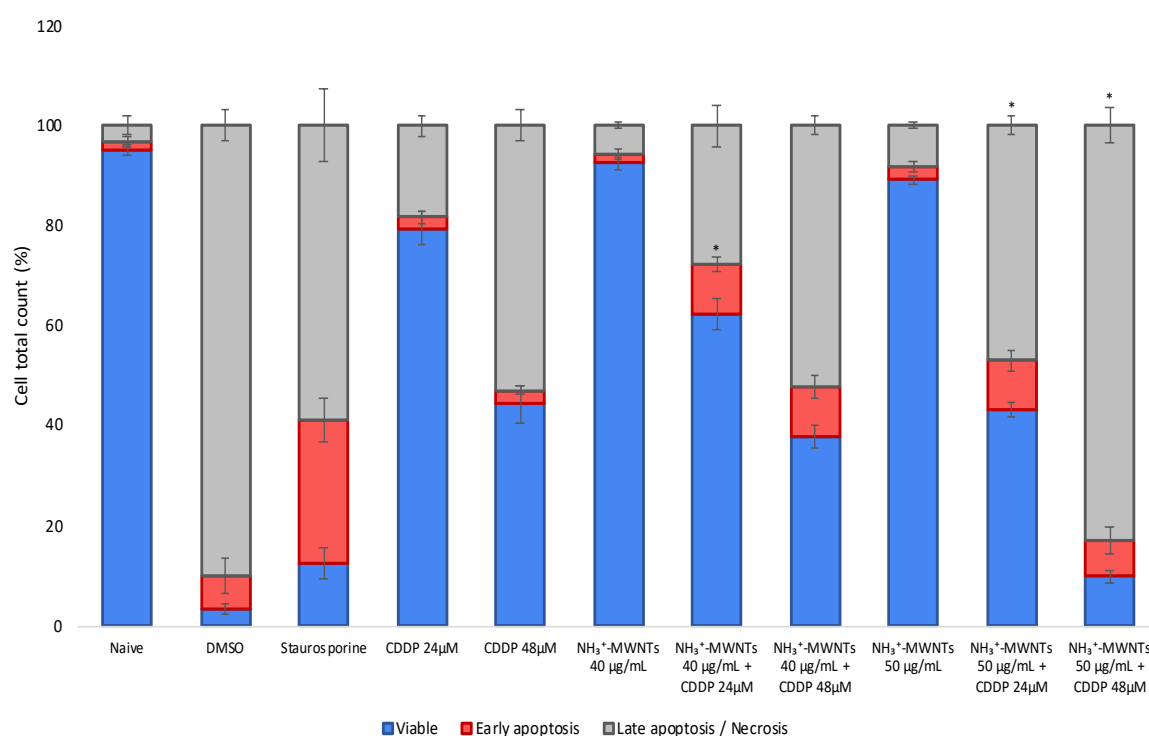


Figure 5.12 | Cell death assessment of amine-functionalised MWNTs + cisplatin complexes in Annexin-V-FITC/PI labelled MCTS. MCF7 spheroids treated with increasing concentrations of free CDDP, single NH_3^+ -MWNTs and NH_3^+ -MWNTs+CDDP were disaggregated and fluorescently stained. Staurosporine and DMSO were used as positive controls of early apoptosis and late apoptosis/necrosis respectively. Percentages of viable cells (**blue**), early apoptosis (**red**), and late apoptosis/necrosis (**grey**) are depicted in each bar. Values represent the mean of 10,000 events ($n=3$) $\pm$ SD. Statistical significance compared to free CDDP at equal concentrations: * $p < 0.05$.

As expected, CDDP-containing treatments caused great damage to the cells, which was mostly induced through late apoptosis/necrosis. Free CDDP 48 μM

inhibited $55.6 \pm 3.6\%$ of cell viability; almost the double effect observed by its lesser concentrated counterpart. In both treatments, the early apoptosis induction was marginal, causing $\sim 2.5\%$. In spite of that, more exciting results were obtained with the NH_3^+ -MWNTs+CDDP combinations. NH_3^+ -MWNTs $40 \mu\text{g/mL}$ + CDDP $48\mu\text{M}$ reduced almost two thirds the viable population, being $52.2 \pm 2\%$ necrotic cells. Interestingly, NH_3^+ -MWNTs $40 \mu\text{g/mL}$ + CDDP $24\mu\text{M}$ and NH_3^+ -MWNTs $40 \mu\text{g/mL}$ + CDDP $48\mu\text{M}$ showed the highest proportions of early apoptotic cells ($\sim 10\%$). Ju et al. showed that similar low concentrations of MWNTs did not depict significant toxicity in A549 cell models as no changes in the cell cycle or apoptosis were detected (543). Only concentrations as high as $300 \mu\text{g/mL}$ resulted in a slight increase from 3% to 7% of apoptosis (543). Nevertheless, other cell lines have proved enormous cytotoxic effects such as human skin fibroblasts, which doubled the apoptosis/necrosis levels with the exposure to concentrations as low as $0.06 \mu\text{g/mL}$ (544), stressing the importance of toxicological analysis.

The NH_3^+ -MWNTs $50 \mu\text{g/mL}$ + CDDP $24\mu\text{M}$ combination caused $56.9 \pm 1.5\%$ of cell death; only 1.3% more than free CDDP $48\mu\text{M}$ but using half the concentration. As for NH_3^+ -MWNTs $50 \mu\text{g/mL}$ + CDDP $48\mu\text{M}$, the nanocomplex confirmed to be the most effective treatment, causing $82.9 \pm 3.4\%$ of necrosis and inhibiting more than 90% of the total cell population. These values indicated that such combination was able to reduce the cell viability 69% more than free CDDP $24\mu\text{M}$ and 34% more than the drug at $48\mu\text{M}$.

It is widely accepted that the DNA-damage induced by CDDP triggers the apoptotic machinery. Nonetheless, CDDP is a nonspecific drug that induces biochemical events interconnecting apoptosis and necrosis. Upon CDDP intercalation in DNA, PARP is activated, diminishing NAD⁺ levels, followed by the eventual ATP depletion and resulting in cell death by necrosis (149, 152). However, in normal energy levels, caspases cleave PARP, inducing apoptosis. In fact, the two types of cell death may happen simultaneously in cultures and tissues (149, 152).

Overall, MWNTs+drug combinations demonstrated a cytotoxicity enhancement compared to free drugs mainly via late apoptosis/necrosis. However, it is important to note a small increase in the early apoptotic cell population, which suggests that cells exposed to the nanocomplexes may have undergone the apoptotic route. Other CDDP-containing nanoformulations have reported a higher proportion of early apoptotic cells that decreases at longer exposure periods while necrosis increase (424). Furthermore, the cytotoxicity of SWNTs and MWNTs were thoroughly examined in in vitro and in vivo by Kavosi et al (545). The researchers not only determined that MWNTs were more biosafe than SWNTs (50% inhibitory concentrations were 400 and 50 µg/mL, respectively), but also high concentrations of the nanomaterials triggered an increase of apoptotic cells that progressed to necrosis over time (545). Furthermore, DLD-1 colon carcinoma cells-derived MCTS were treated with free CDDP and DOX among other drugs (546). In the results, DOX exerted visible size reductions after 48h, whereas CDDP presented a minor size reduction in the same time-point

followed by a static period (546). In addition, the morphology analysis using TEM techniques of CDDP- and DOX-treated spheroids demonstrated the loss of cellular connections and the presence of apoptotic cells and necrotic regions in the surface (546). Interestingly, our microscopic evaluation of MCTS exposed to drug-containing treatments displayed almost identical structural damage, which was further corroborated with fluorescent techniques.

These results may support the idea that drugs and nanocomplexes tested here triggered a transient apoptosis that has progressed to late apoptosis/necrosis by the time the analysis was performed. In order to determine the exact cell death type, a time-course would be additionally needed.

5.2.3 Cytotoxicity assessment by fluorescence imaging

Imaging techniques were used to further sustain findings in 3D cell cultures. Consequently, MCF7 MCTS were treated with the two functionalised MWNTs, both anticancer drugs, and the most relevant nanocomplexes in previous evaluations. Therefore, for this experiment the testing of COOH-MWNTs+DOX was discarded as it showed the poorest therapeutic efficacy. Besides, in order to analyse integral MCTS, we decided to image 24h-treated samples to avoid the disaggregation caused by highly efficient treatments observed at longer time periods. Thus, MCTS were fluorescence-labelled following the protocol described in previous sections. The probe discriminates living and damaged/dying cells based on the membrane integrity. Nonspecific cytosolic esterases present only

in intact-membrane cells convert non-fluorescent calcein AM to green fluorescent calcein (ex/em 488/515nm). On the other hand, cells with compromised membranes are permeable to red fluorescent ethidium homodimer-1 (ex/em 570/602nm); a dye with high affinity for nucleic acids (547). Afterwards, a collection of maximum-intensity 3D projections was acquired and illustrated in figure 5.13, depicting untreated and nanomaterial treated MCTS. Firstly, naïve MCTS demonstrated to be well-defined structures (figure 5.13A). Aggregates were densely compact, comprising only viable cells.

Similar results were obtained in MCTS treated with single COOH-MWNTs, where no signs of cell damage were observed (figure 5.13B). Although scattered red fluorescence was detected, it was due to artefacts and dye aggregates as the cellular structure was intact and firmly packed. Studies have used this fluorescent technique to assess the proportion of live and dead cells in MCTS, reporting a high viability in MCF7 spheroids cultured for less than 12 days (519). In contrast, single NH_3^+ -MWNTs caused a low degree of damage as a small proportion of dead cells were observed in the spheroid structures (figure 5.13C). These results correlated to previous experiments, where the amine MWNTs demonstrated to cause a slight increase of late apoptosis/necrosis on MCTS and minor cell inhibition on cytotoxicity assays. Nonetheless, spheroids were robust with closely attached cells.

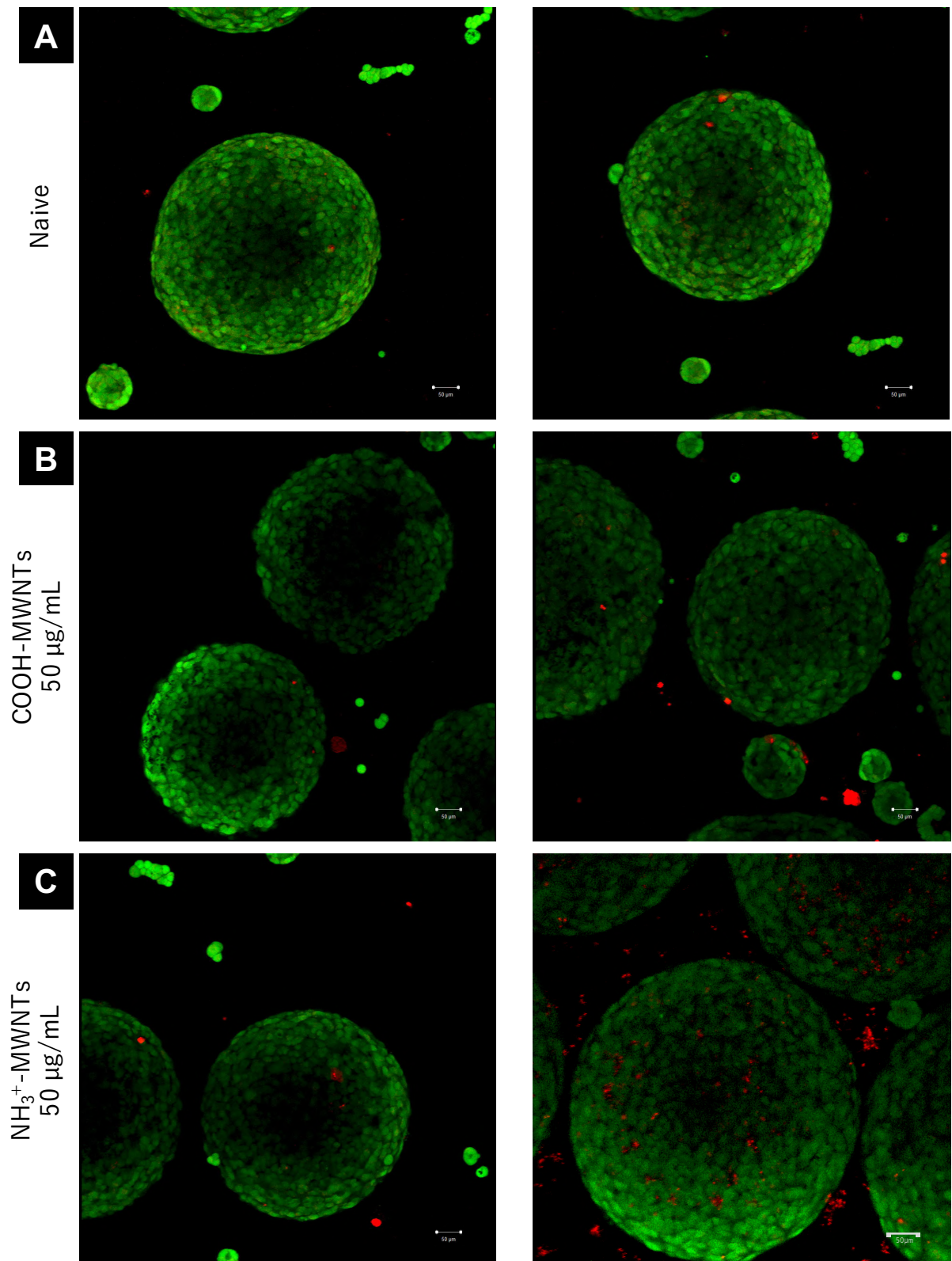


Figure 5.13 | Cytotoxicity assessment of MWNTs in fluorescence-labelled spheroids. 3D projections of treated MCTS depicting: (A) untreated control, (B) COOH-MWNTs and (C) NH_3^+ -MWNTs for 24h. MCTS were stained to differentiate living cells (green) and dead cells (red).

Live/dead fluorescent assays are frequently used to determine the viability of cells in food, environmental and clinical samples as well as a routine research technique (548). Additionally, studies have reported the use of fluorescent dyes as valuable tool to evaluate the cytotoxicity of compounds and nanoformulations in 3D cultures thorough imaging techniques (534, 549).

Turning now to the cytotoxicity analysis of free DOX 48 μ M and combinations of NH₃⁺-MWNTs+DOX, cellular constructs where labelled following the next colour patterns: the inherent fluorescence of DOX was nominated as red; living cells were defined as green and dead ones as blue. While results showed that DOX was easily detected in all samples (figure 5.14 A1-C1), interestingly, free DOX 48 μ M samples depicted a high proportion of living cells, compared to NH₃⁺-MWNTs+DOX combinations, which showed predominantly dead cells (figure 5.14 A2-C2).

NH₃⁺-MWNTs 50 μ g/mL + DOX 48 μ M showed particularly intense blue-stained MCTS, indicating the high cytotoxicity of the treatment; effect that was further confirmed in merged microphotographs (figure 5.14 A3-C3). The mixture of the three colours varied depending on the predominance of the cells. The free anthracycline produced light yellow shades and blue spots, indicating the major proportion of living cells. But then, NH₃⁺-MWNTs 40 μ g/mL + DOX 48 μ M depicted a light purple general coloration, most likely produced by the mixture of the DOX (red) and dead cells (blue).

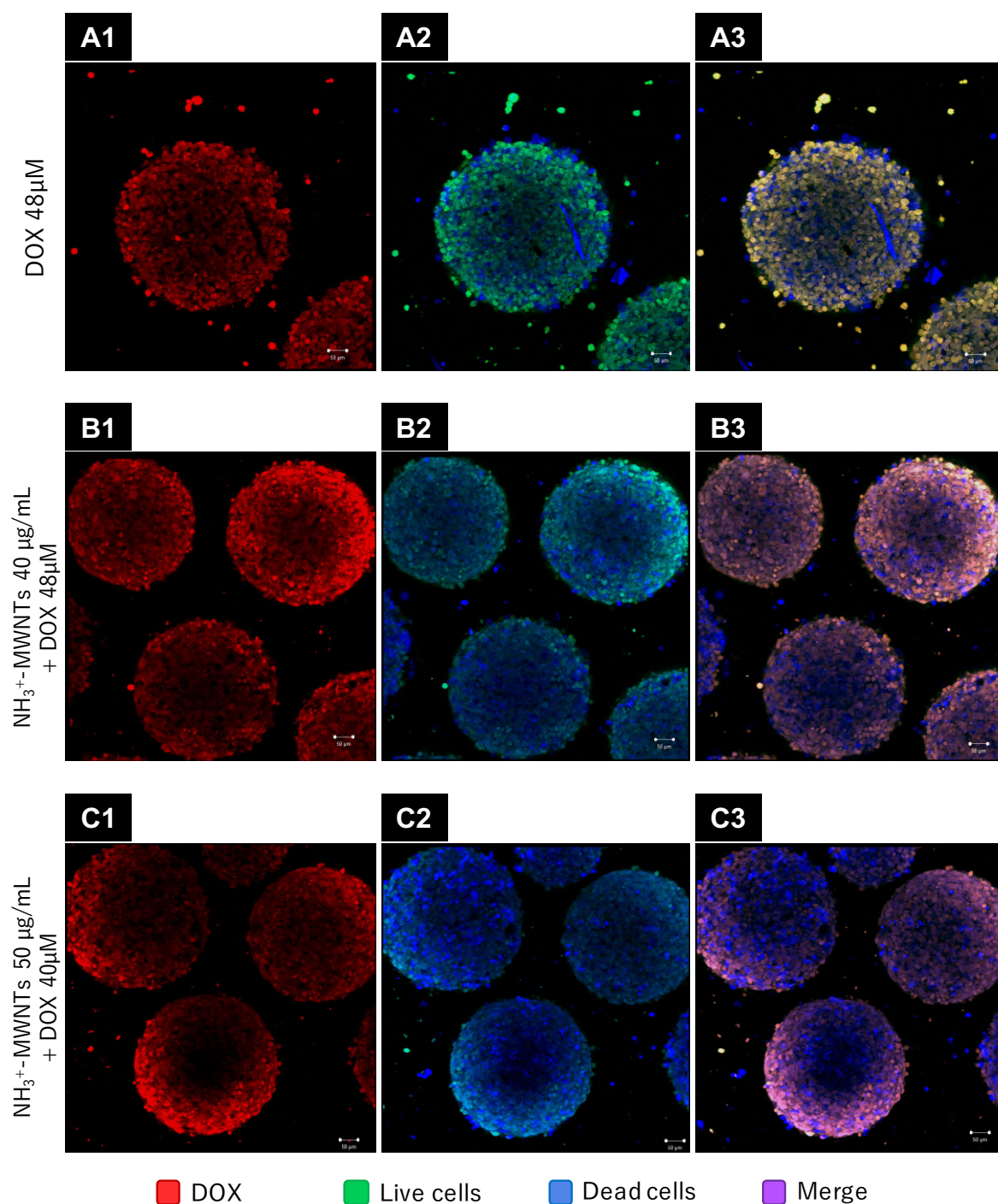


Figure 5.14 | Cytotoxicity assessment of amine-functionalised MWNTs + doxorubicin complexes in fluorescence-labelled spheroids. 3D projections of treated MCTS depicting: (A) free DOX, (B) NH_3^+ -MWNTs 40 $\mu\text{g/mL}$ + DOX 48 μM complex, and (C) NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + DOX 48 μM complex for 24h. MCTS were stained to identify: (1) inherent DOX fluorescence, (2) live and dead cells and (3) merge.

Such effect was enhanced in MCTS treated with NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + DOX 48 μM , depicting intense violet tones, supposing the presence of a high number of dead cells. More importantly, these results added evidence to past studies where NH_3^+ -MWNTs+DOX showed to be more efficient than free DOX.

Evaluation continued with the cytotoxicity assessment of free CDDP and MWNTs+CDDP combination. First, the study of free CDDP and COOH-MWNTs+CDDP was performed. Here, the action of free CDDP during 24h produced mainly healthy cells (figure 5.15A). Also, an important number of dead cells were distributed along the MCTS' surface, yet the structure demonstrated to be firm and dense, suggesting the damage was rather superficial. In contrast, nanocomplexes showed remarkable cytotoxic effects.

COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 48 μM illustrated a marked efficacy enhancement compared to the free anticancer drug (figure 5.15B). The great presence of dead cells produced a dominant red colour that diminished the intensity of the green viable cells. Moreover, even though the spheroids were complete, they showed evident signs of disaggregation.

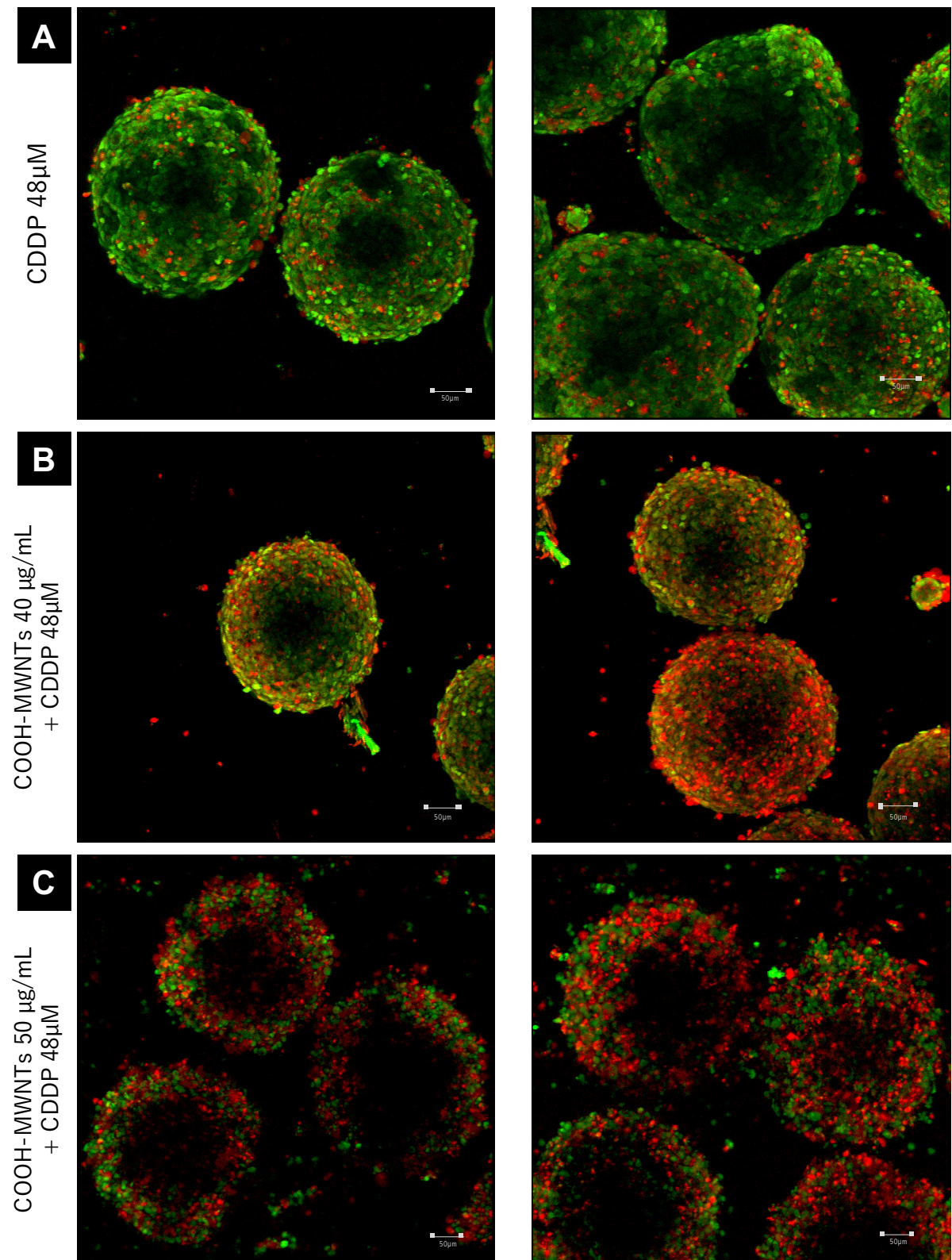


Figure 5.15 | Cytotoxicity assessment of carboxyl-functionalised MWNTs + cisplatin complexes in fluorescence-labelled spheroids. 3D projections of treated MCTS depicting: (A) free CDDP, (B) COOH-MWNTs 40 μ g/mL + CDDP 48 μ M complex, and (C) COOH-MWNTs 50 μ g/mL + CDDP 48 μ M complex for 24h. MCTS were stained to differentiate living cells (green) and dead cells (red).

Lastly, COOH-MWNTs 50 $\mu\text{g/mL}$ + CDDP 48 μM demonstrated the greatest cytotoxicity, producing a predominantly proportion of dead cells (figure 5.15C). Nonetheless, more impressive was the disruption of the cellular structure caused in such short time. The spheroids lost density, showing dead cells detaching. The structural damage was such that cells could not maintain the 3D arrangement. The final therapeutic assessment included NH_3^+ -MWNTs+CDDP combinations. Obtained microphotographs demonstrated consistency and reliability.

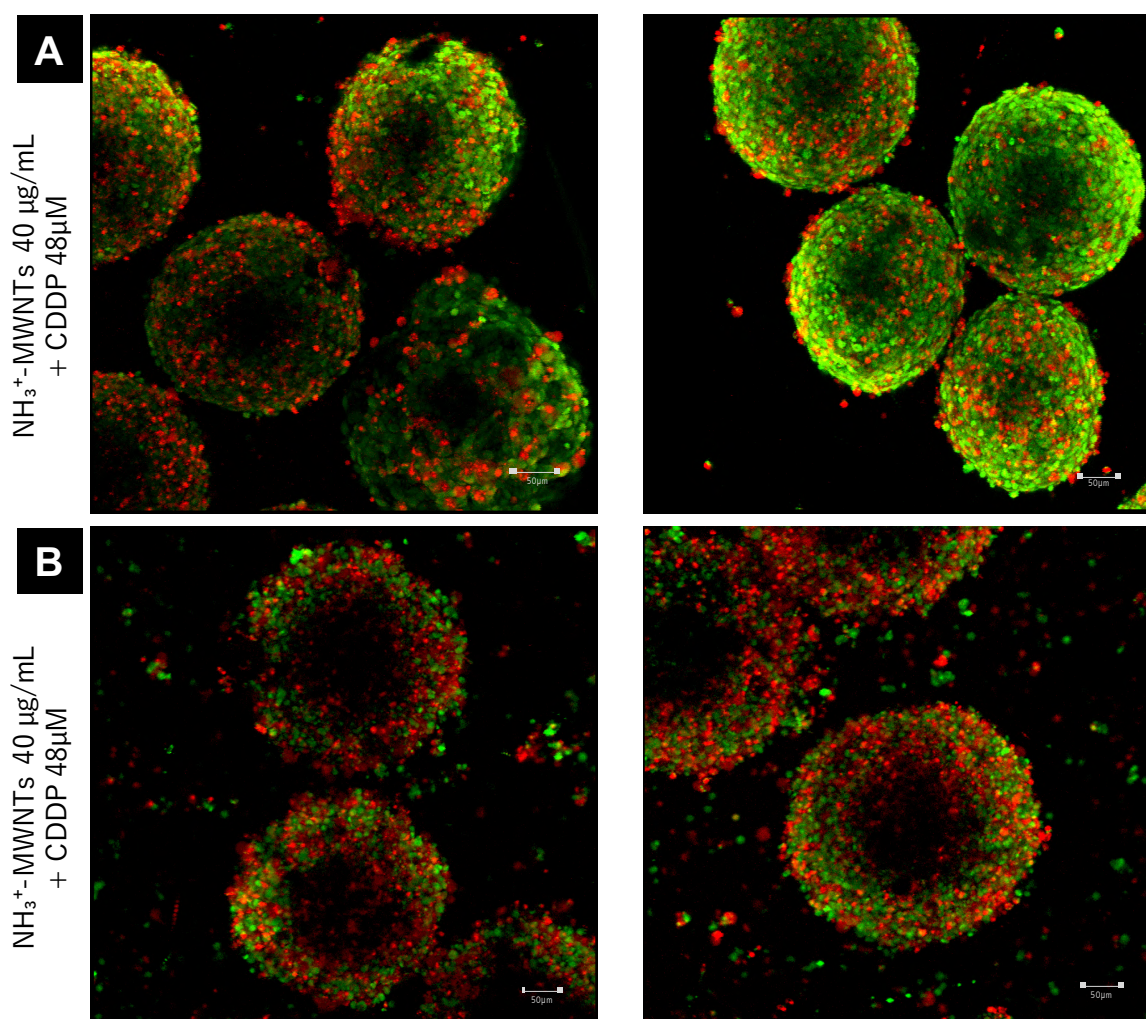


Figure 5.16 | Cytotoxicity assessment of aminated-functionalised MWNTs + cisplatin complexes in fluorescence-labelled spheroids. 3D projections of treated MCTS depicting: (A) NH_3^+ -MWNTs 40 $\mu\text{g/mL}$ + CDDP 48 μM complex and (B) NH_3^+ -MWNTs 50 $\mu\text{g/mL}$ + CDDP 48 μM complex for 24h. MCTS were stained to differentiate living cells (green) and dead cells (red).

Fluorescence-labelled MCTS treated with NH_3^+ -MWNTs 40 $\mu\text{g}/\text{mL}$ + CDDP 48 μM nanocomplex described an important increase of red-coloured dead cells compared to free CDDP (figure 5.16A). Furthermore, the surface injury was clearly demonstrated by the large number of cells detaching. Nevertheless, NH_3^+ -MWNTs 50 $\mu\text{g}/\text{mL}$ + CDDP 48 μM depicted a stronger therapeutic efficacy (figure 5.16B). The addition of this combination caused not only cell disaggregation, but also destruction of the MCTS, causing structural loss.

In conclusion, the presented protocol to generate MCF7-derived MCTS demonstrated to be reproducible for the formation of compact, dense spheroids. The exposure of nanocomplexes to these intricate three-dimensional cultures showed different degree of structural damage. While the effect of MWNTs+DOX combinations was comparable to free DOX, both CDDP complexes depicted exceptional efficacy, diminishing the spheroids' size and even disaggregating them. Moreover, fluorescence-imaging assays were in line with morphological studies. They not only confirmed the disintegration of the MCTS, but also proved that treatments effectively caused cell death. These findings were further validated with Annexin-V-FITC/PI experiments, demonstrating an important decrease in the viable cells proportion whereas early apoptotic and late apoptotic/necrosis were the dominant populations. Limited cytotoxicity was associated with the nanomaterial. Investigations have resolved that the information acquired in 2D and 3D cultures is significantly different (550). Therefore, the therapeutic consistency of nanocomplexes in both systems

strengthens our results and the promise of new anticancer alternatives. Nevertheless, it is important to understand the mechanism of action by which nanocomplexes exert the anticancer activity. Hence, the next chapter will present insights that should aid the comprehension of their enhanced chemotherapeutic efficacy and improve the success probability in further experimentation.

CHAPTER 6

MECHANISM OF ACTION OF MWNTS + CISPLATIN

6.1 Introduction and summary

The development of new therapeutic compounds is accompanied by a complex process, from the discovery and formulation of the active system to the commercialisation. A central step of this process is the characterisation of the mechanisms of action involved in the therapeutic action. The objective of such assessment is not only to unveil the biochemical interactions that produce pharmacological outcome, but also to define and modulate undesired effects.

Preclinical models are useful and necessary to provide insights into cellular processes involved, pathways and cell death. In nanomedicine, the study must include critical aspects of the nanocarrier, such as physicochemical properties and biosafety. Besides, DDSs may offer alternative therapeutic approaches that most probably will modify the mechanisms of the loaded compound.

The mechanistic elucidation of essential features like biocompatibility, pharmacokinetics and pharmacodynamics could lead to the discovery of new

targets such as angiogenesis and metastasis as well as improved therapeutic strategies, combinations for potentiation and more importantly, reduction of the possibility of eventual failure.

Recent advancements in biological models present unique opportunities to elucidate precisely essential features like biocompatibility, drug release, cellular uptake. Besides, the use of 3D models can help to better predict in vivo results, improving new therapeutic approaches and reducing the possibilities of failure.

Therefore, in efforts to unveil the mechanistic phenomenon underlying the enhanced effect of MWNTs + CDDP combinations, several experiments were performed. In this chapter, we focused on the effect of COOH-MWNTs+CDDP, since in previous sections of this work, the nanocomplex presented the greatest therapeutic efficacy, while the nanomaterial proved the lowest cytotoxicity. Therefore, 2D and 3D MCF7 cultures were used to evaluate the cellular uptake, intracellular CDDP concentration, chemosensitisation, cell cycle, ROS and GSH levels, membrane potential and DNA fragmentation.

6.2 Results and discussion

6.2.1 Cellular uptake of cisplatin

The transport through the cellular membrane is one of the most important biological processes. Designed for the efficient exchange of substances, it is rigorously regulated to maintain the balance. Thus, the plasma membrane is the

first barrier that chemotherapeutics and DDSs have to overcome to exert their mechanism of action. There are three main pathways of cellular internalisation: simple diffusion, facilitated diffusion and active transport.

The physicochemical characteristics and surface modifications of NPs are major determinants in their interactions with cells and uptake. It is generally accepted that NPs enter the cell mainly through endocytosis due to the amount of cargo, size, and tendency to agglomerate (551, 552). Endocytosis is an energy-dependant internalisation process and as such, it can be evaluated to deduce the uptake pathway of our nanocomplexes. A simple method to study the energy-dependant traffic of DDSs thorough the cytoplasmic membrane is the inhibition by low-temperature. Several investigations have mentioned that transport mechanisms are diminished at 4 °C incubations (553-556).

Consequently, the previously explained mLDH assay was adapted to infer the participation of active transport in the uptake of free CDDP, COOH-MWNTs and nanocomplexes. The main objective was to detect advantages that the nanocarrier could provide on cellular internalisation over the free drug under adverse conditions, rather than comparing their cytotoxicities at different temperatures. With that purpose, the therapeutic activity of said compounds was measured in MCF7 monolayers incubated at 4 °C for 3 and 6h. The incubation period was shortened to limit the cell death associated with the exposure of low temperature. Obtained data was graphed in figure 6.1.

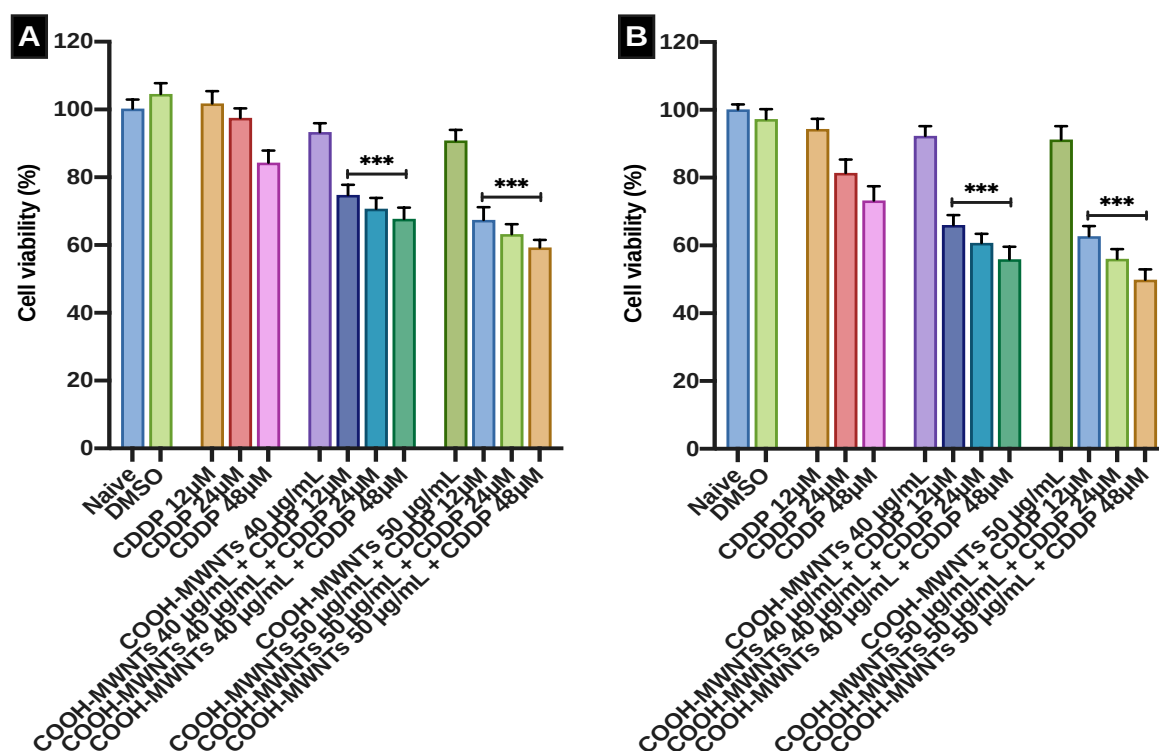


Figure 6.1 | Therapeutic efficacy of carboxyl-functionalised MWNTs and cisplatin complexes incubated at 4 °C. MCF7 cells were treated with increasing concentrations of free CDDP, COOH-MWNTs and combinations of these compounds for (A) 3h and (B) 6h. DMSO was used as positive control. Percentages represent the mean cell viability of 3 individual replicates ($n=3$) $\pm$ SD. Statistical significance compared to free CDDP at equal concentrations: *** $p<0.001$.

Overall, a concentration-dependant inhibition was observed in all CDDP-containing treatments at both time-points. Importantly, all nanocomplexes demonstrated statistically significant greater inhibition rates than free CDDP at equivalent concentrations, while the presence of the nanomaterial alone showed a slight reduction compared to the untreated control. Interestingly, DMSO showed little to none cytotoxic effect, which may be related to its cryoprotective effect (557).

Particularly, after the first 3h of the experiment (figure 6.1A), the lowest inhibition was observed with the addition of free CDDP 24μM, reducing $2.5\pm2.8\%$ the cell

growth; that considering that free CDDP 12 μ M did not show any cytotoxicity. In contrast, CDDP 48 μ M, the highest concentration of the free drug, increased the inhibitory effect to 15.3 $\pm$ 3.5%. Nevertheless, that effect was surpassed by the COOH-MWNTs 40 μ g/mL + CDDP 12 μ M (25.2 $\pm$ 2.9%), which produced the smallest effect of all nanocomplexes. The greatest effect of the complexes based on COOH-MWNTs 40 μ g/mL was that combined with CDDP 48 μ M (32.3 $\pm$ 3.3%), whereas COOH-MWNTs 50 μ g/mL + CDDP 48 μ M produced the highest inhibition of all, reducing 40.8 $\pm$ 2.2% the cell population.

Then, the same trends as the above-explained were observed in 6h-experiments (figure 6.1B). While single COOH-MWNTs practically did not show any modification in respect of the last assay, CDDP-containing treatments increased their therapeutic effect. Again, free CDDP 48 μ M (26.7 $\pm$ 4.1%) depicted greater decrease of cell viability than the less concentrated analogues. Such cell death was 7.3% and 17.4% lower than that observed with COOH-MWNTs 40 μ g/mL + CDDP 12 μ M (34 $\pm$ 2.8%) and COOH-MWNTs 40 μ g/mL + CDDP 48 μ M (44.1 $\pm$ 3.6%). Similarly, COOH-MWNTs 50 μ g/mL + CDDP 12 μ M (37.3 $\pm$ 2.9%) reduced 10% more the viable cells than the most concentrated free CDDP treatment, containing only a quarter of the drug concentration. At equivalent CDDP concentrations, COOH-MWNTs 50 μ g/mL + CDDP 48 μ M (50.1 $\pm$ 3%) inhibited 23.3% more cells.

It is accepted that CDDP passively diffuses into cells. Besides, studies have described that transmembrane transporters such as OCT, CTR1 and CTR2 take

an important part in the internalisation (137, 143). In the case of CNTs, endocytosis and the nanoneedle mechanism have been studied as the main uptake routes (359, 361, 368). Properties like length, type of functionalisation and surface chemistry may dictate the uptake process (242). According to Lacerda et al., 30-50% of derivatised MWNTs enter the cell via non-energy dependant mechanisms (362). Thus, the ability of CNTs to translocate across membranes has been proposed as one of the most important biological advantages in their use as nanovehicles (335, 470).

Nevertheless, our results proved that, regardless the internalisation mechanism, the nanocomplexes managed to inhibit the cell proliferation more efficiently than free CDDP under temperatures that diminished the uptake kinetics. Hence, results suggested that the enhanced effect could be associated with an increased intracellular drug accumulation.

CDDP is the first treatment option for a wide range of human cancers. Unfortunately, after an initial good response, cancers frequently develop resistance, limiting the use of the drug. There are various factors associated with the development of resistance and CDDP therapy failure, being one of the most important, the reduced cellular accumulation.

Altered cellular CDDP accumulation is usually the result of any of two independent routes: reduced uptake and upregulated export. Recently, there has been uncertainty about the mechanisms involved in the CDDP uptake as some

reports have raised doubts about the participation of protein-mediated transport (558, 559). Nevertheless, it is believed that downregulation of transporters correlate with a diminished intracellular CDDP accumulation (196).

Considering that MWNTs provide an alternative internalisation mechanism, the intracellular concentration of Pt, a fundamental ion in the CDDP molecule, was measured. Therefore, monolayer cultures were exposed to the free drug and complexed with COOH-MWNTs and assessed using ICP-MS techniques. The objective was to compare the levels of the metal ion inside cells and determine whether the enhanced cytotoxicity of nanocomplexes is associated to an increased CDDP uptake. Obtained concentrations were graphed in figure 6.2.

In these series of experiments, the intracellular Pt content was directly related to the CDDP exposed concentration. The incubation with free CDDP 12 μ M showed Pt levels of 256 ± 30 ng/mL; practically the same concentration observed after the addition of COOH-MWNTs 40 μ g/mL + CDDP 12 μ M (259 ± 17 ng/mL) and slightly lower than COOH-MWNTs 50 μ g/mL + CDDP 12 μ M (303 ± 24 ng/mL). Furthermore, as predicted, the addition of CDDP 24 μ M doubled the Pt concentration detected with free CDDP (543 ± 86 ng/mL) and COOH-MWNTs 50 μ g/mL + CDDP 24 μ M (604 ± 50 ng/mL). However, COOH-MWNTs 40 μ g/mL + CDDP 24 μ M depicted lower intracellular Pt than the other treatments with 436 ± 46 ng/mL.

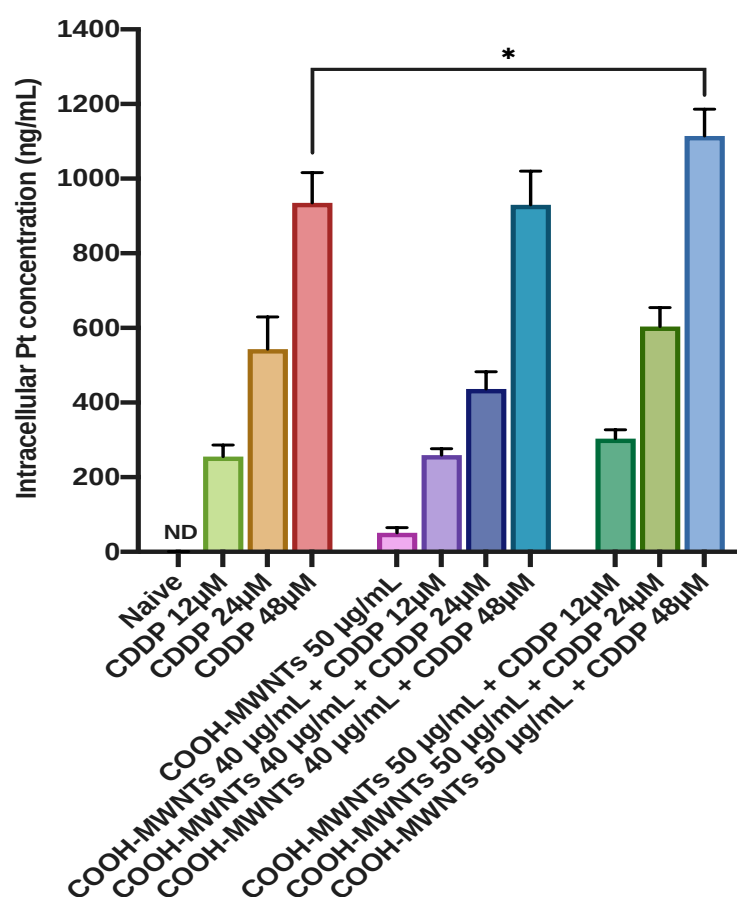


Figure 6.2 | Assessment of cellular uptake of cisplatin. MCF7 cells were treated with increasing concentrations of free CDDP, COOH-MWNTs and combinations of these compounds. After 24h of incubation, cells were acid-digested, and the intracellular content of Pt was determined by ICP-MS. Results represent the Pt concentration in 5×10^5 of 3 individual replicates ($n=3$) $\pm$ SD. Statistical significance compared to free CDDP at equal concentrations: * $p<0.05$. ND: not detected.

Finally, while free CDDP 48µM (935±81 ng/mL) and COOH-MWNTs 40 µg/mL + CDDP 48µM (930±90 ng/mL) displayed almost the same intracellular Pt, COOH-MWNTs 50 µg/mL + CDDP 48µM showed a greater concentration, being the only treatment to show statistically significant difference. Naïve and cells treated with COOH-MWNTs were evaluated as well. The absence of the metal in untreated cells and minimal levels in cells incubated with the nanomaterial (51±14 ng/mL) confirmed that the presence of Pt was due to CDDP. Traces detected with

nanotubes correspond to leftovers of other samples and high sensitivity of the detection system, although it was thoroughly washed between analyses.

It is important to consider an imminent loss of the originally drug concentration due to the sample processing. Since the sample preparation include microfiltration, complexed CDDP could be eliminated and not considered in the analysis. The composition of the nanomaterial prevented its digestion along with cells. Additionally, as presented previously, high concentrations of both the free drug and combinations caused a high cytotoxicity; thus, dying cells and cell debris could be lost.

Publications have reported the use of DDSs to increase the intracellular accumulation of anticancer drugs. Examples are the use of PEG-graphene oxide to increase the intratumor CDDP concentration (560). Similarly, palladium NPs showed greater tumour concentrations of a DOX prodrug (561), while gold NPs were used to downregulate the overexpression of resistance-associated proteins to accumulate paclitaxel (562).

In general, all combinations with COOH-MWNTs 50 µg/mL showed greater cellular CDDP, suggesting that higher concentration of the nanomaterial may incorporate more drug. We hypothesise that the nanomaterial can attach and transport a high number of CDDP that are readily internalised into cells. Uptake via endocytosis can introduce several nanocomplexes simultaneously, whereas the nanoneedle mechanism provides an alternative energy-independent pathway

to deliver the drug into cells. These processes facilitate a greater cytoplasmic accumulation of CDDP in short time, forcing cells to die. Nonetheless, other cellular mechanisms could be involved in the enhanced therapeutic efficacy of combinatory treatments; some of them will be presented next.

6.2.2 MWNTs chemosensitisation

Chemosensitisation refers to the use of one agent to selectively potentiate the anticancer effects of a drug (563) or to improve the susceptibility of cancer cells to the effects of a drug (564).

Numerous compounds have demonstrated chemosensitising effects against several human tumours, for example, hydroxychloroquine in lung cancer (565) and neuroblastoma (566), mifepristone in cervical cancer (567) and glioblastoma (568) or miRNAs in the case of breast cancer (569, 570). In the particular case of CDDP, natural products (571, 572), metformin (573) and PARP inhibitors (574, 575) among others, have demonstrated to sensitise cells for the treatment of CDDP.

Furthermore, NPs have showed chemosensitising activity as well. For instance, the addition of AuNPs in ovarian cancer cultures produced not only cell sensitisation to CDDP, but also reduced acquired chemoresistance and stemness and showed enhanced anticancer efficacy in vivo (576). Similarly, iron oxide NPs sensitised resistance ovarian cancer cells, restoring the efficacy of

CDDP (577). Liposomes have the ability to incorporate different compounds in one nanosystem. Such is the case of CDDP-loaded liposomes with mifepristone intercalated in the lipid bilayer (214). The combination demonstrated potentiated anticancer cytotoxicity and significant tumour volume reduction (214). Moreover, studies indicated that pre-incubation of DOX-resistant MCF7 cells with iron oxide NPs followed by a magnetic field exposure, resulted in increased cytotoxicity efficacy of the drug (578). Taking that into consideration, the ability of COOH-MWNTs to chemosensitise MCF7 cells for the treatment of CDDP was assessed. The objective of these experiments was to determine whether the cellular pre-exposure to the nanomaterial influenced on the therapeutic efficacy of the platinum drug. Hence, 2D cultures were pre-cultured with 50 µg/mL of COOH-MWNTs, a concentration previously used, and a low concentration, 10 µg/mL for 24h and 48h. After each time-point, the nanotubes were completely removed and fresh media containing CDDP at usual concentration was added for further 24h. The mLDH assay was performed and data was illustrated in figure 6.3.

In general, all samples pre-incubated with COOH-MWNTs showed statistically significant enhanced therapeutic efficacy compared to those that were not. It is important to note that the cytotoxicity of single COOH-MWNTs never exceeded 9% of cell death compared to naïve.

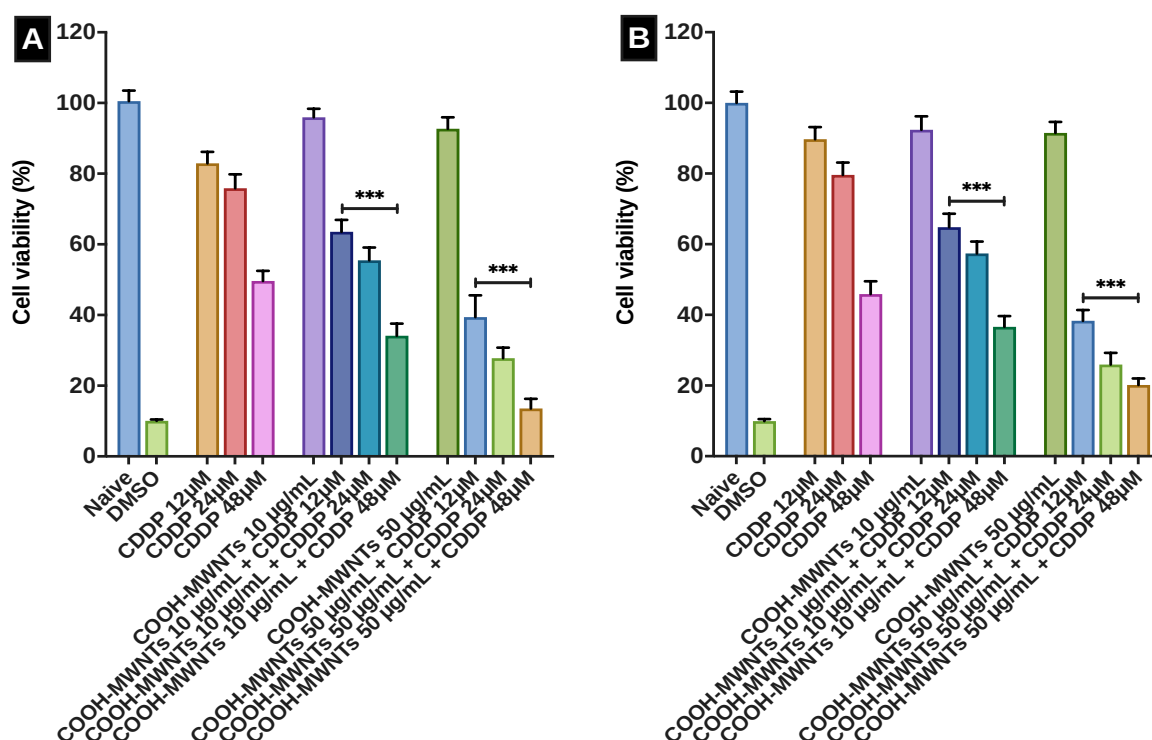


Figure 6.3 | Cell chemosensitisation to cisplatin by carboxyl-functionalised MWNTs. MCF7 cells were pre-treated with two concentrations of COOH-MWNTs for (A) 24h and (B) 48h. Then, the nanomaterial was removed and increasing concentrations of CDDP were replaced for 24h. Such samples are indicated in the graphs with the previous concentration of nanotubes plus the added concentration of CDDP. In samples treated with single COOH-MWNTs, fresh media with the same concentration of nanomaterial was substituted. DMSO was used as positive control. Percentages represent the mean cell inhibition of 3 individual replicates ($n=3$) $\pm$ SD. Statistical significance compared to free CDDP at equal concentrations: *** $p<0.001$.

Samples pre-exposed to 10 $\mu\text{g/mL}$ carboxylated MWNTs for 24h (figure 6.3A), followed by CDDP 12 μM resulted in inhibition of $36.5\pm3.3\%$, whereas CDDP 48 μM showed an impressive cell decline of $65.9\pm3.4\%$. These values were improved in cells pre-incubated with 50 $\mu\text{g/mL}$. The subsequent addition of CDDP 12 μM reduced the cell population only 5% more than COOH-MWNTs 10 $\mu\text{g/mL}$ + CDDP 48 μM . Moreover, the highest CDDP concentration, 48 μM , depicted $86.5\pm2.6\%$ of cell death, the greatest cell inhibition observed in these experiments. In contrast, samples that were not sensitised, did not follow the

same efficacy rates. While the lowest concentration inhibited $17.1 \pm 3.2\%$ of the cells, free CDDP $48 \mu\text{M}$ killed only half of the culture, which was 10% less than COOH-MWNTs $50 \mu\text{g/mL}$ + CDDP $12 \mu\text{M}$, containing four times less CDDP concentration.

In the case of cells pre-treated with the same concentrations of the nanomaterial for two days (figure 6.3B), COOH-MWNTs $10 \mu\text{g/mL}$ followed by CDDP $12 \mu\text{M}$ inhibited $35.2 \pm 3.8\%$ of the cells, whereas the subsequent addition of $48 \mu\text{M}$ produced $65.9 \pm 3.4\%$ cell death. Such percentage proved the enhancing effect of the nanomaterial pre-exposure as it was 9% greater than free CDDP $48 \mu\text{M}$, again, with only a quarter of the drug concentration. Impressively, the therapeutic effect depicted by COOH-MWNTs $10 \mu\text{g/mL}$ + CDDP $48 \mu\text{M}$ was practically the same of that resulted from the pre-incubation of COOH-MWNTs $50 \mu\text{g/mL}$ followed by CDDP $12 \mu\text{M}$. The highest concentration, COOH-MWNTs $50 \mu\text{g/mL}$ + CDDP $48 \mu\text{M}$, reduced $79.9 \pm 1.8\%$ the population.

Based on our results, the pre-exposure of breast cancer cells to COOH-MWNTs demonstrated a chemosensitising effect for the treatment of CDDP. More importantly, pre-incubation for short periods of 24h with concentrations as low as $10 \mu\text{g/mL}$ of carboxylated nanotubes showed the best cell growth inhibition.

The vast majority of studies focus on the use of CNTs as delivery systems for active molecules. Nonetheless, until recently carbon-based nanomaterials have shown the potential to sensitise cells to different chemotherapeutics.

Nanofibers and MWNTs showed the ability to chemosensitised prostate and bladder cancer cells to the treatment of platinum drugs (487). The effect resulted in increased cell death and clonogenic survival inhibition, mainly mediated through apoptosis (487). To corroborate, the same research group tested the sensitising potential of the nanomaterials in DU-145 cells (579). The experiment consisted in pre-treating the cancer cells with both carbon NPs at concentrations ranging from 1 to 200 $\mu\text{g/mL}$ for 22h; then, docetaxel (1.5 – 2.5 ng/mL) or mitomycin C (0.3 – 0.5 $\mu\text{g/mL}$) were added for 2h extra. After treatment removal, the cells were incubated for further 72h, proving improved cytotoxic and pro-apoptotic effects compared to the single treatments (579).

Zhang et al. described a sensitisation effect to paclitaxel when OVCAR3 cells were co-treated with non-functionalised SWNTs (580). The mechanistic analysis demonstrated that the addition of the nanomaterial increased 2-fold the percentage of apoptosis (580), depicting the chemosensitising ability of CNTs regardless the functionalisation. Shen et al. explained that one possible explanation of the CNTs' chemosensitising effect is the activity inhibition of the plasma membrane transporters (581). Their studies demonstrated that SWNTs, regardless the length and functionalisation type, significantly increased the cytotoxicity of arsenic trioxide, a substrate of the ABC membrane transporters (581). These reports could also add information to the increased intracellular concentration of CDDP presented previously.

Furthermore, oxidised SWNTs conjugated with chitosan and FA effectively chemo- and radiosensitised MDA-MB-231 and ZR-75-1 cells to 5-fluorouracil, epirubicin, pirarubicin, paclitaxel and carboplatin (582). Likewise, oxidised SWNTs improved the efficacy of paclitaxel both in vitro and in vivo, increasing the apoptosis rate and improving the hypoxia status (348).

Some of the mechanism associated were cell cycle arrest at G2/M-phase (487), ROS increase (488), downregulation of proteins related to the apoptosis regulation such as Bcl-2 and survivin, and upregulation of various caspases, LC3 and beclin-1 as well as reduction in the expression of HIF-1 and P-gp, linked with cellular resistance (348, 582). Add more here or in the next paragraph.

The DDSs presented in this thesis are promising anticancer treatments; however, of great interest as well is the potential development of multimodal therapies that may offer different solutions for patients. Our findings suggest an alternative to enhance the efficacy of conventional anticancer agents, while reducing the concentrations used. It has been speculated that chemosensitising may decrease the probabilities of cancer recurrence and resistance. Thus, possible sensitisation to other anticancer drugs and even in radiotherapy should be further studied.

6.2.3 Cell cycle assessment

The cell cycle is a sequential process that leads cells to replication. It is typically divided in four stages: (G1), cell growth and content duplication; (S), DNA synthesis; (G2), cell growth and final preparations to divide; and mitosis (M), chromosome condensation, alignment and separation for cell division. Non-dividing cells may exit the cell cycle to the quiescent phase (G0) (583).

The orderly progression of the cell cycle is dictated by extracellular events and is highly regulated by monitoring points (584). The checkpoints are signalling pathways regulated by cyclin-dependant kinases that evaluate the DNA integrity to avoid the proliferation of mutations; determinant for the transition or arrest of the cycle (585, 586). Alterations in the cell cycle are often associated with oncogenesis; therefore, the use of regulators and treatments that impact the cycle are promising anticancer therapies (585). DNA damage is usually accompanied by cell cycle arrest. G1 arrest involves the induction of p21 and the characteristic upregulation of p53, whereas G2 arrest correlates to the phosphorylation of cyclin complexes (587). p53 is also responsible for blocking the S-phase progression (399). Several conventional chemotherapeutics block the cell cycle, interfering with the proliferation of cells. For instance, studies suggest that DOX exerts the cytotoxicity via cell cycle arrest (588). Siu et al. observed the ability of DOX to prolongedly arrest G1 during the transition from G0, with a subsequent exit associated with the downregulation of p53; however, the G2 arrest was predominant (587). NPs have also suggested cell cycle affectations when used at toxic concentrations. AgNPs showed a concentration-

dependant increase of cells arrested in G2/M-phase (589); the cellular exposure to titanium oxide NPs also arrested the cycle in G2/M-phase (590), whereas graphene oxide and reduced graphene oxide depicted dose- and time-dependant arrest in G1-phase (591).

Considering the importance in the cellular mechanism, the cell cycle of MCF7 2D and 3D cultures exposed to CDDP, COOH-MWNTs and nanocomplexes was assessed. The objective was to determine whether the cytotoxic activity of the treatments impacted the cell cycle and compare such effects. Thus, the mean intensity of PI in treated cells (figure 6.4) and disaggregated MCTS (figure 6.5) was evaluated using flow cytometry techniques. Due to the number of compounds tested, to facilitate the comparison, the phases of the cell cycle were graphed separately.

MCF7 monolayers were the first culture to be analysed. Herein, CDDP-containing samples produced an evident concentration-dependant increase of the G1-phase proportion (figure 6.4A). Samples exposed to CDDP 12 μ M and 24 μ M depicted similar percentages. Free CDDP 12 μ M accumulated 24.3 $\pm$ 0.7% of the cells in G1-phase, whereas COOH-MWNTs 40 and 50 μ g/mL + CDDP 12 μ M values were 23.1 $\pm$ 0.7% and 22.7 $\pm$ 0.6% respectively. Similarly, free CDDP 24 μ M, with 31.1 $\pm$ 2.3% of the cells in G1 showed a difference of less than 1% to COOH-MWNTs 40 μ g/mL + CDDP 24 μ M and COOH-MWNTs 50 μ g/mL + CDDP 48 μ M.

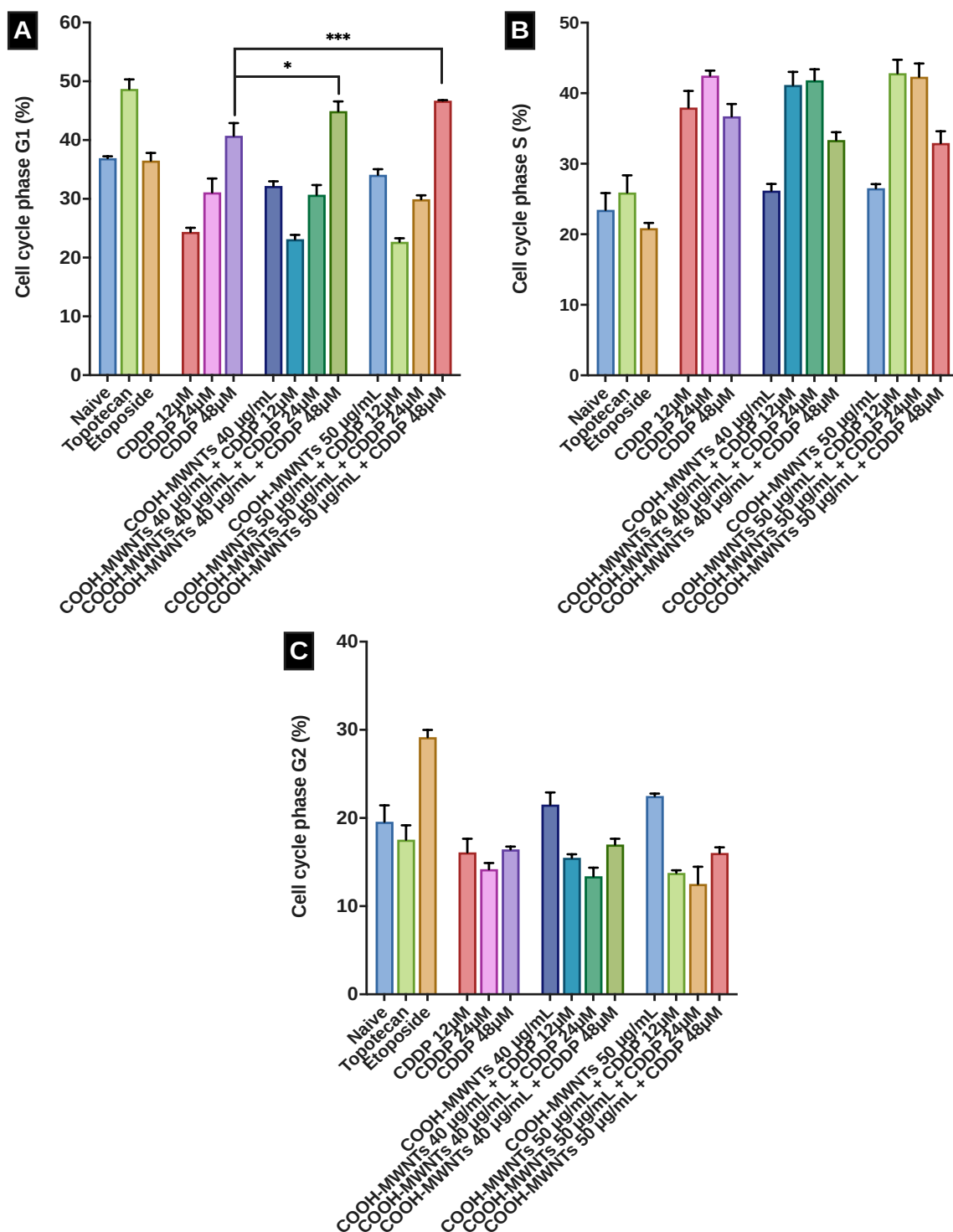


Figure 6.4 | Cell cycle distribution of 2D cultures exposed to free CDDP, carboxyl-functionalised MWNTs and nanocomplexes. Synchronised MCF7 cells were treated with increasing concentrations of free CDDP, COOH-MWNTs and combinations of these compounds for 24h. Cell cycle stages are represented as (A) G1-phase, (B) S-phase and (C) G2-phase. Topotecan and etoposide were used as positive controls for arrest in G1 and G2 phases respectively. Values represent the mean cell percentages of 3 individual replicates (n=3) $\pm$ SD. Statistical significance compared to free CDDP at equal concentrations: *p<0.05, ***p<0.001.

In contrast, cells treated with COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 48 μM ($44.9\pm1.6\%$) and COOH-MWNTs 50 $\mu\text{g/mL}$ + CDDP 48 μM ($46.7\pm1\%$) demonstrated statistically significant increase compared to the free drug at equal concentration ($40.7\pm2.1\%$). Interestingly, only cells treated with CDDP 48 μM presented a higher accumulation in this phase compared to the untreated control. The opposite was observed in the S-phase, where all samples exposed to CDDP revealed an arrest as compared to naïve (figure 6.4B). However, no significant difference was observed between these treatments at equivalent drug concentrations. The greatest cell accumulation in S-phase was produced by COOH-MWNTs 50 $\mu\text{g/mL}$ + CDDP 12 μM ($42.8\pm1.9\%$), very close value to COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 12 μM ($41.1\pm1.8\%$) and free CDDP 24 μM ($42.5\pm0.7\%$) with the double drug concentration. A drop in the S-phase arrest was observed at the highest CDDP concentration. COOH-MWNTs 50 $\mu\text{g/mL}$ + CDDP 12 μM depicted the greatest decrease to $32.9\pm1.6\%$. Nevertheless, most probably this reduction is related to the arrest in G1-phase.

Finally, no significant effects were observed over the G2-phase (figure 6.4C). Even though, a higher proportion of cells were found after incubating cells with COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 48 μM ($17\pm0.6\%$) and COOH-MWNTs 50 $\mu\text{g/mL}$ + CDDP 48 μM ($16\pm0.6\%$), no major difference was determined when compared to free CDDP. The only statistically significant difference detected in the addition of single COOH-MWNTs compared to naïve was at 50 $\mu\text{g/mL}$, with 3% higher accumulation in the G2-phase.

The different rate of proliferation, nutrients and oxygen diffusion in MCTS produce heterogeneous cell population. Spheroids tend to present dispersed cell cycle distributions as opposed to 2D cultures, where cells are likely in the same stage (509). Therefore, the assessment continued with disaggregated cells from MCTS exposed to the same treatments, concentrations and time. In these experiments, the predominant phase was G1; however, a slight S-phase arrest was detected in CDDP-containing samples (figure 6.5A).

The greatest accumulation in this phase depicted by free CDDP was at 24 μ M with 64.2 $\pm$ 4.8, while the COOH-MWNTs 40 μ g/mL + CDDP 48 μ M was the complex with highest effect with 61.3 $\pm$ 2.3%. Opposite to that, free CDDP produced the lowest percentage of cells in G1 (55 $\pm$ 2.3), which was compensated with the greatest proportion observed in the S-phase with 31 $\pm$ 2.2% (figure 6.5B). An increasing percentage in the sub-G1 population was observed as concentrations of CDDP-containing treatments increased, which is related to the presence of apoptosis. Also, an inverse concentration-dependant in cells treated with the free drug. The greatest S-phase arrest described by free CDDP was not too different from the combinatory treatments. COOH-MWNTs 40 μ g/mL + CDDP 24 μ M resulted in 26.7 $\pm$ 2.9% and COOH-MWNTs 50 μ g/mL + CDDP 12 μ M showed 25.7 $\pm$ 2.3% accumulated in S-phase. Lastly, G2-phase illustrated the lowest percentages of all samples (figure 6.5C).

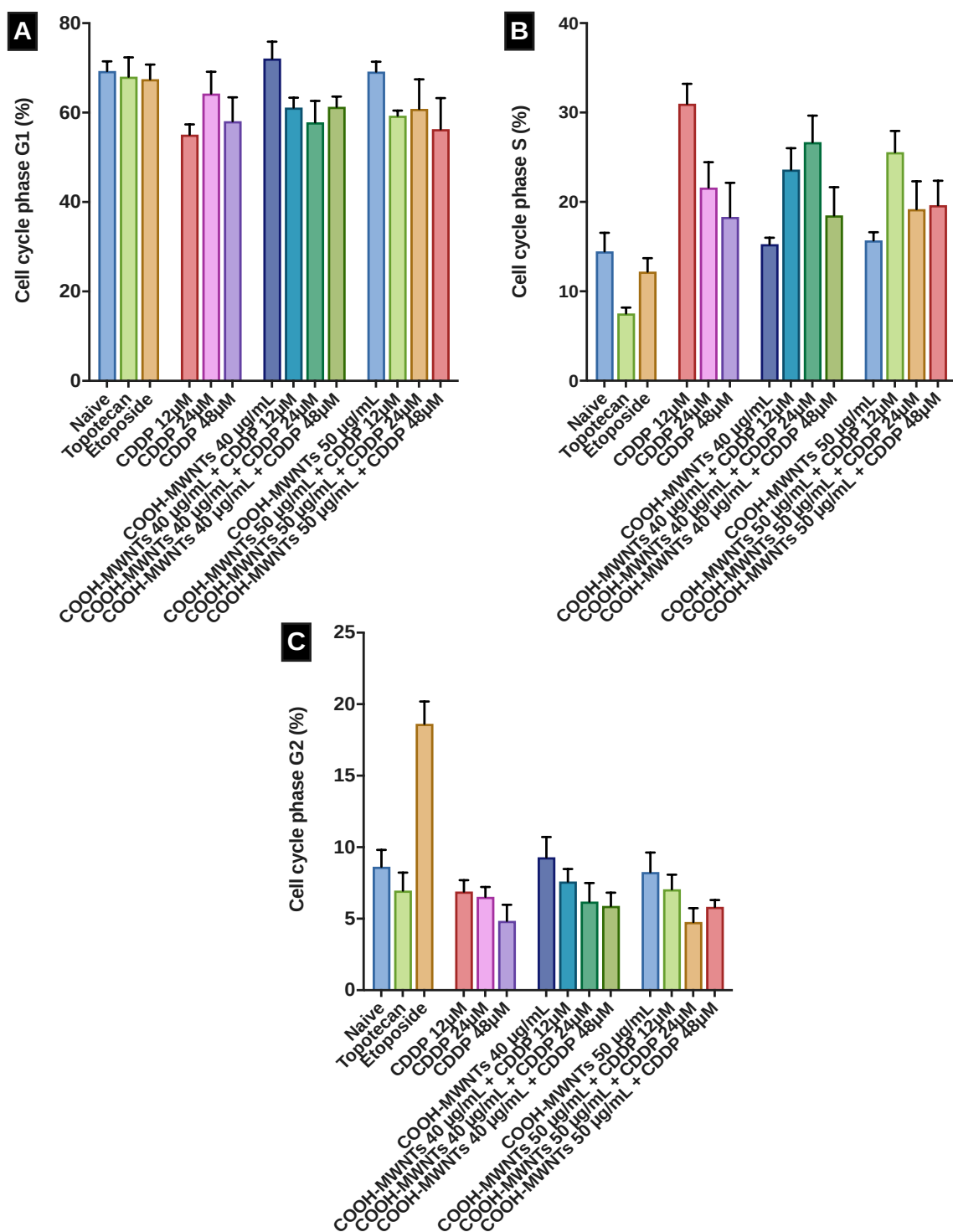


Figure 6.5 | Cell cycle distribution of disaggregated MCTS exposed to free cisplatin, carboxyl-functionalised MWNTs and nanocomplexes. MCF7 MCTS were treated with increasing concentrations of free CDDP, COOH-MWNTs and combinations of these compounds for 24h and disaggregated for their assessment. Cell cycle stages are represented as (A) G1-phase, (B) S-phase and (C) G2-phase. Topotecan and etoposide were used as positive controls for arrest in G1 and G2 phases respectively. Values represent the mean cell percentages of 3 individual replicates (n=3) $\pm$ SD.

Constant percentage diminishes were observed as CDDP concentrations augmented. Although no significant differences were calculated between free CDDP and nanocomplexes, the latter described greater proportions than free CDDP. Nonetheless, all values were similar to the untreated control. Moreover, all samples treated with the COOH-MWNTs alone produced comparable cell cycle distributions to those obtained with naïve, corroborating the safety of the nanomaterial. An arrest in G1/S-phase indicates that DNA has lost the integrity, so the cell machinery stops the cycle progression for DNA repair; S-phase arrest indicates the presence of unrepaired DNA areas, whereas an arrest in G2/M-phase prevent irregular cells from entering mitosis and division, mainly under the control of Cdk1/Cyclin B1 complex (592, 593).

Since the mechanism of action of CDDP damages the DNA, researchers have also described the irruption of the cell cycle. The DNA adducts that CDDP produce are mainly responsible for the affectation on the cell cycle, which induces apoptosis (151). However, all mechanisms implicated are not completely elucidated. CDDP may affect different cell cycle checkpoints. Typically, the drugs depicts a transitory S-phase arrest, followed by a lasting G2 phase arrest due to the inhibition of Cdc2-cyclin A and B kinases (150). Although infrequent, the cell cycle may progress due to the checkpoint sequence promoted by p16 and arrest cells in G1-phase (150). Moreover, investigations have suggested that SWNTs gradually downregulates the cell cycle genes *cdk2*, *cdk4*, *cdk6* and *cyclin D3*, arresting cells in G1-phase and inducing apoptosis (594). Increasing concentrations of the nanomaterial resulted in a concentration-dependant G1-

phase arrest that progressed to S-phase and G2/M-phase over time, with an increase in the sub-G1 peak (594). Likewise, other studies determined that MWNTs arrest cells in G1/S transition, with slight affectations in G2-phase (595). However, the removal of the nanomaterial rapidly retuned cells to cycle, proving that nanotubes only retard the cell proliferation. More importantly, MWNTs alterations to cell cycle were p53 and apoptosis independent (595). Experiments in A549 cells demonstrated that only concentrations as high as 300 µg/mL showed important affectations on the cell cycle and increased the early apoptotic population (399). It has also been described that G2/M arrest increases the cell sensitivity to CDDP (150).

Based on the results obtained, treatments demonstrated to induce a predominant cell cycle arrest in S-phase compares to untreated cells. Interestingly, 2D cultures exposed to free CDDP and nanocomplexes followed a concentration-dependant arrest in G1-phase, whereas MCTS depicted an increase cell population in sub-G1, suggesting apoptosis induction. Interruptions in the cells cycle are mainly associated with DNA damage and alterations in the expression of the checkpoint proteins such as p53 (150). Nonetheless, an imbalance in the ROS production and mitochondria damage may also cause cycle arrest (399, 596). Thus, complementary studies were conducted and presented next in this chapter.

6.2.4 Assessment of oxidative stress

ROS is a term used to designate several reduced and highly reactive molecules and free radicals derived from O_2 , such as peroxide, hydrogen peroxide, superoxide and hydroxyl radicals, among others (597). ROS are known to play a relevant role in pathways involving cell growth and differentiation, immune responses and, depending on the signalling, survival and cell death (598). Furthermore, oxidative reactions participate in essential biological processes like cellular respiration, metabolism, lipid synthesis and phagocytosis (599). Nonetheless, the imbalance of ROS has been associated with a number of pathologies like cardiovascular disease, neurogenerative conditions, metabolic disorders and oncogenesis (600). Such imbalance is defined as oxidative stress and occurs when the production of ROS exceeds the cell antioxidant mechanisms.

The relation between oxidative stress and cancer is not fully understood. However, since the mitochondrion is the principal source of intracellular ROS, it is believed that aberrant ROS levels impair the mitochondria machinery, which in turn is related to autophagy dysfunction and molecular changes, leading to cancer initiation, progression, chemoresistance and radioresistance (601, 602).

Oxidative stress may interact with several transcription factors and activate more than 500 different genes connected to growth factors as well as cell cycle and pro- and anti-inflammation mediators (603). For instance, peroxidases may arrest the cell cycle in G1-phase, by inhibiting cycle E/Cdk2 complex, whereas

hydrogen peroxide inhibits the progression of G2 to S-phase by blocking cyclin B/Cdc2 complex (604). Excessive intracellular ROS produce a global alteration, resulting in, among other effects, mitochondrial membrane damage, cell cycle irruption and apoptosis through cytochrome C translocation and activation of caspases (605).

Ironically, while ROS has been linked to tumorigenesis and cancer progression, various chemotherapeutics rely on ROS production as part of their mechanism of action. Such is the case of DOX, which generates high ROS levels by downregulating antioxidant enzymes, upregulating of pro-ROS proteins and disturbing the mitochondria electron transport chain (606). Moreover, elevated ROS levels present in cancer cells may be an important target of anticancer therapies focused on exploiting the redox alterations. In this context, several NPs can stimulate ROS imbalance, acting both as ROS scavengers and promoters (607). Having in mind the altered levels of ROS in cancer cells and effects of anticancer drugs and NPs, the total intracellular ROS was assessed in MCF7 2D and 3D models exposed to CDDP, COOH-MWNTs and nanocomplexes. The objective was to determine and compare the ROS generation between the different treatments. Thus, the mean intensity of fluorescence-labelled cells was evaluated using flow cytometry techniques (figure 6.6).

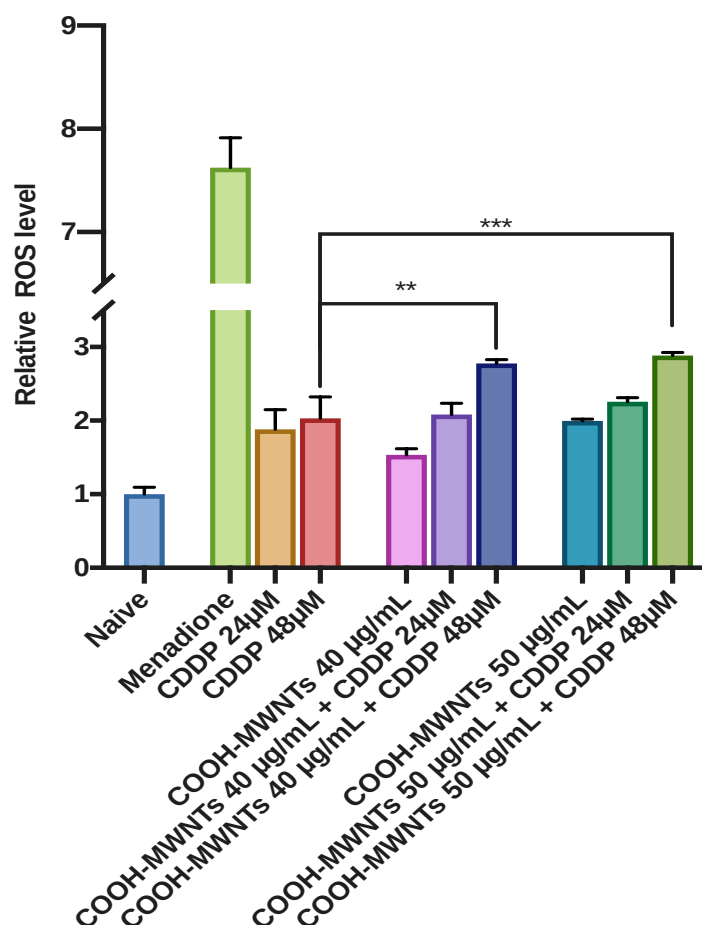


Figure 6.6 | Reactive oxygen species assessment in MCF7 cells exposed to CDDP, carboxyl-functionalised MWNTs and nanocomplexes. 2D cultures were treated with increasing concentrations of free CDDP, COOH-MWNTs and combinations of these compounds for 24h. Menadione was used as positive control for ROS generation. Cells were fluorescence-labelled and 20,000 events per sample were analysed by flow cytometry. Results represent the mean values of 3 independent replicates ($n=3$) $\pm$ SD. Statistical significance compared to free CDDP at equal concentrations: ** $p<0.01$, *** $p<0.001$.

Our results demonstrated a clear concentration-dependant increase of intracellular ROS in all samples. Particularly, the exposure to free CDDP depicted 1.88 ± 0.26 at $24\mu\text{M}$, increasing to 2.03 ± 0.29 at $48\mu\text{M}$. The free drug produced the smallest concentration-dependant relation. In contrast, nanocomplexes showed high ROS induction. COOH-MWNTs $40\mu\text{g/mL}$ + CDDP $24\mu\text{M}$ elevated the

cellular ROS to 2.08 ± 0.15 , whereas its combination with CDDP $48 \mu\text{M}$ resulted in 2.77 ± 0.05 . The latter effect was similar to that observed with the addition of COOH-MWNTs $50 \mu\text{g/mL}$ + CDDP $48 \mu\text{M}$ (2.88 ± 0.04) and significantly higher than free CDDP at equal concentration. Both COOH-MWNTs 40 and $50 \mu\text{g/mL}$ + CDDP $24 \mu\text{M}$ combinations showed higher ROS induction than free CDDP $48 \mu\text{M}$, with half the drug concentration, demonstrating the enhanced production of ROS.

Although the ROS levels generated by COOH-MWNTs were smaller than those detected by CDDP-containing treatments, they were significantly greater than the untreated control. While $40 \mu\text{g/mL}$ of nanomaterial displayed slightly higher intracellular ROS than naïve (1.53 ± 0.08), $50 \mu\text{g/mL}$ result was closer to that of free CDDP $24 \mu\text{M}$ (1.99 ± 0.02).

It has been described that ROS-inducer chemotherapeutics such as DOX and CDDP present difficulties to cause cytotoxicity in MCTS (517). Some of the mechanisms associated with drug resistance of MCTS are cellular arrangement, intracellular communication, improved repair (537), presence of hypoxic region and apoptosis resistance (608). Hence, to corroborate findings in 2D cultures, spheroids exposed to free CDDP $48 \mu\text{M}$, COOH-MWNTs $50 \mu\text{g/mL}$ and nanocomplexes were imaged to detect the presence of total ROS (red), whereas MCTS were also counterstained with a cell-permeant dye (blue) (figure 6.7 – 6.8).

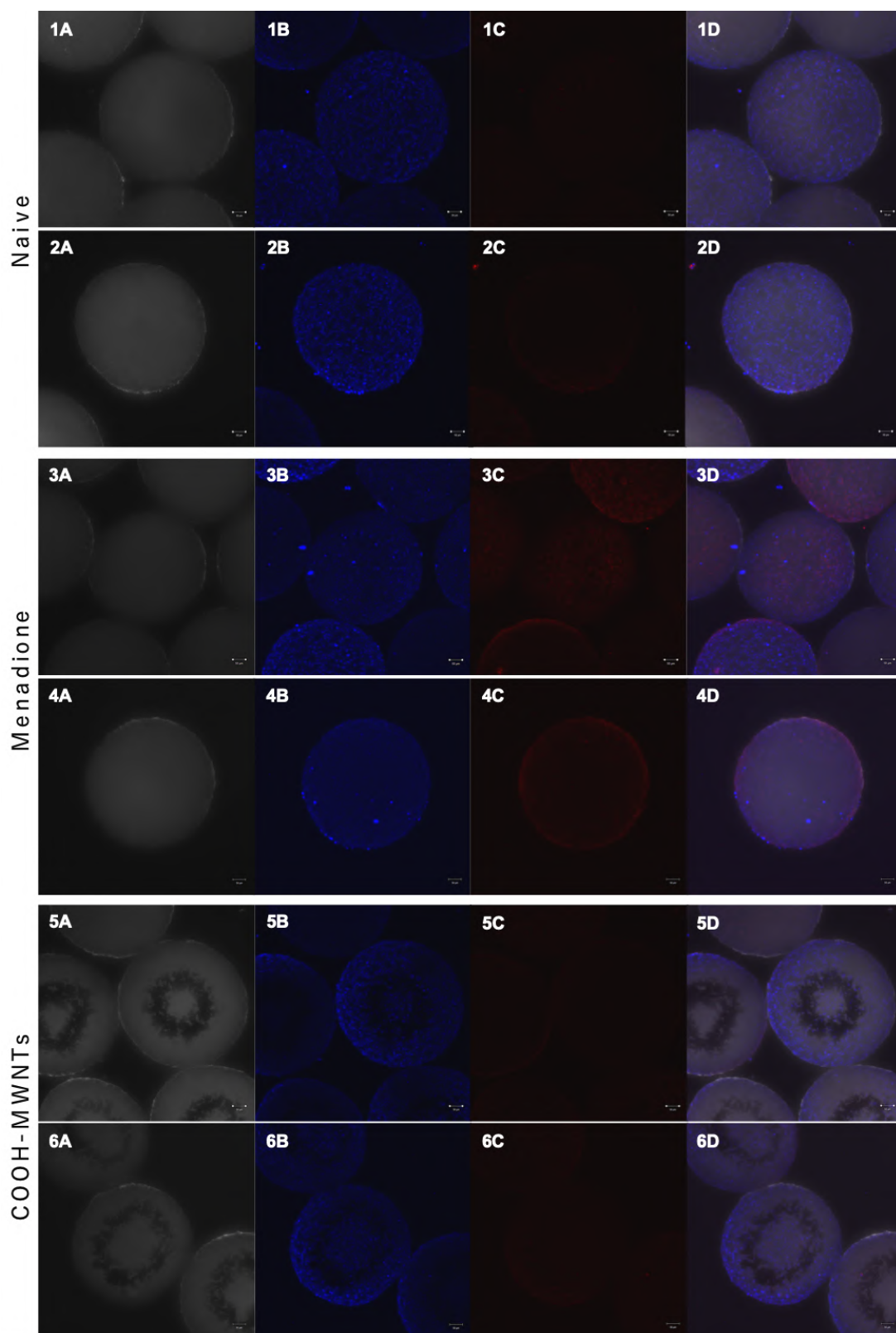


Figure 6.7 | Reactive oxygen species assessment of fluorescence-labelled MCF7 spheroids. Representative 3D projections of MCTS depicting: (1-2) untreated control, (3-4) menadione as positive control and (5-6) COOH-MWNTs treated for 24h. 3D cultures were labelled to represent: (A) transmitted light, (B) nuclear counterstain, (C) ROS and (D) merge.

The analysis of the controls was followed by the assessment of COOH-MWNTs. The transmitted light channel clearly illustrated the internalisation of the nanomaterial (figure 6.7, 5-6), which was confirmed by the quenching of the counterstain's blue fluorescence (figure 6.7, 5B-6B). Additionally, ROS presence was detected in small areas, especially in the outer layers (figure 6.7, 5C-6C). Similar findings were obtained after the addition of free CDDP 48 μ M. While the blue fluorescence described firmly attached cellular constructs (figure 6.8, 7-8), ROS was detected only in small sections of the spheroids. Finally, the exposure to the nanocomplex produced the greatest ROS-associated fluorescence of all tested compounds, confirmed by the purple tone in the merged microphotograph (figure 6.8, 9-10). Moreover, uptake of the nanomaterial into MCTS was evident. These results corroborated the quantitative ROS evaluation presented above, showing comparable ROS generation after the addition of free CDDP and the nanomaterial and high ROS induction of the combinatory treatment.

Maximum intensity 3D projections of untreated MCTS depicted compact cellular structures, uniformly dyed blue (figure 6.7, 1-2). Only very few red spots were observed, product of the normal redox processes. In contrast, the positive control menadione, a ROS-inducer agent, showed an overall red tone (figure 6.7, 3-4), which was confirmed in the merged image with purple colour, produced from the mixture of the ROS positivity and the blue counterstain.

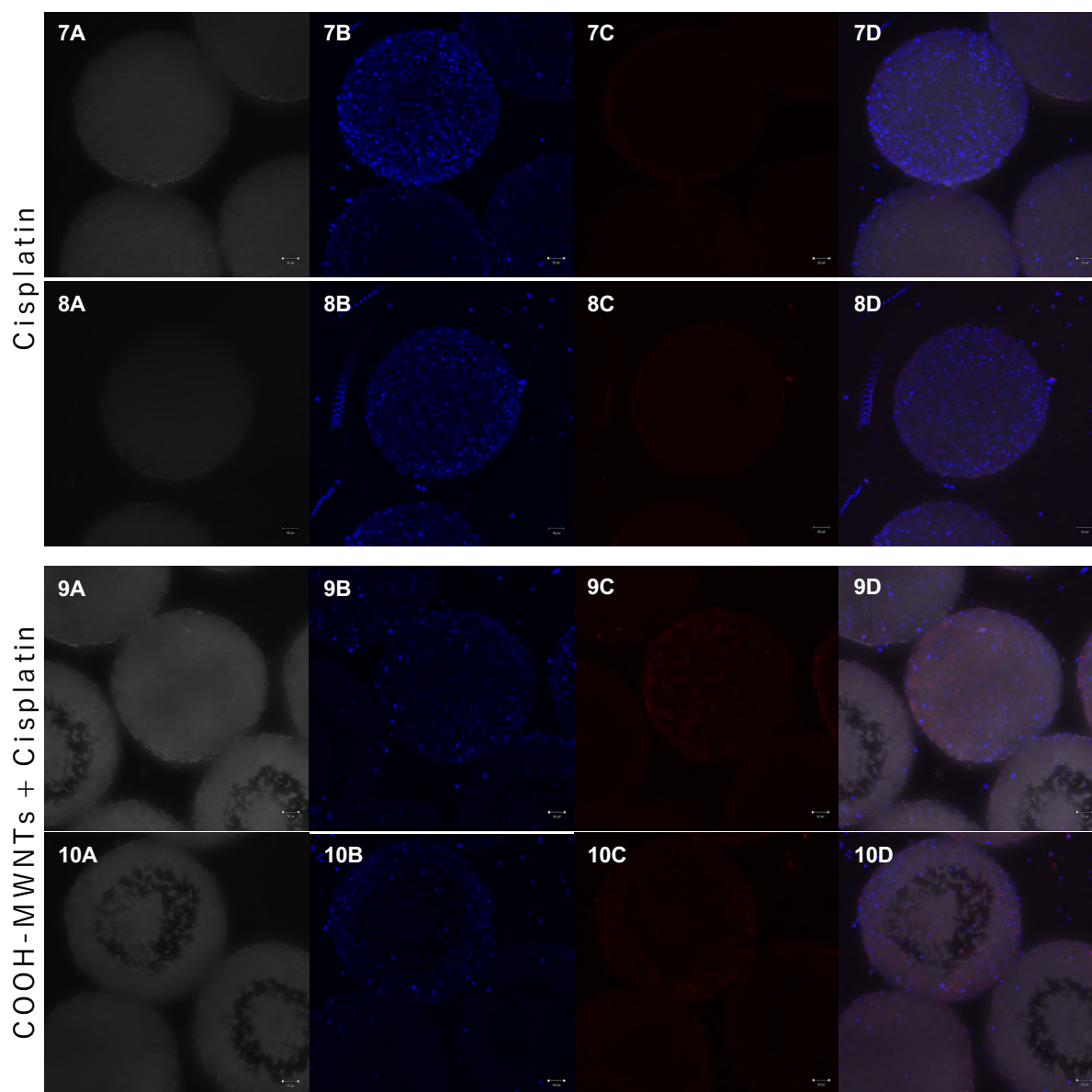


Figure 6.8 | Reactive oxygen species assessment of fluorescence-labelled MCF7 spheroids exposed to cisplatin and nanocomplexes. Representative 3D projections of MCTS depicting: (7-8) CDDP and (9-10) COOH-MWNTs + CDDP treated for 24h. 3D cultures were labelled to represent: (A) transmitted light, (B) nuclear counterstain, (C) ROS and (D) merge.

Reports have stated that oxidative stress is an important part of CDDP's cytotoxicity. There are various mechanisms involved in the ROS production, possibly being the mitochondrial damage the most important. Mitochondrion impairment results in inhibition of calcium uptake and energy production as well

as diminish of membrane potential and mitochondrial sulfhydryl-proteins (157). The latter effect is important as CDDP main targets are DNA and GSH.

GSH is a reducing peptide; crucial in the maintenance of the redox homeostasis (156). The tripeptide is a major protectant against damaging oxidants; besides, it is a critical co-factor for hydrogen peroxidase and takes part in the neutralisation of reactive metabolites produced in redox reactions (609). During detoxification GSH is oxidised to glutathione disulphide, catalysed by glutathione peroxidase in a cycling process that regenerates GSH by glutathione reductase (610).

Since oxidative stress depends not only on the ROS levels, but also on the antioxidant defence, and GSH has been referred to as vital antioxidant, we continued the investigation with the assessment of reduced GSH. Moreover, alterations in the GSH balance have been linked to development and progression of diseases (156); thus, MCF7 cells were exposed to free CDDP, COOH-MWNTs and nanocomplexes. Passed 24h, the samples were labelled, and the mean fluorescence intensities were collected, graphed and compared in figure 6.9.

In these experiments, the cell exposure to CDDP-containing treatments caused evident concentration-dependant GSH decrease trends. Free CDDP depicted limited effect over the GSH content; while 24 μ M produced the lowest reduction (84.4 $\pm$ 1.6%), 48 μ M showed was similarly efficient (79.3 $\pm$ 1.6%). As opposed to those results, nanocomplexes induced marked GSH inhibitions. The percentage

obtained after the addition of COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 24 μM combination was 2.2 less than the free drug at equal concentration. COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 48 μM showed a significantly deeper effect than free CDDP, lowering the intensity to $68.6 \pm 2.9\%$.

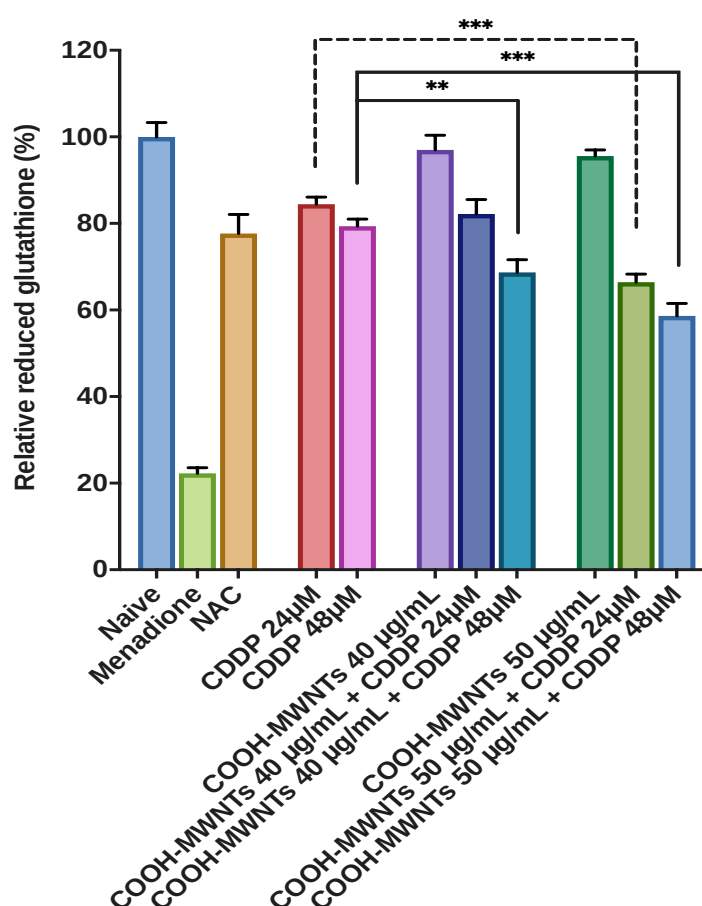


Figure 6.9 | Assessment of reduced glutathione in MCF7 cells exposed to CDDP, carboxyl-functionalised MWNTs and nanocomplexes. 2D cultures were treated with increasing concentrations of free CDDP, COOH-MWNTs and combinations of these compounds for 24h. Menadione was used as positive control for ROS generation, whereas NAC was added as reducing agent. Cells were fluorescence-labelled and 20,000 events per sample were analysed by flow cytometry. Results represent the mean values of 3 individual replicates ($n=3$) $\pm$ SD. Statistical significance compared to free CDDP at equal concentrations: ** $p < 0.01$, *** $p < 0.001$.

Furthermore, COOH-MWNTs 50 $\mu\text{g/mL}$ + CDDP 24 μM reduced the GSH concentration 15.7% more than COOH-MWNTs 40 $\mu\text{g/mL}$ + CDDP 24 μM and

almost 20% more than free CDDP, with half the drug concentration. COOH-MWNTs 50 µg/mL + CDDP 48µM produced the highest GSH inhibition with a reduction to $58.6 \pm 2.8\%$. Such value represented more than 20% higher effect than free CDDP 48µM, with 2-fold less drug concentration and more than 40% the GSH levels observed in the untreated control. The incubation with single COOH-MWNTs had insignificant GSH changes compared to naïve, with less than 5% decrease. As for controls, the addition of NAC, a reducing compound, showed a protective action against the ROS-generation of menadione.

As observed in the results, the presence of CDDP is definitive for the inhibition of reduced GSH. The nanomaterial alone depicted a minimal but increasing effect. However, COOH-MWNTs 40 µg/mL + CDDP 24µM and free CDDP 24µM showed the only comparable results between nanocomplexes and free drug, since all other combinations demonstrated higher GSH reduction. Therefore, we hypothesise that the presence of the nanomaterial is key to enhance the GSH inhibition.

The binding of CDDP to cytosolic GSH is considered an important mechanism of resistance as the drug is inactivated, avoiding DNA lesions, and effluxed through GS-conjugated export pumps (611) and MRP1 and MRP2, members of the ABC transporters family (156). Importantly, elevated ROS levels have been associated with the diminish of multidrug resistance by conversion of NADH to NAD in redox imbalance states to reduce the cell energy and thus block the ATP-dependant ABC transporters (612). Moreover, studies found that overexpression of these

transporters could be linked to lower levels of valuable molecules such as GSH, leading to a higher susceptibility of oxidative stress (613). In contrast, high levels of GSH along with overexpressed DNA repair mechanisms to overcome oxidative stress have been detected in brain and breast cancers as well as metastasis (538, 614), suggesting that GSH could also be a therapeutic target in certain situations.

Reports have pointed the possibility that CNTs generate ROS. The nanomaterial has affinity for the cell membrane, lysosome, mitochondria and nucleus, whose damage produce the increase of ROS levels (615). In vitro studies showed concentration-dependant ROS induction and decrease of GSH that led to intrinsic apoptosis after the addition of pristine SWNTs (616). Moreover, the in vivo administration of COOH-SWNTs concluded that the toxicity observed was related to oxidative stress (617). Nonetheless, the ROS cytotoxicity is mainly dictated by the surface functionalisation, physicochemical characteristics and purity of CNTs (383). For example, oxidised CNTs could be prone to generate ROS in the presence of reducing agents like NADH (618).

Based on the findings presented here, we concluded that the nanocomplexes not only induce ROS generation, but also, diminish the GSH levels, which most probably will lead to oxidative stress. Additionally, evidence suggests that the cell exposure to nanocomplexes cause cell death via apoptosis. Previously, cytotoxicity assessment assays showed that nanocomplexes cause cell death

mainly through late apoptosis and necrosis. Therefore, we continued to evaluate the mitochondria function and DNA integrity.

6.2.5 Assessment of apoptosis induction

Evidence indicates that toxic levels of ROS generated by chemotherapeutics like CDDP trigger cell death mainly through intrinsic apoptosis. The proposed mechanism suggests that free radicals interrupt the integrity of mitochondrial permeability transition pores (MPTP). The resulting augmented permeability releases and translocates the cytochrome C, which forms the apoptosome in complex with Apaf-1 and pro-caspase 9, followed by the activation of effector caspases, DNA fragmentation and death (612). Additionally, GSH levels can regulate apoptosis via S-glutathiolation (619).

Mitochondrial-generated ROS produce DNA injury, which can cause multiple mutations associated with irregular cell proliferation, survival and development of resistance. Chemoresistance has been linked to alteration in receptor tyrosine kinases and mitochondria malfunction adaptations to hypoxia, damage on the electron transport chain (605).

Several cellular stresses can trigger intrinsic apoptosis, including hypoxia, heat shock and mitochondrial DNA injury among others. Particularly, mitochondrial damage is considered a universal sign of apoptosis (620), which can block the synthesis of electron transport chain-related proteins, impairing the respiratory

chain and leading to further ROS generation and loss of mitochondrial membrane potential ($\Delta \Psi m$) (621).

Mitochondria function is essential in the apoptotic process, playing a crucial role in the control of energy production, mitochondria membrane potential and release of apoptotic effectors (622). The $\Delta \Psi m$ is produced during the oxidative phosphorylation in the ATP generation. In many systems, the loss of $\Delta \Psi m$ is considered an early marker of apoptosis (622). Alternatively, membrane depolarisation may lead to ATP depletion due to the impaired synthesis. Apparently, under decreased $\Delta \Psi m$, the inner mitochondrial membrane becomes permeable, this is accompanied with ATP decrease levels, higher mitochondrial calcium content and ROS production, involving the MPTP (621).

Flow cytometry-based assays for the study of $\Delta \Psi m$ use cationic lipophilic dyes that concentrate in high protonated sites such as the inner mitochondrial membrane. Probes are derivatives of rhodamine, carbocyanine and MitoTracker, whose fluorescence intensity is proportionally direct to the proton concentration (620).

All considered, as an important indicator of apoptosis and due to the relation with oxidative stress, we evaluated the effect of free CDDP, single COOH-MWNTs and nanocomplexes on the mitochondrial membrane depolarisation. Thus, using flow cytometry techniques, the fluorescence intensity and shifting of 24h-treated cells were determined. Figure 6.10 illustrates the relative percentage of $\Delta \Psi m$.

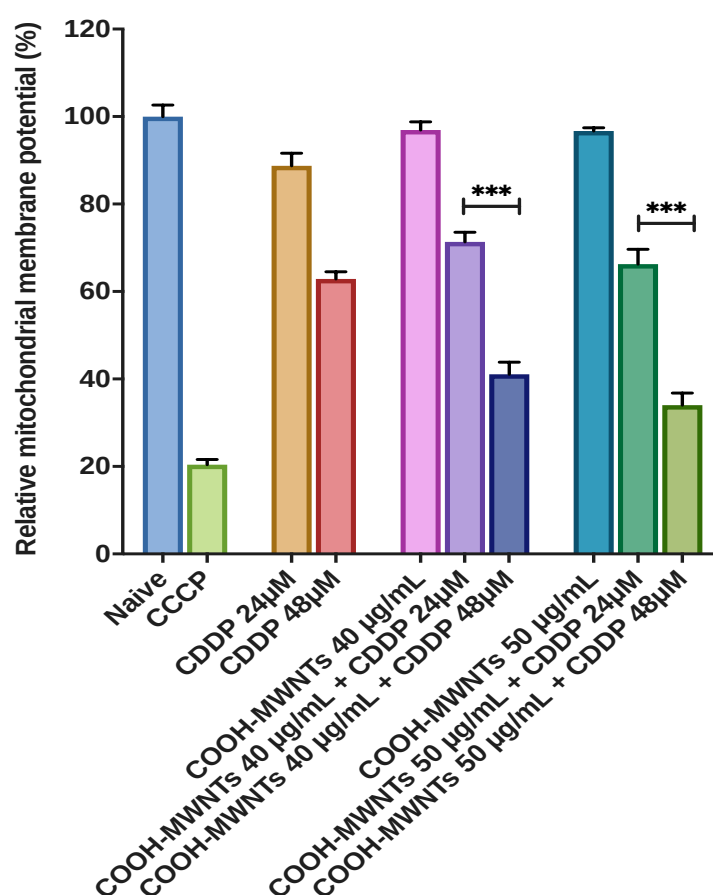


Figure 6.10 | Mitochondrial membrane potential assessment in MCF7 cells exposed to CDDP, carboxyl-functionalised MWNTs and nanocomplexes. MCF7 cells were treated with increasing concentrations of free CDDP, COOH-MWNTs and combinations of these compounds for 24h. The mitochondrial uncoupling agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as positive control. Then, cells were fluorescence-labelled and 20,000 events per sample were analysed by flow cytometry. Results represent the mean values of 3 individual replicates ($n=3$) $\pm$ SD. Statistical significance compared to free CDDP at equal concentrations: *** $p<0.001$.

Herein, the presence of CDDP produced a notable mitochondrial membrane depolarisation in a concentration-dependant manner. Firstly, the impact of single COOH-MWNTs was almost unnoticeable. Both tested concentrations reduced the relative percentage less than 4%, suggesting limited damage on the mitochondria. Opposite to that, free CDDP 24µM induced lower membrane potential with $88.7\pm2.8\%$, while the higher concentration reduced the relative

percentage to $62.9 \pm 1.6\%$. Overall, nanocomplexes caused significantly higher mitochondrial depolarisation. COOH-MWNTs $40 \mu\text{g/mL}$ + CDDP $24 \mu\text{M}$ resulted in a reduction to $71.3 \pm 2.2\%$; such value was 5% greater than COOH-MWNTs $50 \mu\text{g/mL}$ + CDDP $24 \mu\text{M}$. Nonetheless, more pronounced effect was observed following the addition of combinations containing $48 \mu\text{M}$ of CDDP. Particularly, COOH-MWNTs $50 \mu\text{g/mL}$ + CDDP $48 \mu\text{M}$ showed an important depolarisation of $34 \pm 2.7\%$; the complex depicted an enhanced effect of almost 30% greater than free CDDP at same concentration and an amazing 2.6-fold more than free CDDP $24 \mu\text{M}$. Once again, the use of the carboxylated nanomaterial demonstrated to potentiate the effect of CDDP.

These results demonstrated a clear loss of $\Delta \Psi_m$, which most probably is indicating that cell exposed to tested compounds underwent apoptosis. A correlation between ROS-related cytotoxicity of CDDP with membrane depolarisation has been established. Studies have demonstrated that CDDP induces mitochondrial ROS, causing oxidative stress, mitochondrial DNA injury, collapse of $\Delta \Psi_m$, alterations in the respiratory chain and apoptotic cell death. (623). Moreover, CDDP causes mitochondrial damage leads to metabolic dysfunction, causing ADP-ATP imbalance and glycolysis inhibition (623). As for CNTs, a comparative analysis proved that both SWNTs and MWNTs showed concentration-dependant toxicities in skin mitochondria (624). However, SWNTs generated greater levels of mitochondrial ROS, $\Delta \Psi_m$ loss, ATP reduction and cytochrome C release, causing apoptosis (624). It is important to mention that

the nanomaterials used in such experiment were long, contained a considerable percentage of impurities and were coated with Triton X-100.

Importantly, CNTs-induced membrane depolarisation is dependent on surface chemistry and hydrophobicity (618). Investigators explained a marked membrane depolarisation as result of a rapid ROS increase after the incubation of macrophages with pristine, functionalised and annealed MWNTs were observed. Nonetheless, such effects were successfully balanced due to cellular antioxidant activity (618). Another principal feature of apoptosis is the DNA fragmentation into nucleosomal-size fragments. Considered a late apoptotic process, double-stranded breaks are the result of caspase-dependant cascade that activates the DNA fragmentation factor and, with the help of high-mobility group proteins and histone H1, will cleave genomic DNA into units of about 180bp (625, 626). Although caspase-3 is described as a primary activator of DNA fragmentation, the MCF7 cell line, which lacks such apoptotic effector, can activate the DNA degradation via caspase-7 (627).

The TUNEL assay is a frequently used method to detect double- and single-stranded DNA breaks in situ samples or cell suspension by flow cytometry. The probe catalytically incorporates fluorescein-12-dUTP-modified terminal deoxynucleotidyl transferase in free 3' OH DNA ends of apoptotic cells to generate a polymeric tail that can be analysed by fluorescence microscopy (628). In this work, such assay was used to evaluate DNA damage in MCF7 MCTS. Hence, spheroids treated with high concentrations of free CDDP, COOH-MWNTs

and a complexation of these compounds were cryosectioned, fixed and labelled. Figures 6.11 and 6.12 illustrate representative microphotographs of samples analysed using confocal imaging techniques.

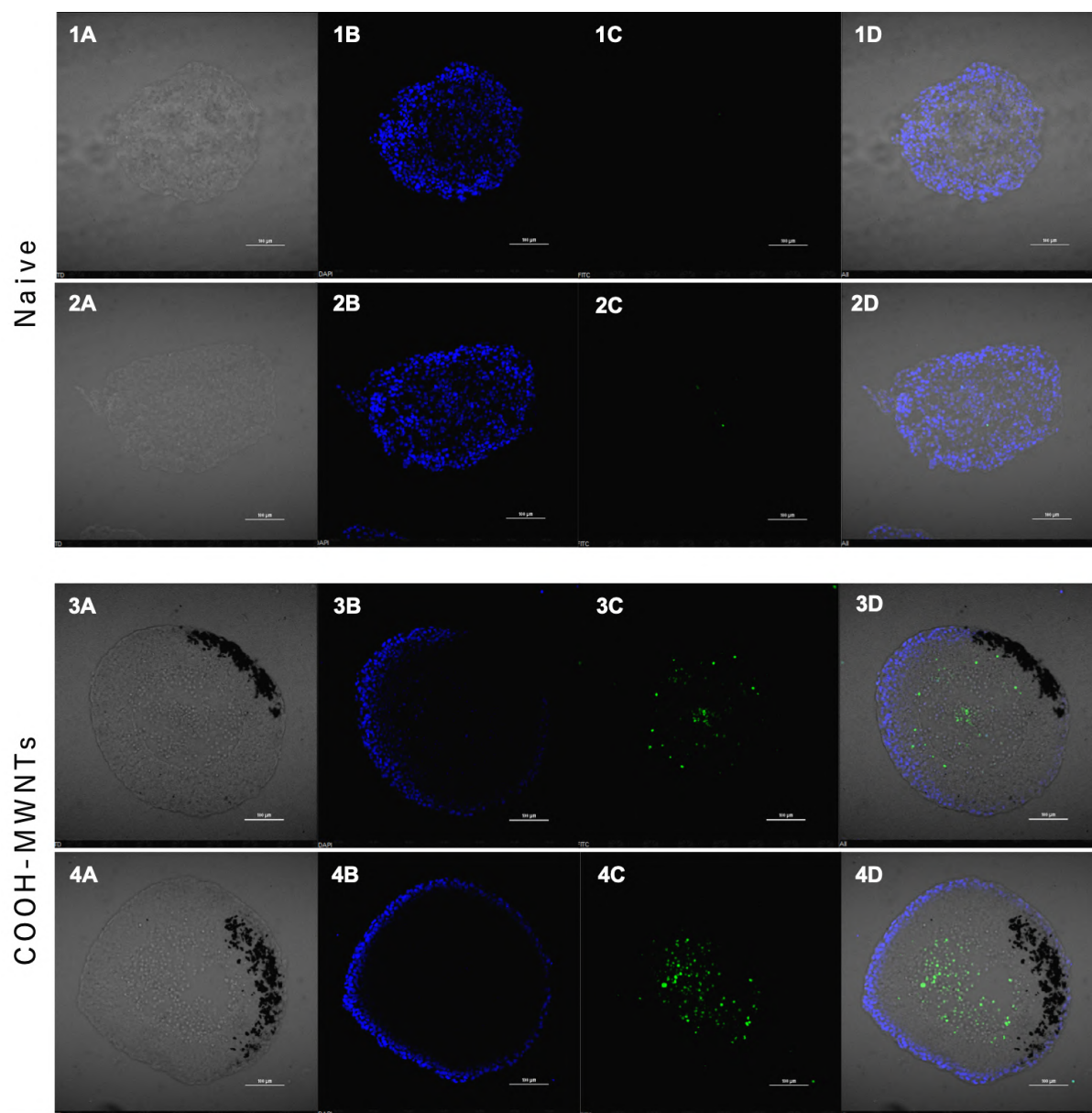


Figure 6.11 | DNA fragmentation assessment of fluorescence-labelled cryosections from MCF7 spheroids. Representative maximum projections of sectioned MCTS depicting: (1-2) untreated control and (3-4) COOH-MWNTs exposed for 24h. Cryosections were labelled to detect: (A) transmitted light, (B), Nuclei counterstain, (C) DNA fragmentation and (D) merge.

Resulting sections obtained from untreated spheroids (figure 6.11, 1-2) depicted compact cellular structures with few to complete absence of DNA fragmentation (figure 6.11, 1-2C). In contrast, the 24h incubation with COOH-MWNTs showed DNA damage, particularly localised in the centre of the MCTS (figure 6.11, 3-4C).

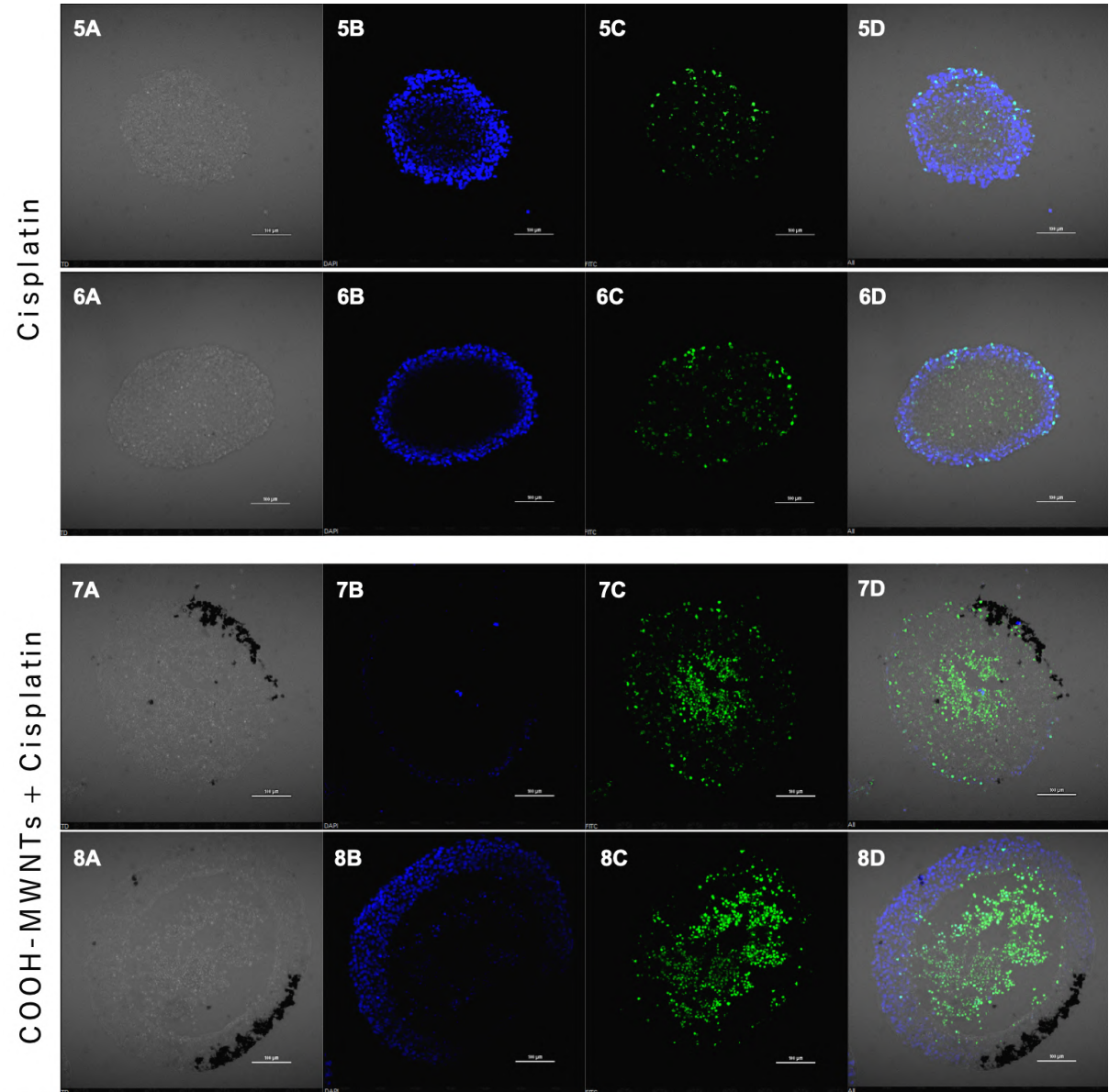


Figure 6.12 | DNA fragmentation assessment of fluorescence-labelled cryosections from MCF7 spheroids exposed to cisplatin and nanocomplexes. Representative maximum projections of sectioned MCTS depicting: (5-6) free CDDP and (7-8) COOH-MWNTs + CDDP treated for 24h. Cryosections were labelled to detect: (A) transmitted light, (B), Nuclei counterstain, (C) DNA fragmentation and (D) merge.

These results agree with results from cytotoxicity assays, where limited inhibition was observed. Moreover, the damage could be related to the increased levels of ROS observed in previous experiments. Besides, the counterstain dye failed to thoroughly label the 3D structures (figure 6.11, 1-2B), possibly due to the quenching of the nanomaterial that was clearly internalised into the spheroids. We can speculate that the degree of penetration may be related to the exposure time, increasing at longer periods. Then, the analysis with free CDDP and nanocomplex followed.

As expected, the presence of CDDP notably increased the DNA fragmentation compared to previous samples. MCTS treated with free CDDP 48 μ M were, in general, condense and well counterstain. As opposed to samples treated with the nanomaterial, where fluorescence was localised in specific areas, herein, DNA breaks were found dispersed in the whole spheroid, especially in the outer layers (figure 6.12, 5-6C).

Nevertheless, the exposure to COOH-MWNTs 50 μ g/mL + CDDP 48 μ M displayed the highest proportion of DNA fragmentation. Damage was detected throughout the multicellular cellular, with greater effect in the core (figure 6.12, 7-8C). These results corroborate past findings, which demonstrated an enhanced therapeutic efficacy of nanocomplexes compared to free CDDP. Additionally, mechanistic experiments constantly showed increased effects. The DNA fragmentation distribution on MCTS, with particular damage in inner zones, could explain the disaggregation observed at longer treatment exposures. In this samples, the

presence of COOH-MWNTs was spotted, evidently quenching the fluorescence of the counterstain (figure 6.12, 7-8A and 7-8B respectively). Besides, inconsistent cell distribution was noticed in certain areas of the sections, which could be the initiation of the disaggregation.

In summary, this chapter presented the mechanistic characterisation of COOH-MWNTs, free CDDP and combinations of these compounds in 2D and 3D cell models. With the objective of providing insights about cellular processes involved in the enhanced therapeutic efficacy observed in previous chapters, comparisons were performed in all experiments, paying particular attention to nanocomplexes. A higher intracellular concentration of CDDP was detected after the exposure to nanocomplexes. A greater accumulation is probably due to the nanoneedle mechanism; an energy independent uptake pathway characteristic of CNTs. Furthermore, short-time pre-incubation with low concentrations of COOH-MWNTs demonstrated the ability to sensitise cells to the platinum drug, which could be due to activation or downregulation of cellular pathways involved in the response to chemotherapy. One of these processes is the cell cycle that followed an arrest after treatments. Besides, a significant increase in the ROS generation was measured, accompanied by a diminish of GSH levels. The imbalance produced most probably led to cellular damage and apoptosis induction. Indicators of early and late apoptosis were found altered, confirming that nanocomplexes caused enhanced cytotoxicity via the programmed cell death.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

7.1 General conclusions

Over the last years, NPs have attracted enormous attention due to the increasing applications in the biomedical field. The unique characteristics of CNTs have opened a wide range of potential opportunities in the areas of energy, electronics, and materials among others (279, 282). Moreover, in cancer research, CNTs have proved great potential to conjugate/incorporate a diversity of molecules and transport them preferentially to their site of action due to the EPR effect (358). A fundamental objective of this project was the development of nanocomplexes. One of the drawbacks of non-covalent conjugation of drugs to CNTs is the low efficiency of attachment and the possibility of early release before reaching the target. On the other hand, covalent attachment of drugs, although a feasible and highly efficient procedure, it can lead to chemical modifications in the drugs, reducing the efficacy (394). Hence, all MWNTs+drug conjugations were based on non-covalent interactions.

MWNTs can be widely functionalised, providing different particularities and features that are associated with their effects, interactions and toxicity (384). Thus, the development of new therapeutic approaches must be accompanied with a thorough examination of the inherent properties of both nanocarrier and DDSs. Herein, physicochemical characterisation of pristine MWNTs, copolymer-coated MWNTs and chemically derivatised MWNTs were performed to determine the surface derivatisation, particle length, superficial charge, composition and stability. Results corroborated the effects of functionalisation on the nanomaterial purity, aqueous-dispersibility and length reduction. Zeta potential values were related to the functional groups attached to the MWNTs' surface; the addition of CDDP shifted the charge to neutrality. However, the modification did not alter the cytotoxicity.

An essential step of the development process was the cytotoxicity evaluation of nanocarriers and therapeutic assessment of nanocomplexes. For these purposes, MCF7 breast adenocarcinoma cells were used to determine the cytotoxicity related to the nanomaterials and antitumour drugs, proving that COOH-MWNTs was safest nanomaterial, while F127-MWNTs showed the highest toxicity (proliferation inhibition). These tests were useful to optimise the concentrations of the components incorporated into combinations. The MWNTs+DOX therapeutic analysis demonstrated comparable cell inhibition rates of those observed by free DOX. NH_3^+ -MWNTs+DOX was the exception, resulting in significant cell viability decrease. In contrast, all MWNTs+CDDP conjugations displayed an impressive anticancer activity enhancement compared to the

platinum drug alone. The reason behind the efficacy difference between the two chemotherapeutic conjugations could be the interactions between components dictated by their chemical structures and resulting in different rates of drug release.

These results were corroborated using an inexpensive method to produce size-homogeneous MCF7 MCTS developed herein. Microphotographs obtained from spheroids treated with COOH-MWNTs and NH_3^+ -MWNTs illustrated the uptake of the nanomaterial without damage to the cellular structure. On the other hand, although MWNTs+DOX showed clear structural damage to the 3D cultures, such results were only slightly better than those obtained with free DOX. Although free CDDP demonstrated an important growth inhibition in treated MCTS, the effects were far from nanocomplexes'. MWNTs+CDDP not only caused significantly greater damage to 3D cultures, but also it was such that spheroids were disaggregated after 48h of exposure to high concentrations of the nanocomplexes. The structural damage of treated spheroids was further studied using fluorescence-labelling probes. Whereas the emission observed using imaging techniques confirmed the enhanced cytotoxicity of combinatory treatments, Annexin-V-FITC/PI analysis established that the mechanism of cell death involved was mainly late apoptosis/necrosis. The anticancer potentiation is not only important to inhibit the proliferation of cancer cells and MCTS' size, but also could lead to a reduction of the drug administration and diminish the side effects associated, which is a major limitation in current therapies.

Further studies were performed to provide insights about the mechanism of action underlying the potentiated effect. One of the main mechanisms associated with diminished cytotoxic response is the reduced intracellular accumulation of anticancer drugs. Consequently, the quantification of Pt, the metal atom of CDDP core, revealed a significantly greater accumulation of the drug in cells exposed to COOH-MWNTs+CDDP compared to those incubated only with CDDP. Furthermore, concentrations as low as 10 mg/mL of carboxylated nanomaterial proved the ability to chemosensitise cells to CDDP, resulting in increased cell inhibition. We speculate that the characteristic nanoneedle mechanism of CNTs, an energy-independent cell uptake pathway tested in this thesis, can increase the drug internalisation and thus promote a higher intracellular concentration. Subsequent examinations of the damage caused by such cell accumulation, established an oxidative imbalance state, result of the augmented ROS generation contrasting the declined reduced GSH levels. These results were associated with the induction of DNA damage, leading to S-phase cell cycle arrest, an increasing G1-phase block at high concentrations of nanocomplex and concentration-dependant sub-G1 population in MCTS. These events concluded in mitochondrial depolarisation and cell death via apoptosis.

Although this thesis focused on the development of MWNTs-based complexes integrating CDDP and DOX directed to the treatment of BC, conjugation methods can serve as basis of new therapeutic approaches of other cancers and even other pathological conditions that may influence beyond the oncology field. Furthermore, the method to produce mammospheres proved success on the

development of spheroids of other cancer and non-cancer cell lines (data not presented). Affordable 3D in vitro models that mimic the in vivo environment may lead to predictive screening models, refinement and reduction in the use of experimental animals and enhance the success rates of new therapies. Finally, the mechanistic insights provided herein offer contributions to future explorations. Altogether, these findings could lead to alternative strategies and advances of current anticancer therapies, helping to reach the ultimate goal of improving the patients' lives.

7.2 Future work

This thesis described a diversity of studies, analyses, and comparisons that are essential for subsequent evaluation stages. Nonetheless, throughout this research project, important aspects were detected that would deserve further investigation to fulfil the expectations generated.

The characterisation of MWNTs presented attributes inherent to the nanomaterial and covalent or non-covalent derivatisation. It has been described that these features influence the toxicity and interactions with cells and other components (384, 571, 615). However, toxicity reports on CNTs are contradictory, so additional evaluation of microscopic and spectroscopic techniques like x-ray photoelectron spectroscopy and SEM, computational modelling and molecular simulations could bring a better understanding of the composition and behaviour. Besides, the study of protein corona and MWNTs inclusion in other biomedical

relevant media such as plasma could help expand the understanding of interactions, stability and possibly predict effects and outcomes.

Several functionalisation reactions have been reported mainly to improve the dispersibility and individualisation of CNTs, but also to couple compounds (256, 358). Worthwhile modifications of developed nanocomplexes could include the attachment of antibodies for targeted cargo delivery, pH- and temperature-sensible moieties and prolonged circulation systems.

The antitumour activity assessment of nanocomplexes was focused on MCF7 2D and 3D cultures. However, CDDP and DOX are not only first-line treatments for breast cancer, but standard therapy for several human cancers. Therefore, the efficacy evaluation on other breast cancer cell lines as well as additional cancer models or even complex cell organisations like patient-derived organoids can bring clarity about the therapeutic spectrum. Equally significant, is the cytotoxicity and mechanism evaluation on chemoresistant cell lines. A major goal of new anticancer approaches is to overcome resistance. Hence, success in this matter would offer key information in the search for solutions.

Supplementary studies of the mechanism of action may include detailed analysis of cellular uptake, signalling pathways, characterise the drug release, more precise imaging techniques to assess cell-nanocomplex interactions, and deepen the understanding of cell death process. For instance, the Organisation for Economic Co-operation and Development (OECD) suggests a battery of in vitro

assays to determine the genotoxicity of nanomaterials; benchmark tests for MWNTs are the micronucleus assay and comet assay (618, 629). Furthermore, subsequent in vivo studies could include the therapeutic efficacy assessment in xenografts and metastasis models, as well as toxicological and pharmacokinetics evaluation.

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APPENDIX

CHAPTER 5 SUPPLEMENTARY DATA

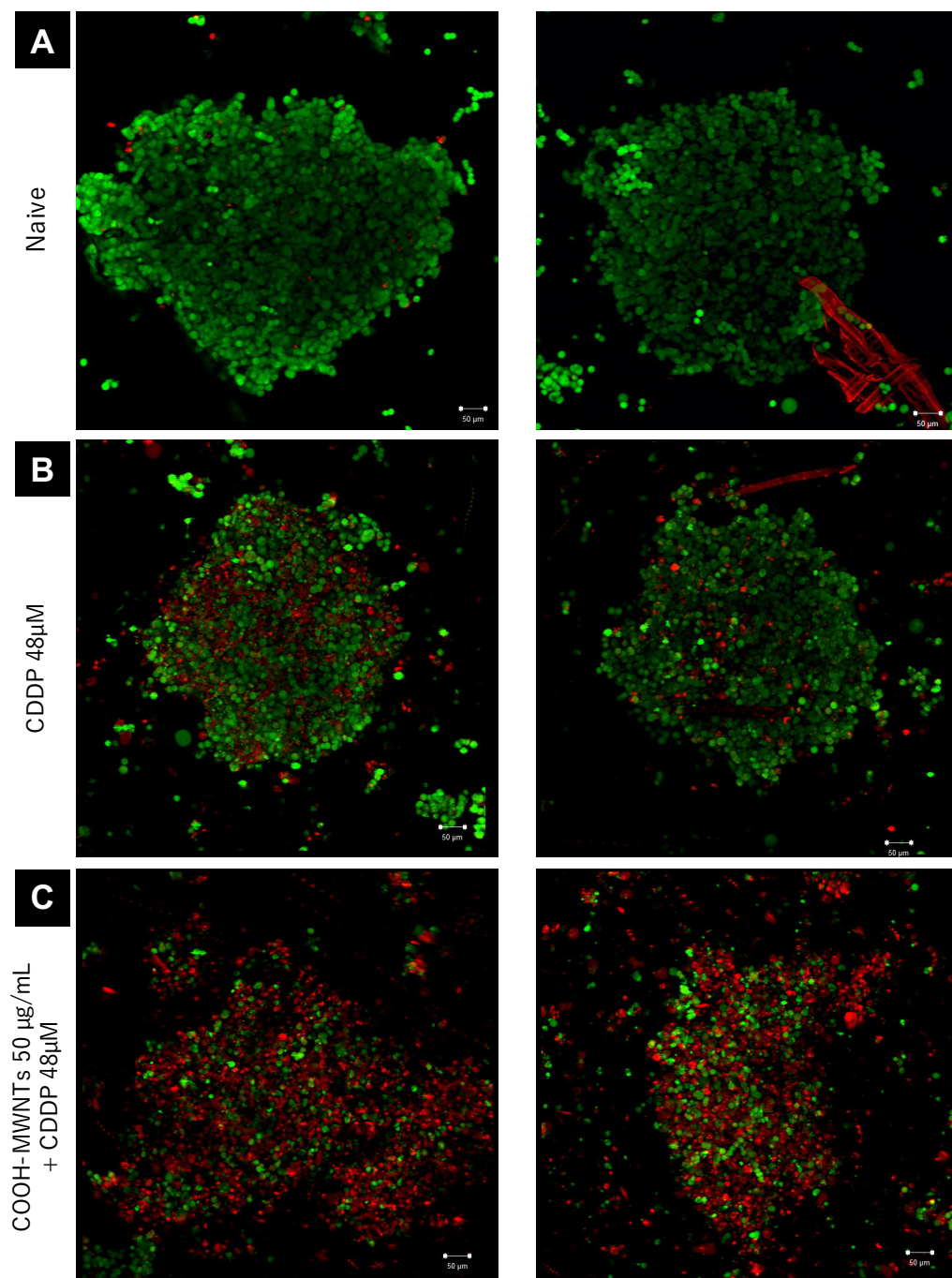


Figure A1 | Cytotoxicity assessment of aminated-functionalised MWNTs + cisplatin complexes in fluorescence-labelled spheroids. 3D projections of treated A549 MCTS depicting: (A) naive, (B) free CDDP, and (C) NH_2 -MWNTs 50 µg/mL + CDDP 48µM complex for 24h. A549 MCTS were stained to differentiate living cells (green) and dead cells (red).

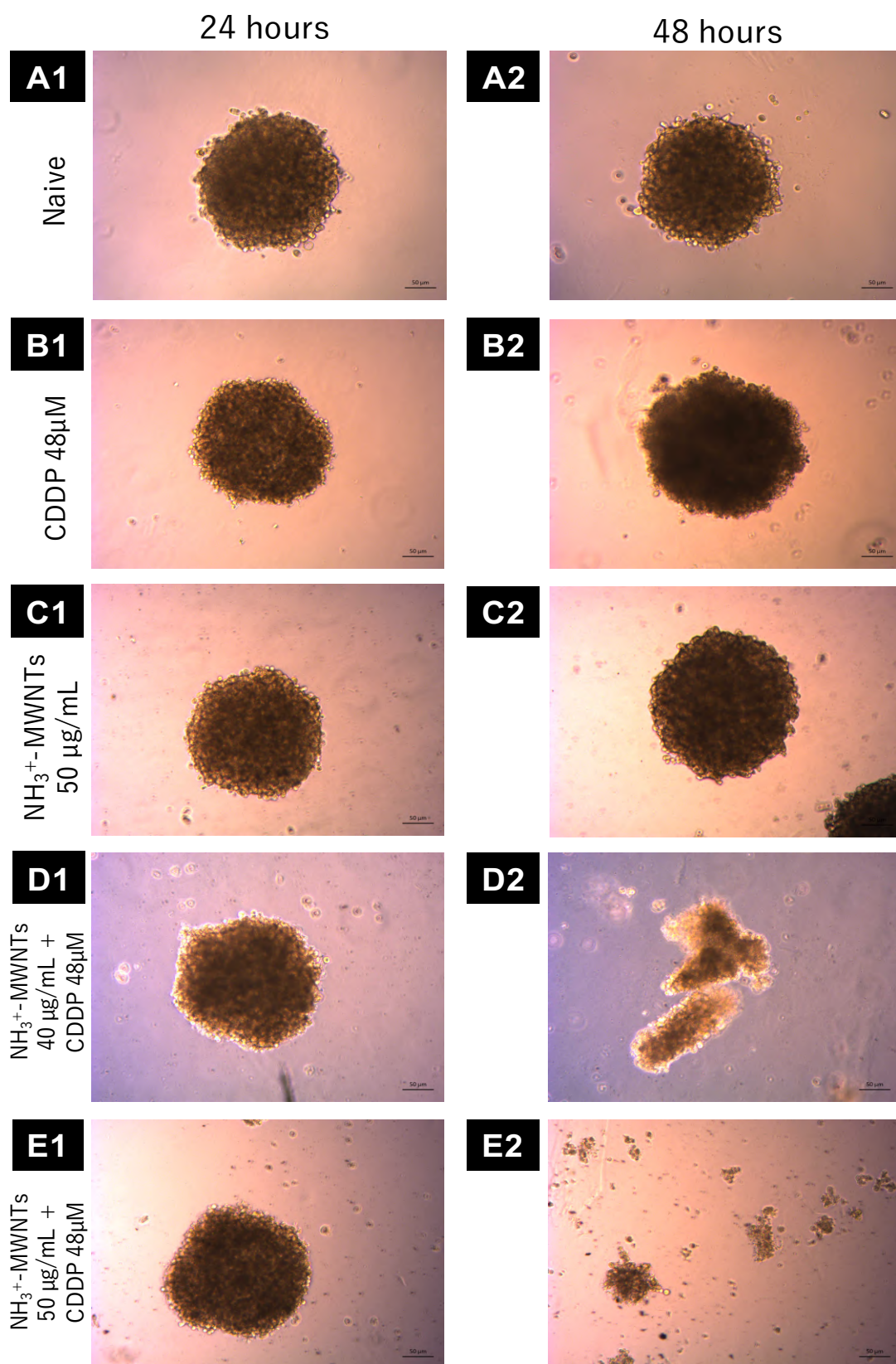


Figure A2 | Microphotographs of A549 MCTS treated with amine-functionalised MWNTs + cisplatin complexes. Images of A549 MCTS depicting: (A) untreated control, (B) free CDDP, (C) single NH₃⁺-MWNTs, (D) NH₃⁺-MWNTs 40 μg/mL + CDDP 48μM complex, and (E) NH₃⁺-MWNTs 50 μg/mL + CDDP 48μM complex for (1) 24h and (2) 48h post-treatment.

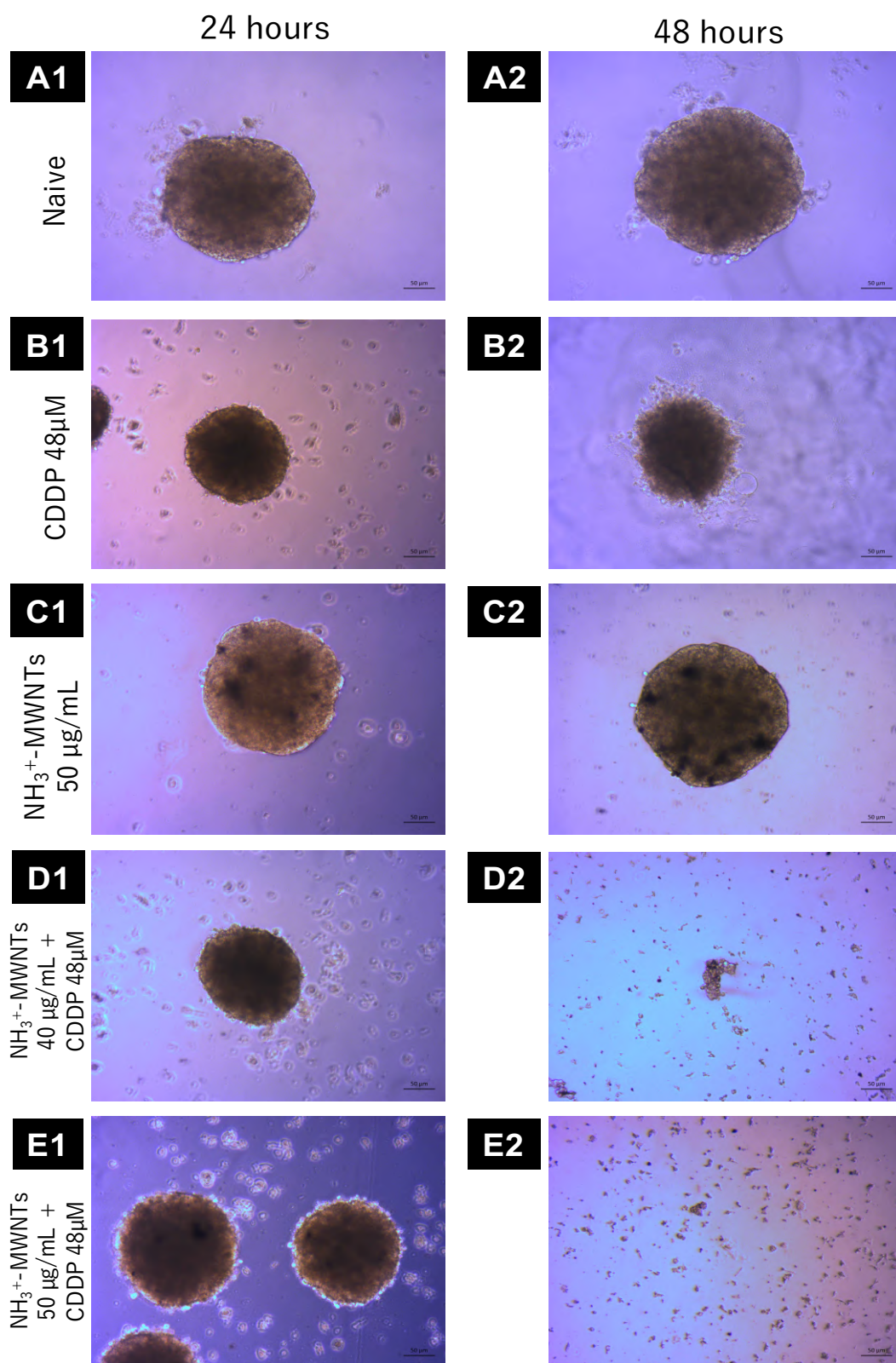


Figure A3 | Microphotographs of SKOV3 MCTS treated with amine-functionalised MWNTs + cisplatin complexes. Images of SKOV3 MCTS depicting: (A) untreated control, (B) free CDDP, (C) single NH₃⁺-MWNTs, (D) NH₃⁺-MWNTs 40 μg/mL + CDDP 48μM complex, and (E) NH₃⁺-MWNTs 50 μg/mL + CDDP 48μM complex for (1) 24h and (2) 48h post-treatment.

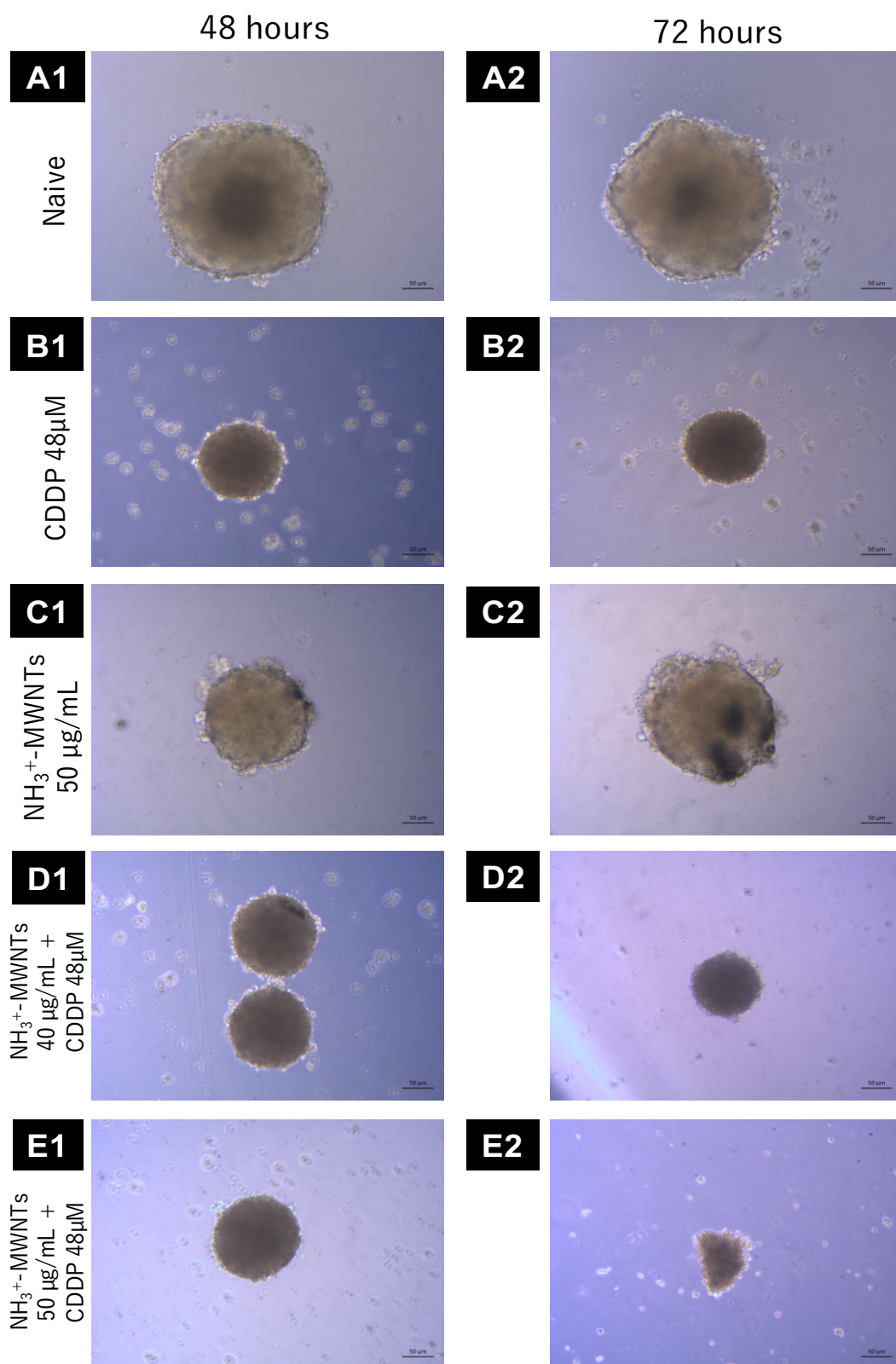


Figure A4 | Microphotographs of HCT 116 MCTS treated with amine-functionalised MWNTs + cisplatin complexes. Images of HCT 116 MCTS depicting: (A) untreated control, (B) free CDDP, (C) single NH₃⁺-MWNTs, (D) NH₃⁺-MWNTs 40 µg/mL + CDDP 48µM complex, and (E) NH₃⁺-MWNTs 50 µg/mL + CDDP 48µM complex for (1) 48h and (2) 72h post-treatment.