



rijksuniversiteit  
 groningen



UNIVERSITA' DEGLI  
 STUDI DI TORINO

## Master thesis Pharmacy

*Nrf2 siRNA as a tool to  
 overcome chemo-  
 resistance in bladder  
 cancer cells*

---

**A B S T R A C T:** The transcription factor Nrf2 (NF-E2-related factor 2) is the master regulator of antioxidant and cytoprotective systems. Nrf2 activation is critical for resistance to drugs in various tumors, including bladder cancers. Thereby, cancer cells often depend on high Nrf2 activity for survival. For this reason, it has been postulated that Nrf2 could represent an interesting target to combat chemo-resistance. Several inhibitors of Nrf2 are already identified. However, lack of potency and selectivity of these compounds makes the use of a specific small interfering RNA (siRNA) against this gene an attractive possibility. Since siRNAs are unstable in blood and have very poor ability to cross lipophilic cell membranes, a carrier is needed. The use of nanostructures as carriers for nucleic acid delivery could overcome these obstacles, as they can protect siRNA from degradation during systemic circulation, and transport siRNA to target cells avoiding nonspecific delivery.

This project aimed to evaluate the biological activity of cationic carbosilane dendrimers containing siRNA against Nrf2 in reducing cisplatin resistance and tumor growth in bladder cancer cell lines with a high level of Nrf2. We found that the used delivery system was able to enhance the uptake of anti-Nrf2 siRNA with a downregulation of the Nrf2 protein expression and its target protein GSTA4 as a result. Furthermore, the inhibition of the Nrf2 expression resulted in an increase in cisplatin sensitivity and apoptosis induction, and a decrease in the migration and growth of resistant bladder cancer cells. These findings indicate that anti-Nrf2 siRNA carried by dendrimers is a potential new therapy for patients diagnosed with chemotherapy resistant (bladder) cancer.

---

**KEYWORDS:** *Chemo-resistance, bladder cancer cells, Nrf2, gene silencing, siRNA, nano-delivery system*

---

## TABLE OF CONTENTS

<b>1. INTRODUCTION .....</b>	<b>3</b>
1.1 Mechanism of drug resistance.....	3
1.1.1 Cisplatin: working mechanism and resistance.....	3
1.1.2. ROS and its role in cancer .....	4
1.1.3 CDDP resistance in bladder cancer .....	5
1.1.4 The role of Nrf2 in gaining resistance .....	5
1.2 Inhibition of Nrf2 signaling pathway .....	6
1.2.1 Gene silencing by siRNA .....	7
1.3 Carriers for siRNA delivery .....	8
1.3.1 Carbosilane dendrimers .....	8
<b>2. AIM OF THE STUDY AND HYPOTHESIS.....</b>	<b>10</b>
2.1 Plan of action .....	10
<b>3. MATERIALS AND METHODS.....</b>	<b>11</b>
3.1 Cells, culture conditions and treatments .....	11
3.2 Induction and maintenance of CDDP resistance .....	11
3.3 Preparation of delivery systems .....	11
3.3.1. Preparation of improved anti-Nrf2 siRNA-loaded dendrimers .....	11
3.4 Chemical characterization of delivery systems .....	12
3.5 Cell treatments .....	12
3.6 RNA interference .....	12
3.7 MTT assay .....	13
3.8 Lysate preparation and western blot analysis.....	13
3.9 Colony forming assay.....	14
3.10 Crystal violet assay .....	14
3.11 Apoptosis assay .....	14
3.12 Wound-healing assay .....	15
3.13 Statistical analysis.....	15
<b>4. RESULTS AND DISCUSSION.....</b>	<b>16</b>
4.1 Characterization of CCD formulations.....	16
4.2 Optimizing nanocarrier-siRNA complex based on toxicity tests.....	18
4.2.1. Cytotoxicity CCDs – preparation 1 .....	18
4.2.2. Cytotoxicity CCDs – preparation 2 .....	21
4.2.3. Cytotoxicity S002-CCD – preparation 3 .....	22
4.3 Biological effect of siRNA-loaded CCDs .....	23
4.3.1. Transfection efficiency of loaded CCD.....	23
4.3.2 Effect of Nrf2 silencing, alone or with CDDP, on viability .....	25
4.3.3 Effect of Nrf2 silencing on cell growth and colony forming .....	26
4.3.4 Effects of Nrf2-silencing, alone or with CDDP, on apoptosis .....	27
4.3.5 The effect of Nrf2 silencing on migration of bladder cancer cells .....	29
<b>5. SUMMERIZING DISCUSSION AND CONCLUSION.....</b>	<b>31</b>
<b>AKNOWLEDGEMENT.....</b>	<b>33</b>
<b>REFERENCES.....</b>	<b>34</b>

## 1. INTRODUCTION

According to the World Health Organization, cancer is responsible for nearly a staggering one in six deaths worldwide. It could therefore be considered as one of the most lethal diseases in the present world. Globally, every family is affected by this heterogeneous disease and, to reduce suffering and deaths by cancer, more effective and reliable treatments are urgently needed.<sup>1-2</sup> At this moment chemotherapy, radiotherapy and surgery are the most widely used therapies to fight cancer.<sup>3</sup> Chemotherapy still plays an important role in the treatment of cancer,<sup>2-6</sup> despite several disadvantages that are associated with its use, such as: impairment of quality of life of cancer patients by the severe side effects (such as: vomiting, diarrhea and nausea), varying pharmacokinetics of the chemotherapeutic agents (caused for example by poor solubility and non-specific distribution throughout the body<sup>4</sup>), and development of resistance by the tumor cells, which compromises the effectiveness of chemotherapy.<sup>4</sup>

In the past decade, a lot of research has been done to overcome certain limitations. One of the most promising solutions has been the use of nanocarriers as a drug delivery system. Those particles encapsulate the drugs and improve the pharmacokinetics of the chemotherapeutic agents, as they help to bypass some resistance mechanisms and reduce the severe systemic side effects.

### 1.1 Mechanism of drug resistance

Cells, whether healthy or not, are capable of adapting to new situations to increase their survival rate. One of the ways tumor cells use this enormous adaptability is by developing strategies to increase their resiliency to chemotherapy to avoid death.<sup>6-9</sup> This phenomenon is called acquired resistance: resistance to therapy can be intrinsic or acquired. In both of them the heterogeneity of tumors plays a crucial role.

In case of intrinsic resistance, the initiating potential exists already before treatment: a part of the cells of heterogenous tumor has a selective advantage and is able to grow while being treated. Cells without this advantage, the so-called sensitive subpopulations, cannot overcome the applied treatment and will die. Eventually the sensitive cells will be supplanted by resistant cells. When tumor cells progressively develop new genetic mutations under the pressure of therapy (and thus exhibit different behavior than before treatment) it is called acquired resistance. The genome of the cells evolves during treatment, whereby the occurred mutations provide the cell with characteristics to increase the survival rate. For example, the elevated expression of the energy-dependent efflux pumps on the cell membrane. It is one of the main mechanisms whereby tumor cells obtain resistance to a broad spectrum of anticancer drugs: an increase in quantity of those efflux pumps results in a decrease of the intracellular concentration of the administered drugs, which hinders the treatment, and which is a typical form of acquired resistance.<sup>6,10</sup> More examples of this form of resistance are: modification of the drug target site, increased activation of anti-drug-induced apoptotic and drug detoxifying pathways and an upregulation of repair mechanisms.<sup>11-13</sup>

In case of resistance to cisplatin (a well-known and frequently used chemotherapeutic drug), multiple mechanisms can be involved.

#### *1.1.1 Cisplatin: working mechanism and resistance*

Cisplatin or cis-diamminedichloridoplatinum (CDDP) is used to treat several types of human cancers, including bladder, head and neck, lung, ovarian and testicular cancers.<sup>12</sup> CDDP is a heavy metal complex

which, after entering the nucleus, binds to macromolecules such as DNA. In the affected cells, the DNA is damaged by the formed adducts, which blocks the replication and transcription, causing cell death induced by apoptosis.<sup>12-14</sup> Traditionally, the toxicity of CDDP was thought to be the result of the formed adducts with the nuclear DNA. However, more recently, an increase in reactive oxygen species (ROS) is been reported in cells when they were exposed to CDDP, and which can contribute to the anti-tumor effect of CDDP.<sup>14</sup>

Resistance to CDDP (and other platinum-based therapeutics) occurs regularly and all involved mechanisms can be categorized into four classes of mechanisms: pre-target, on-target, post-target and off-target.

- Pre-target resistance: Before CDDP can exert its effect, cancer cells can evade the cytotoxic activity by modification of the intracellular concentration. This can be obtained by decreasing the drug uptake, by increasing the drug efflux and by increased intracellular detoxification.
- On-target resistance: DNA damage as a result of the initiated adduct forming by CDDP, can be overcome by DNA repair mechanisms whereby specific polymerases are involved.
- Post-target resistance: Defects in the signal transduction pathways (which will normally elicit apoptosis in response to the DNA damage) or flaws within the cell death executioner machinery itself may contribute to post-target resistance to CDDP. The loss of function of p53 is one of the most common mechanisms of post target resistance and occurs in approximately half of all human neoplasms. It is demonstrated that various proteins which are involved in apoptosis modulate the response to CDDP *in vitro*. Therefore, post-target resistance to CDDP is characterized by the aberrant expression of those proteins.
- Off-target resistance: Pathways which are not directly involved in the working mechanism of CDDP may compensate the cytotoxic effect of the drug.

### 1.1.2. ROS and its role in cancer

ROS are constantly generated by various physiological oxidative processes in the biological system and drive certain pathways. Under normal circumstances the level of ROS is balanced by a scavenging system, built from various antioxidant enzymes and molecules. A misbalance between generating and eliminating of ROS can lead to higher levels of ROS, which cause damage to proteins, lipids, and DNA and eventually could initiate carcinogenesis.<sup>15-18</sup> However, an excessive production of ROS can also induce cell aging and mediates cell death by triggering oxidative stress (intracellular increase of ROS).<sup>19-20</sup>

Several types of cancer exhibit, when compared to normal cells, higher levels of ROS because of an abnormal metabolism, malfunction in mitochondrial activity and stimulation of oncogenes. And therefore, those cancer cells are more vulnerable to the damage caused by a further increase ROS production, which occurs after administration of cytotoxic agents as CDDP.<sup>18-20</sup> Based on this, several ROS-generating compounds are in clinical trial. Although, it is already known that some cancer cells become less sensitive to intrinsic or drug-induced oxidative stress by up-regulating their glutathione (GSH) antioxidant system.<sup>20</sup>

GSH is one of the molecules which can regulate the level of oxygen species. Its abundant in all mammalian tissues and therefore, it is considered to be one of the most important scavengers. The antioxidant effect is caused by the oxidation of GSH to GSSG, which is mediated by the enzyme GSH peroxidase. GSH can be

regenerated from GSSG by the enzyme GSH reductase in the presence of NADPH, and continuously detoxify ROS.<sup>21</sup>

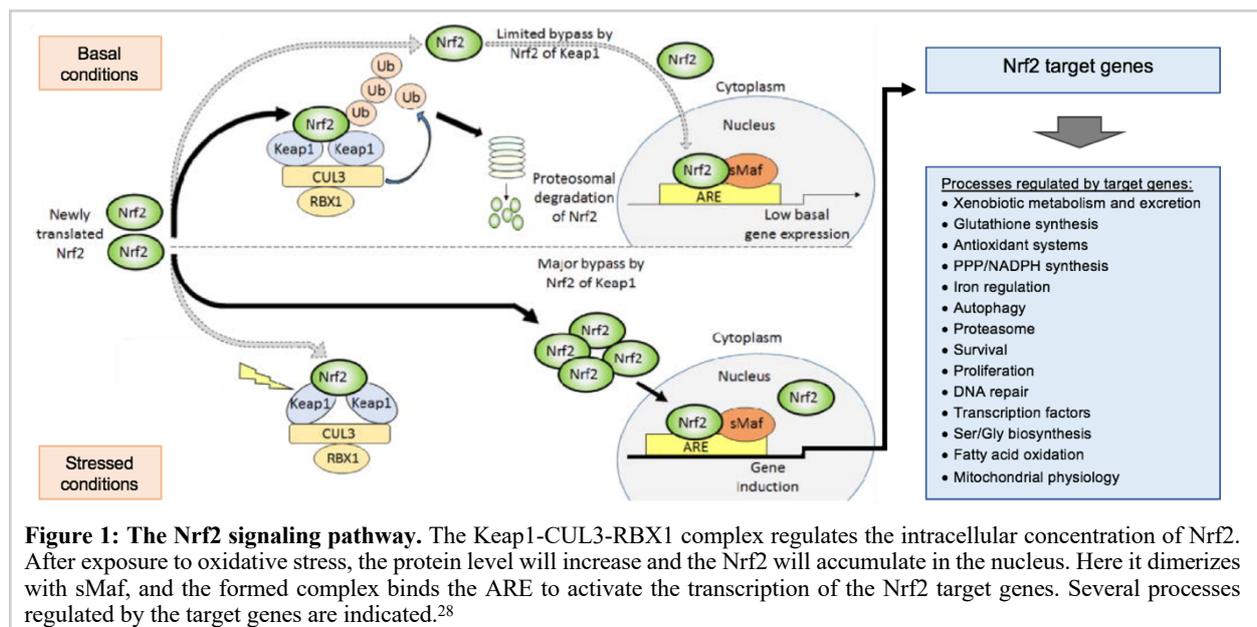
### 1.1.3 CDDP resistance in bladder cancer

Bladder cancer cells acquire resistance to CDDP (and its derivatives) as a result of the upregulation of antioxidant capacity, cellular detoxification, changes in drug uptake and efflux of compounds and evading the apoptotic pathway. This resistance is a major clinical problem; bladder cancer is one of the top five frequently diagnosed cancers in the European Union (according to the estimates of the International Agency for Research on Cancer) and is a heterogeneous disease with a high morbidity and mortality.<sup>22-24</sup>

Urothelial bladder cancer is the most common subtype. Since 1980, the standard treatment for urothelial carcinoma is a CDDP-based chemotherapy and despite the severe side effects of chemotherapy (as discussed before), it used to provide a survival advantage in bladder cancer.<sup>22-24</sup> Unfortunately, relapses occur regularly and are often accompanied with resistance to the standard therapy, and thus a poor prognosis. And although, the involved biological processes are studied widely, we still know very little of the underlying genetic mechanisms inducing resistance in bladder cancer.<sup>25</sup>

### 1.1.4 The role of Nrf2 in gaining resistance

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is known as one of the main regulators of the cellular antioxidant response and is for this reason an interesting target to combat the resistance. Originally, Nrf2 was recognized as a target for chemo-preventive compounds; to help preventing cancer and other diseases. Recent studies show, however, that the transcription factor has a ‘dark side’ and that it creates an environment which promotes the survival of cancer cells.<sup>26-27</sup>



Nrf2 is expressed in all cell types, but under physiological conditions the protein level is low.<sup>27</sup> The level of Nrf2 is mainly regulated by Kelch-like ECH-associated protein 1 (Keap1). Keap1 binds as a dimer Nrf2 and is a substrate for cullin 3 (CUL3), which contains an E3 ubiquitin ligase. At its turn, CUL3 is a target for the

E3 ligase RBX1. Under unstressed conditions newly synthesized Nrf2 is rapidly ubiquitylated by this Keap1-CUL3-RBX1 complex causing degradation by proteasomes in the cytoplasm. Only a small fraction of the translated Nrf2 find its way to the nucleus where it heterodimerizes with small Maf proteins (sMaf) and brings antioxidant response element (ARE) target genes at a low basal level to expression.<sup>27-28</sup> Cellular oxidative stress leads to a dramatical increase in the cellular level of Nrf2. This increase is a result of oxidative modification of Keap1 by electrophiles. After the oxidation of certain cysteine residues, Keap1 is no longer active, preventing the ubiquitylation of Nrf2 and, at this point, newly synthesized Nrf2 can escape the degradation.<sup>20,27-29</sup> Then, Nrf2 accumulates in the nuclei and activate target genes for cytoprotection via ARE.<sup>28</sup> See also *figure 1: The Nrf2 signaling pathway*.

Nrf2 is an interesting therapeutic target in cancer, because on one hand, it protects the cells from chemical carcinogenesis by detoxify ROS, and on the other hand, it provides cytoprotection after prolonged and uncontrolled activation.<sup>27,29</sup>

In various types of cancer, including CDDP resistant bladder cancer, an intrinsically high Nrf2 activity is seen. In those cancers Nrf2 protects the cancer cells by increasing the cellular detoxifying capacity and inducing the expression of efflux pumps, as well as reprogramming metabolic pathways (see *figure 1: The Nrf2 signaling pathway; processes regulated by target genes*).<sup>29-30</sup> For these cancers, Nrf2 inhibiting compounds could be useful in combination therapy: they may increase the sensitivity of the cancer cells to chemotherapeutic agents and therefore improve the clinical outcome.

## **1.2 Inhibition of Nrf2 signaling pathway**

As mentioned before, an abnormal activation of Nrf2 has been observed in many cancers and is involved in multi drug resistance (MDR). Silencing the Nrf2-ARE pathway in those cancer cells results, indeed, in an increase in sensitivity to anticancer drugs, which confirms the involvement of Nrf2 in resistance.<sup>31-33</sup>

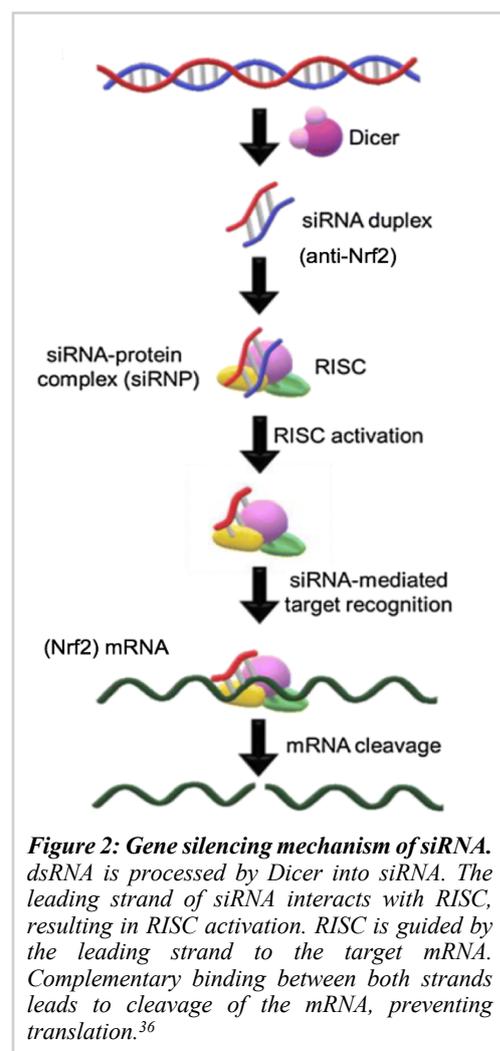
In the Nrf2 pathway there are several potential target sites and various strategies to achieve blockage of the signaling; such as affecting the Nrf2 translation or by acting on the Nrf2 degradation by increasing the level Keap1. A mode of action could also be preventing the translocation of Nrf2 to the nucleus or alter the binding of Nrf2-sMaf-complex to the DNA.<sup>31</sup> A variety of small molecular Nrf2 inhibitors have been identified. Unfortunately, the potency, selectivity and/or pharmacokinetics of those modulators form an obstacle to overcome. For this reason, the use of small interfering RNA (siRNA) against the Nrf2-gene is an attractive possibility.<sup>31,34-35</sup>

### 1.2.1 Gene silencing by siRNA

Within a cell there are several mechanisms to regulate gene expression; the process by which the coded information in genes is used for the synthesis of a gene product (mostly proteins) in time. Gene regulation gives the cell control over cellular metabolism, growth and differentiation.<sup>36</sup> A dysregulation in gene expression could have a variety of consequences and could lead to cancer, autoimmune diseases and several other undesired conditions.<sup>36</sup>

At the end of last century, it was discovered that genes could be silenced by administering double stranded RNA (dsRNA).<sup>37</sup> When introduced to the cell, dsRNA is spliced into shorter strands by an enzyme called Dicer. Those short dsRNA molecules are known as siRNA. One of the strands of the siRNA (also known as the leading strand) merges with a protein called RNA-induced silencing complex (RISC) and targets the complementary mRNA. The target messenger RNA (mRNA) is cleaved by the siRNA-protein complex, preventing translation of the mRNA into functional protein (*figure 2: Gene silencing mechanism of siRNA*).<sup>36-38</sup> Activated RISC then will be 'released' and can cleave additional complementary mRNAs. The leading strand only binds to mRNA that is complementary to it, causing the inhibition of only that specific mRNA and silencing of a specific gene.<sup>38</sup>

However, delivery of long dsRNAs in mammalian cells elicits the activation of the interferon (IFN) pathway; since dsRNAs are considered as a byproduct of viral replication. Activation of this pathway has a nonspecific mRNA degradation and inhibition of protein synthesis as consequence, which jeopardizes the desired silencing.<sup>39</sup> Studies in human cell cultures showed that the administration of synthetic siRNA results into an effective interference. In this way the cells are not exposed to dsRNA (*figure 2*), bypassing the antiviral immune response.<sup>38</sup>



**Figure 2: Gene silencing mechanism of siRNA.** dsRNA is processed by Dicer into siRNA. The leading strand of siRNA interacts with RISC, resulting in RISC activation. RISC is guided by the leading strand to the target mRNA. Complementary binding between both strands leads to cleavage of the mRNA, preventing translation.<sup>36</sup>

Based on these findings, anti-Nrf2 siRNA could be a useful tool to combat the CDDP resistance; considering the involvement of Nrf2 in acquiring resistance. Introducing anti-Nrf2 siRNA to CDDP resistant cancer cells will result into the cleavage of the mRNA of Nrf2, preventing the formation of functional Nrf2 protein, which will silence its effect (effect is displayed in *figure 1*) and possibly re-sensitize the cancer cells.

However, siRNAs are unstable in blood and because of their intrinsic properties – siRNAs are hydrophilic, negatively charged and relatively high in molecular weight – diffusion across the cell membrane on their own is preposterous.<sup>38</sup> The success of siRNA treatment relies on efficient delivery to the target site. For these reasons the use of a delivery system could provide a solution: it is possible to design a delivery system which targets cancer cells, in this way the Nrf2 level in healthy tissue will be unaffected and the protective role of Nrf2 activation in healthy remains.<sup>27</sup>

### 1.3 Carriers for siRNA delivery

The ideal delivery system enhances the cellular uptake of siRNA at their target sites, by protecting the nucleic acids from degradation by serum endonucleases, by evading immune detection and renal clearance, by preventing interaction with non-target cells and by allowing extravasation to reach the target cells.<sup>40</sup>

Roughly, the gene delivery vectors can be categorized as viral and non-viral vectors. Viruses have proved to be highly efficient in delivering genetic material into the cells, due to their natural characteristics. However, the use of those vectors is limited by potential toxicities and immunogenicity.<sup>41-43</sup> For this reason, non-viral vectors attracted much interest as carriers. Due to the development of nanotechnology, progress has been made and nowadays, nanocarriers can be designed according to the needs, whilst avoiding an immuneresponse.<sup>36,43</sup>

A nanocarrier is a nano-scaled system capable of transporting a variety of substances; from small anticancer drugs to macromolecules. Nanocarriers can be formulated to penetrate tumors from the bloodstream by the Enhance Permeation and Retention (EPR) effect: the accumulation of molecules of a certain size in tumor cells, more than in healthy tissue. The newly generated blood vessels, to supply the tumor with nutrients and oxygen, are usually made of poorly aligned endothelial cells with wide fenestrations and lacking a smooth muscle layer. Furthermore, in most tumor tissue effective lymphatic drainage is absent. These factors contribute to abnormal transport dynamics and allows accumulation of nanocarriers. Moreover, the nanocarrier can be easily modified with functional components to target specific cells and improve their delivery efficiency; allowing even more accumulation and achieving cytotoxic concentrations of the entrapped agent in the targeted cells, whilst reducing exposure of non-targeted (healthy) tissue with less side effects as result.<sup>44,45</sup> In addition, a nanocarrier protects its content from degradation and reduces the renal clearance, and therefore, encapsulating may improve the pharmacokinetics of its content.<sup>43</sup> To avoid provoking the immune system (and getting captured by it), the carrier size must not exceed 400 nm, whereas diameters below 200 nm allow extravasation.<sup>45</sup>

For gene therapy, many delivery systems have been introduced and all of them can be grouped into three categories: polymer, lipid or inorganic based nanocarriers.<sup>4</sup> Only a few of them are or have been under clinical trial.<sup>40,41</sup> It is often reported that a delivery system worked *in vitro*, but failed *in vivo*. Till date, toxicity problems remain an issue, as well as the nonspecific uptake of the carriers and/or provoking immune responses.<sup>46</sup> Therefore, the search for a suitable delivery system carries on. In this study two different nanocarriers are analyzed.

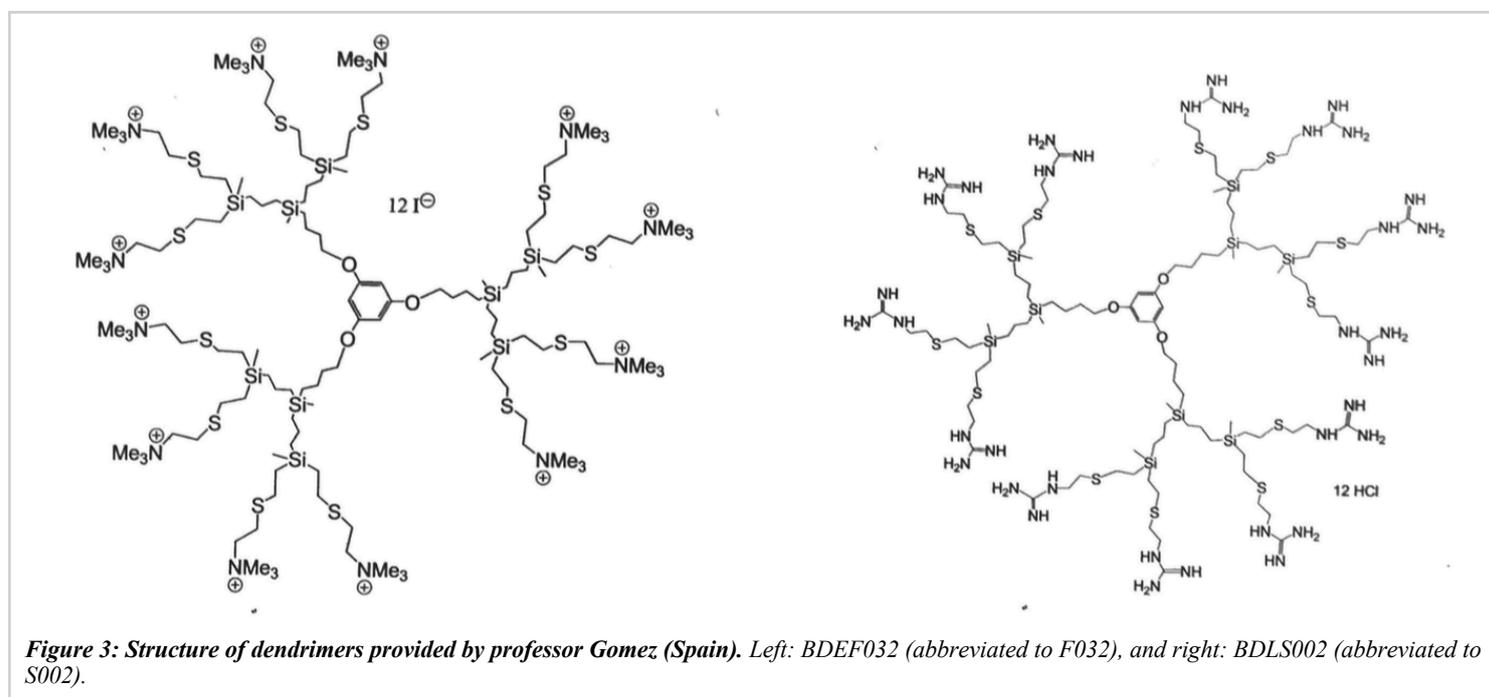
#### 1.3.1 Carbosilane dendrimers

Dendrimers are hyper branched three-dimensional synthetic polymeric macromolecules with a size of 1 to 100 nm.<sup>44-45</sup> They consist of three components – a central core, repeated branches and functional groups at the terminal of each branch – and are synthesized by controlled polymeric reactions. This step-by-step approach provides control over size, shape and flexibility; moreover, it allows adjustments in the pharmacokinetics of the macromolecule by choosing different branching units and terminal functional groups.<sup>44-47</sup>

Research is done towards the use of several types of cationic dendrimers containing different skeletons for gene therapy,<sup>43</sup> among them cationic carbosilane dendrimers (CCDs). It is proven that this kind of dendrimer is useful as a non-viral vector for gene therapy and transport its cargo into various cell types.<sup>43,48</sup> Dendrimers can interact in different ways with the agents they carry; first of all, dendrimers possess internal cavities and the open conformation allows encapsulation. Usually, these cavities have hydrophobic characteristics and additionally, they present nitrogen or oxygen atoms. Those characteristics make hydrophobic interaction with poorly soluble drugs, as well as the formation of hydrogen bonds possible.<sup>47</sup> Secondly, the terminal groups on the surface of the carrier are available for interaction. Therapeutic agents can be bound covalent or by electrostatic interaction to the groups. In particular to opportunity to form nanoconjugates through electrostatic interaction is interesting for gene therapy, nucleic acids (like the anti-Nrf2 siRNA) are after all negatively charged.

For this work two types of CCDs are prepared, shown in *figure 3: structure of CCDs*. The prepared CCDs possess positively charged terminal groups. An electrostatic interaction can be formed between the ammonium or guanidinium extremities and the negatively charged backbone of siRNA.<sup>48</sup> Besides that, the charge of blood vessels and cells must be taken into account. Both contain negatively charged constituents whose might repel nanocarriers with a negative charge. The use of nanocarriers with a positive surface could be a good choice to enhance the uptake.<sup>45</sup>

Till date, regarding dendrimers, there are no FDA approvals due to their unresolved toxicity.<sup>45</sup> For now, their toxicity is associated with their polycationic charge that should be minimized in the human body.<sup>49</sup> Therefore, we need to find an optimum balance between its needed charge and the undesirable side effects.



## **2. AIM OF THE STUDY AND HYPOTHESIS**

This study focuses on the redox adaptation in tumor cells resistant to chemotherapy and how to possibly overcome this resistance; it has been widely demonstrated that tumor cells over time increase the production of antioxidant molecules as a result of the intrinsically increased ROS production (which is caused by their malfunction). Due to this adaptation, tumor cells become moderately less sensitive (and eventually insensitive) to antineoplastic drugs (such as CDDP), which's working mechanism is partly based on inducing ROS production.

It is determined that the transcription factor Nrf2, and its targets, are involved in this particular type of resistance. An increase in Nrf2 expression is seen in chemotherapy resistant cells. It is believed that silencing of this gene can lead to sensitizing the tumor cells and therefore to an improvement in clinical outcome.

### **2.1 Plan of action**

The aim of this work is to evaluate the characteristics and biological activity of anti-Nrf2 siRNA loaded nanocarriers. For this purpose, different nanocarriers are obtained and compared. The protocol for preparing the nano-delivery systems will be optimized based on toxicity tests. With the least toxic delivery system biological test can be performed.

It is hypothesized that incorporating the siRNA against Nrf2 within the optimized nanocarriers will enhance the uptake of the nucleic acid. Therefore, a downregulation of the expression of Nrf2 and its effectors is expected in tumor cells which possess a high level of Nrf2. This is expected to lead to a reduction of CDDP resistance and a repression of the tumor growth.

Administration of siRNA-loaded nanocarriers should lead to a downregulation of Nrf2 compared to untreated cells, which can be detected by a western blot. If Nrf2 downregulation is seen the vulnerability of cancer cells to CDDP treatment will be checked by studying viability, proliferation, apoptosis and migration. Based on these findings it could be possible to conclude whether Nrf2-silencing can improve clinical outcome.

### 3. MATERIALS AND METHODS

#### 3.1 Cells, culture conditions and treatments

For this study three human bladder cancer cell lines were used; T24 (purchased from ATCC, Manassas, Virginia, United States of America, CDDP resistant), 253J B-V sensitive to CDDP and the 253J B-V resistant to CDDP sub-clone (253J B-V C-r). Both, 253J B-V and 253J B-V C-r cell lines were kindly provided by Dr. Roberto Pili from Roswell Parc Cancer Institute, Buffalo, United States of America. All cell lines were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml of penicillin and 100 µg/ml streptomycin) in a 5% CO<sub>2</sub>, 37°C incubator.

#### 3.2 Induction and maintenance of CDDP resistance

Resistance to CDDP in 253J B-V C-r was obtained in Dr. Pili's laboratory, by exposing 253J B-V cells to increasing concentrations of CDDP (0.5-0.8-1-1.3-1.5 µg/ml. Each concentration was maintained for at least six weeks, followed by verification of the viability of the resistant phenotype. After exposure to a certain concentration of CDDP, cells were cultured for at least one month without the drug. If the cells regained the ability to proliferate, cells were exposed to a higher concentration of CDDP. Induction of resistance required a year and the gained resistance was verified by MTT and colony forming assays.<sup>20</sup> CDDP resistance of the 253J B-V C-r sub-clone was maintained by treating them once a week with 0.1 mg/ml CDDP.

#### 3.3 Preparation of delivery systems

For this study two types of CCDs were enriched with anti-Nrf2 siRNA; one decorated with twelve ammonium groups (BDEF032, abbreviated as F032) and the other one having twelve guanidinium groups (BDLS002, abbreviated as S002), see *figure 3*. In both cases, the groups are located at the surface of the CCD, see also *figure 3*. The dendrimers, both kindly provided by Prof. R. Gomez (University of Alcalá, Madrid, Spain), were suspended in a HEPES-buffered saline solution (150 mM NaCl, 25 mM HEPES) to a concentration of 1 mg/ml. Dilutions were made to obtain the desired concentration of dendrimers (blank-CCDs).

Anti-Nrf2 siRNA-loaded dendrimers (siNrf2-CCDs) were prepared by adding the anti-Nrf2 siRNA (SI03246950, Qiagen) dropwise and under magnetic stirring to the previous obtained dendrimer suspension in various N/P ratios (dendrimer/siRNA ratio). Then, the samples were incubated under magnetic stirring and on ice for 30 minutes, to establish the binding between the siRNA and the dendrimer. Subsequently, every sample was characterized, see *3.4 Chemical characterization of delivery systems*.

##### 3.3.1. Preparation of improved anti-Nrf2 siRNA-loaded dendrimers

During the study it was determined that the CCDs were cytotoxic. To reduce the toxicity Pluronic F68<sup>®</sup> was added after establishing the binding between the siRNA and the dendrimer (or in case of the blank-CCDs Pluronic F68<sup>®</sup> was added after obtaining the desired concentration of dendrimer suspension). Initially the (loaded) CCDs were enriched with Pluronic F68<sup>®</sup> in a concentration of 0.1%. After running several experiments, the concentration of Pluronic F68<sup>®</sup> was increased to 0.15% due to persistent problems with the toxicity of dendrimers.

### 3.4 Chemical characterization of delivery systems

Electrophoresis was used to confirm complexation between the anti-Nrf2 siRNA and the dendrimers: samples were loaded on an agarose gel on which an electric field of approximately 15V/cm<sup>2</sup> was applied.

Thereby, every batch of prepared samples was characterized by pH, osmolarity, diameter, polydispersity and  $\zeta$ -potential. The average diameter and polydispersity index of formulations were determined by photon correlation spectroscopy using a 90 Plus instrument (Brookhaven, New York) at a fixed scattering angle of 90° and at 25°C. The polydispersity index indicates the size distribution within a CDD population.<sup>50</sup> The  $\zeta$ -potential was measured by electrophoretic mobility using the 90 Plus instrument and is an indication for the charge of the observed molecule. Each reported value is the average of ten measurements of at least three different formulation batches.

Preparation and characterization of the delivery systems was carried out in collaboration with by Prof. R. Cavalli and her team.

### 3.5 Cell treatments

Cells were seeded in 96, 12 or 6 wells plates (depending on test, all plates: transparent, flat bottom). After 24 hours of incubation (compound-free environment), cells were treated with ‘naked’ anti-Nrf2 siRNA or siNrf2-CCDs, with and without the presence of CDDP. Final concentration of siRNA: 0.08  $\mu$ M or 0.04  $\mu$ M depending on test. Transfection with naked siRNA was accomplished through the use of the HiPerFect<sup>®</sup> Transfection Reagent (301705, Qiagen), see 3.6 RNA interference. If siRNA treatment was combined with CDDP treatment, compounds were administrated simultaneously.

In *table 1* an overview of the treatments is given, also numbers of seeded cells are shown. Experiments to review the biological activity of siNrf2 loaded CCDs are treated according this plan. Toxicity tests did not follow this scheme, tested concentrations will be mentioned in the results.

**Table 1: An overview of used treatments.** All experiments regarding evaluation of the biological activity of siNrf2-loaded CCDs are treated according this plan.

Assay	Cells seeded (cells/well)			Treatment (final concentration in well)				
	T24	B-V 253J	B-V 253J C-r	blank CCD ( $\mu$ g/ml)	siNrf2-CCD ( $\mu$ M)	CDDP ( $\mu$ g/ml)	siNrf2-CCD + CDDP ( $\mu$ M + $\mu$ g/ml)	naked siNrf2 (w. HiPerFect <sup>®</sup> , $\mu$ M)
<b>MTT</b>	1500	2500	2500	8	0.08	2.5	0.08 + 2.5	-
<b>Western blot</b>	120.000	-	200.000	-	0.08	-	-	0.08
<b>Colony</b>	1000	1000	1000	4	0.04	0.1	0.04 + 0.1	-
<b>Apoptosis</b>	120.000	-	200.000	8	0.08	2.5	0.08 + 2.5	-
<b>Wound-healing</b>	125.000	-	225.000	4	0.04	0.1	0.04 + 0.1	-

Cells were exposed to the different treatments for the full duration of incubation as described in following chapters.

### 3.6 RNA interference

To determine the biological activity of the used nanoparticles, cells were transfected with Nrf2-specific siRNA (SI03246950, Qiagen) in all mentioned experiments. The uptake of siRNA was enhanced by the used nanoparticles. The effect on Nrf2 expression after transfection of siRNA in the nanoparticle was compared

with the one obtained with ‘naked’ siRNA using HiPerFect® Transfection Reagent (301705, Qiagen). Transfection with HiPerFect® was carried out in culture medium containing serum, but not antibiotics. To allow complexation between the siRNA and the HiPerFect® reagent, the acquired volume of both substances was diluted in culture medium without serum and without antibiotics, and incubated for 10 minutes at room temperature. Complexes were drop-wise added onto the cells. See also instructions of the manufacturer (*Appendix 1*). Transfection enhanced by nanoparticles was carried out in culture medium enriched with serum and antibiotics.

### 3.7 MTT assay

The MTT assay is a colorimetric assay and used to determine the cytotoxicity of the delivery systems and CDDP. Dissolved 3-(4,5-di-methyl-thiazol-2-yl) – 2,5-diphenyltetraolium bromide (MTT) gives a yellow aqueous solution which can be reduced by dehydrogenases in metabolically active cells to water insoluble violet-blue formazan crystals.<sup>51</sup> The amount of formed formazan is an indication of the mitochondrial integrity and activity, which may be interpreted as a measure of the cell viability as of the cell proliferation.<sup>52</sup>

MTT analysis was carried out in a transparent 96-wells flat bottom plate (EuroClone, ET3096). Cells were seeded (1000-4000 cells/well, depending on cell line) in 200 µl of serum- and antibiotics- supplemented medium. After 24 hours of incubation, cells were treated with different (concentrations) compounds and/or drugs. Untreated cells were used as a control.

After a certain exposure time (24, 48 or 72 hours), the medium with treatment was removed and the cells were washed with PBS 1x. MTT (M2128-SG, Sigma-Aldrich) was added to all the cells with a final concentration of 0.5 mg/ml for two hours. Then the MTT was removed, and the cells were lysed with 100 µl dimethyl sulfoxide (DMSO, EuroClone). Absorbance was determined at 595 nm by a 96-wells-plate ELISA reader.

The viability of cells after a specific treatment was calculated from the measured absorption using the following formula:

$$Viability = \frac{absorbance\ treated}{absorbance\ relative\ untreated\ cells} \cdot 100\%$$

The higher the calculated viability, the less toxic the used compounds are.

### 3.8 Lysate preparation and western blot analysis

The western blot is a biochemical technique which is used to detect specific proteins in a sample. Cells were seeded in 6-wells plates (EuroClone, ET3006), see 3.5 *Cell treatments*. 24-48 hours after treatment, cells were collected and resuspended in a lysis buffer composed of 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% v/v Triton X-100, 150 mM NaCl, and phosphatase (Sigma-Aldrich P0044) and protease inhibitor cocktail (Sigma-Aldrich P8340). The samples were incubated on ice for 30 minutes and mixed by vortexing every 5 minutes. Then, samples were centrifuged at 12.000 rpm for 25 minutes at 4°C. The supernatants were collected, and the protein concentration was determined using the Bio-Rad Protein Assay kit. Western blot

analysis was performed using homemade 7.5 or 12% SDS-polyacrylamide gels (7.5% if Nrf2 was protein of interest, 12% for GSTA4).

20 µg of protein was mixed with 10 µl of solubilization buffer composed of SDS, Tris base, glycerol, purified water and 10% 2-mercaptoethanol, then boiled for 5 minutes at 100°C and loaded onto a gel together with a molecular weight marker (EPS025500, EuroClone). The run was performed at a constant voltage of 150 V. The proteins and marker were transferred onto a nitrocellulose membrane with the TransBlot®Turbo™ (Bio-Rad, Germany). Afterwards, proteins were blocked through placing the membranes in a solution of 5% nonfat dry milk dissolved in TBS-Tween 20 for 1 hour. Incubation with the primary antibody against the protein of interest occurred overnight at 4°C. After three washes with TBS-Tween 20, membranes were incubated with the HRP-conjugated secondary antibody for 1 hour at 4°C. The detection of the bands was carried out after reaction with chemiluminescence reagents (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare) by the instrument ChemiDoc™ XRS+ (Bio-Rad, Germany).

Following antibodies were used: β-actin (#4970S, Cell Signaling Technology), Nrf2 (sc-722, Santa Cruz Biotechnology) and GSTA4 (SAB1401164, Sigma-Aldrich).

### **3.9 Colony forming assay**

The colony forming assay is an assay based on the ability of single cell to proliferate into a colony.<sup>53</sup> Cells were seeded into a 6-well plate (1000 cells/well) and left overnight to adhere to the surface. After 24 hours, the cells were treated with (loaded-)CCDs and CDDP, or a combination of both. This to determine the effect of the compounds on the growth of the cells. Cells were cultured for 9 to 11 days and then fixed and stained with a solution of crystal violet (Sigma-Aldrich) and methanol (9:1 ratio). The colonies were photographed and counted with the colony counter of ImageJ, colonies consisting of 50 cells or more were counted. Untreated cells were used as control.

### **3.10 Crystal violet assay**

Another way to determine the quantity of cells after treatment with the different compounds as mentioned in *3.7 Colony forming assay*, is by a crystal violet assay. The crystal violet dye binds to proteins and DNA; this means that the amount of staining increases when the number of adherent cells (and thus proliferation rate) increases.

After counting and photographing the colonies, the violet staining was dissolved in 10% acetic acid. Plates were placed for at least 15 minutes on a shaker to destain all the cells. Samples were diluted with purified water (1:4) and measured with a 96-well-plate ELISA reader at 595 nm.

### **3.11 Apoptosis assay**

Cells were seeded (120.000 – 200.000 cells/well, depending on cell line) in transparent flat bottom 6-wells plates (EuroClone, ET3006). After 24 hours, cells were treated with (loaded-)CCDs and CDDP, or a combination of both. This to determine if the loaded-CCD increases the fraction of cells going into apoptosis compared to untreated cells and cells treated with only CDDP.

Adherent and non-adherent cells were harvested 24 or 48 hours (for B-V 253J C-r and T24 respectively) after treatment. Cells were washed in PBS 1x and after centrifugation (3000 rpm for supernatant, 1000 rpm for monolayer), the pellets were resuspended in Annexin V binding buffer 1x (BB10x; ANXVKF-100T, immunostep). The samples were supplemented with 1:100 APC-conjugated Annexin V (555563, ANXVKf-100T, immunostep) and 1 µg/ml propidium iodide (307782; ANXVKF-100T, immunostep). Then the samples were incubated for at least 5 minutes in the dark at room temperature. Just before analyzing the samples with the BD Accuri™ C6 Flow cytometer (Becton Dickinson) 300 µl of Annexin V binding buffer 1x was added and samples were gently mixed by flicking.

### **3.12 Wound-healing assay**

The wound-healing assay is a method to study the cell migration in vitro. A wound in the cell monolayer is created after which the healing of this gap by cell migration is monitored.<sup>54</sup>

Cells were seeded (T24: 125.000 cells/well – B-V 253J C-r: 225.000 cells/well) in transparent 12-well-plates (ET3012, EuroClone) and grown to ~90% confluence overnight. The cell monolayers were wounded by scratching with a pipette tip across the center of the well. After scratching, the wells were gently washed with serum free medium to remove the detached cells. In order to monitor cell migration, several areas of each wound were photographed immediately after applying the scratch (=  $t_0$ ). Then, cells were replenished with fresh medium containing 2% of serum to encourage migration, and the to be tested compounds (e.g. blank-CDDs, loaded-CDDs, CDDP and a combination of CDDP and loaded-CDDs). 24 hours after adding the treatment, the wound was photographed again (=  $t_1$ ).

The width of the wound after 24 hours was compared to the width at  $t_0$ , which allows to say something about the migration capacity of the cells after treatment. The width at  $t_0$  was set as maximum and therefore at 100%. The area of wound was calculated by using ImageJ software.

### **3.13 Statistical analysis**

Data is expressed as mean  $\pm$  standard deviation from at least three independent experiments. Significance between experimental groups was determined by a paired T-test, a p-value  $\leq 0.05$  was considered statistically significant. Deviations are mentioned in the caption of the figures which show the results, as in the discussion.

## 4. RESULTS AND DISCUSSION

During the study the CCDs were prepared in three different ways (corresponding with the numbers 1-3 in *table 2*):

1. CCDs dissolved in HEPES-buffer, after obtaining the desired concentration of CCD, anti-Nrf2 siRNA was added and incubated for 30 minutes to establish complex forming.
2. After complexation between CCDs and anti-Nrf2 siRNA Pluronic F86<sup>®</sup> was added – final concentration: 0.1%.
3. The concentration of Pluronic F68<sup>®</sup> was increased to 0.15%

For more details see *3.3 Preparation of delivery system, page 10*.

### 4.1 Characterization of CCD formulations

The prepared CCDs were characterized after preparation, the average diameters, polydispersity,  $\zeta$ -potential and pH before and after loading with siRNA, are reported in *table 2: Physico-chemical characteristics of CCDs formulations*.

**Table 2: Physico-chemical characteristics of CCDs formulations.** Blank = unloaded CCD (either F032 or S002), siNrf2-CCD = loaded, P = Pluronic F68<sup>®</sup> concentration, N/P = CCD/siRNA ratio.

Preparation	Formulation	Average diameter ± SD (nm)	Polydispersity	$\zeta$ -potential ± SD (mV)	pH
1	Blank-F032	980.4 ± 12.4	0.112	12.55 ± 3.04	6.0
	siNrf2-F032 (N/P = 10)	385.6 ± 10.9	0.065	2.64 ± 0.56	6.0
	siNrf2-F032 (N/P = 20)	391.2 ± 9.8	0.047	2.87 ± 0.99	6.0
	Blank-S002	734.5 ± 13.6	0.123	11.89 ± 2.76	6.0
	siNrf2-S002 (N/P = 10)	352.4 ± 11.5	0.052	2.56 ± 0.94	6.0
	siNrf2-S002 (N/P = 20)	366.1 ± 10.6	0.048	2.77 ± 0.61	6.0
2	Blank-F032 + P 0.1%	995.2 ± 22.4	0.190	13.23 ± 1.21	5.9
	siNrf2-F032 + P 0.1% (N/P = 10)	365.5 ± 25.2	0.186	2.55 ± 0.88	5.8
	Blank-S002 + P 0.1%	715.8 ± 20.8	0.112	13.01 ± 0.84	6.0
	siNrf2-S002 + P 0.1% (N/P = 10)	346.8 ± 22.3	0.055	2.42 ± 1.24	6.2
3	Blank-S002 + P 0.15%	720.2 ± 20.6	0.096	12.25 ± 0.43	6.0
	siNrf2-S002 + P 0.15% (N/P = 10)	410.5 ± 10.3	0.045	2.50 ± 1.42	6.0

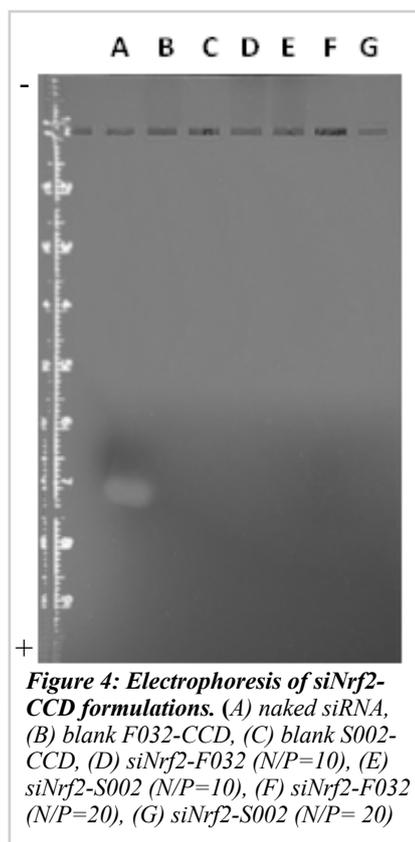
When we compare the different preparations (marked with 1, 2, 3), no really big differences are observed. A closer look learns that the average diameter, polydispersity and  $\zeta$ -potential decreases when CCDs are loaded with anti-Nrf2 siRNA.

The unloaded CCDs had an average diameter of less than 1000 nm and 750 nm (resp. average diameter of blank F032 and blank S002). After incubation with siRNA a decrease in size is observed. This decrease marks a condensation of the polymer chains via electrostatic interactions.<sup>50</sup>

A decrease in the  $\zeta$ -potential (blank-CCD: 12-13 mV versus siNrf2-CCD: 2.5-3 mV) indicates electrostatic interactions between the negative phosphate groups of siRNA and the positively charged terminal groups of CCDs (both CCDs present positively charged groups at a pH of ± 6.0). The siRNA complexation with dendrimers was confirmed by electrophoresis on an agarose gel, *figure 4*: fading of the

siRNA band is observed (A compared to B-G), indicating complex forming between the siRNA and the indicated CCD. The amount of anti-Nrf2 siRNA loaded onto the CCDs was not sufficient to saturate all the positive charges presented at the functional groups: naked anti-Nrf2 siRNA (A) runs towards the positive pole whereas the combination of siNrf2 and dendrimers (D to E, *figure 4*) are pulled to the negative pole as the net charge is positive (confirmed in *table 2*:  $\zeta$ -potential = 2-3 mV for both CCD and in both ratios).<sup>55</sup>

A  $\zeta$ -potential  $\geq 2$  mV is considered as toxic and undesired; molecules with a positive charge can interact with the lipids of cell membranes and cause cell death by disrupting the cell wall. A further decrease in  $\zeta$ -potential can be obtained by increasing the amount of the nucleic acid (decrease the N/P ratio). However, lower zeta potentials might favor the formation of aggregates of the CCDs and affect the stability of the nanosuspension. Also, as mentioned before, blood vessels and cell membranes contain negatively charged constituents. Therefore, a positive charge could enhance the uptake of the nanocarrier and its loadings.<sup>45</sup> For those reasons, other ways decrease cytotoxicity caused by the positive charges were studied.

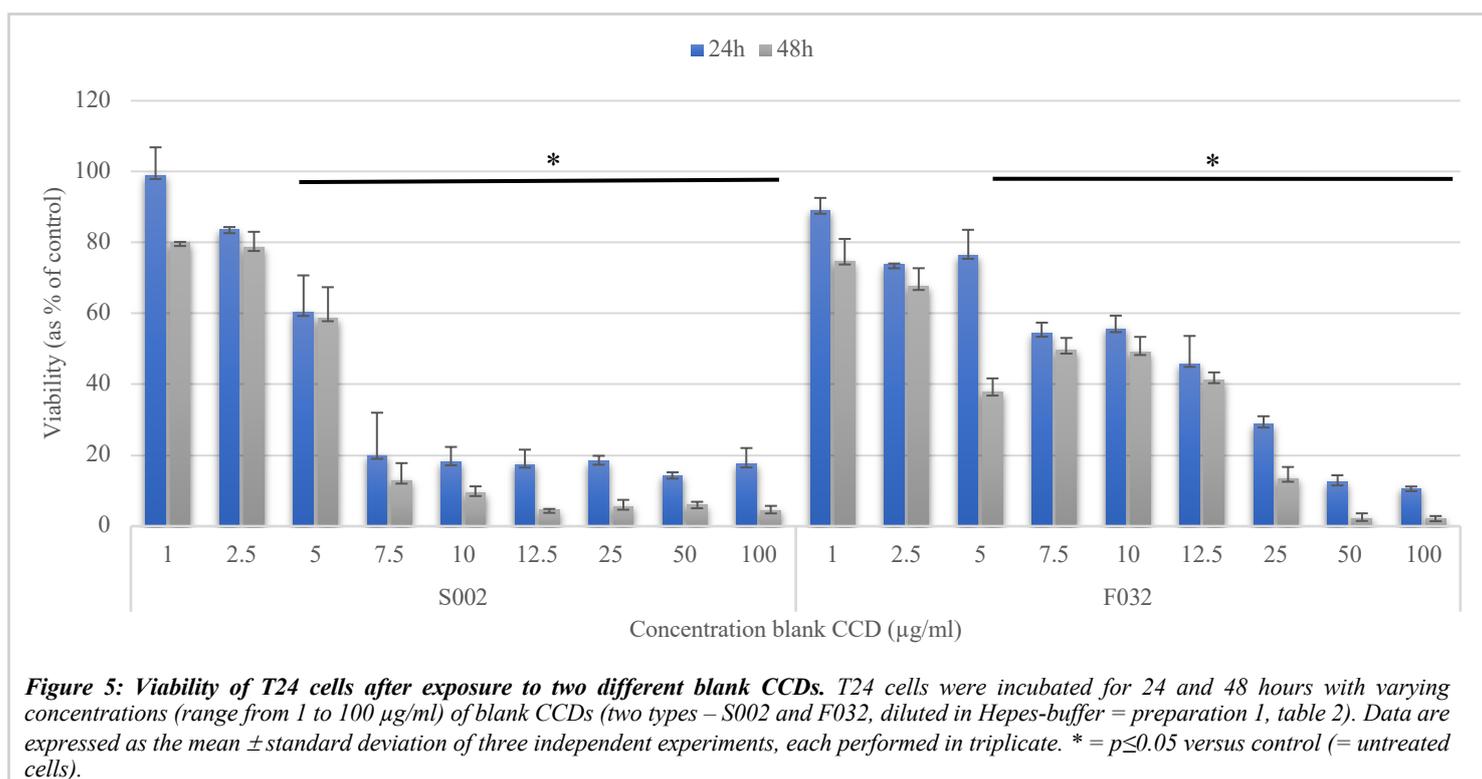


## 4.2 Optimizing nanocarrier-siRNA complex based on toxicity tests.

With the blank CCDs (unloaded dendrimers, either F032 or S002) toxicity tests were done. To test the cytotoxicity of both dendrimers, cells were exposed to different concentrations of CCDs.

### 4.2.1. Cytotoxicity CCDs – preparation 1

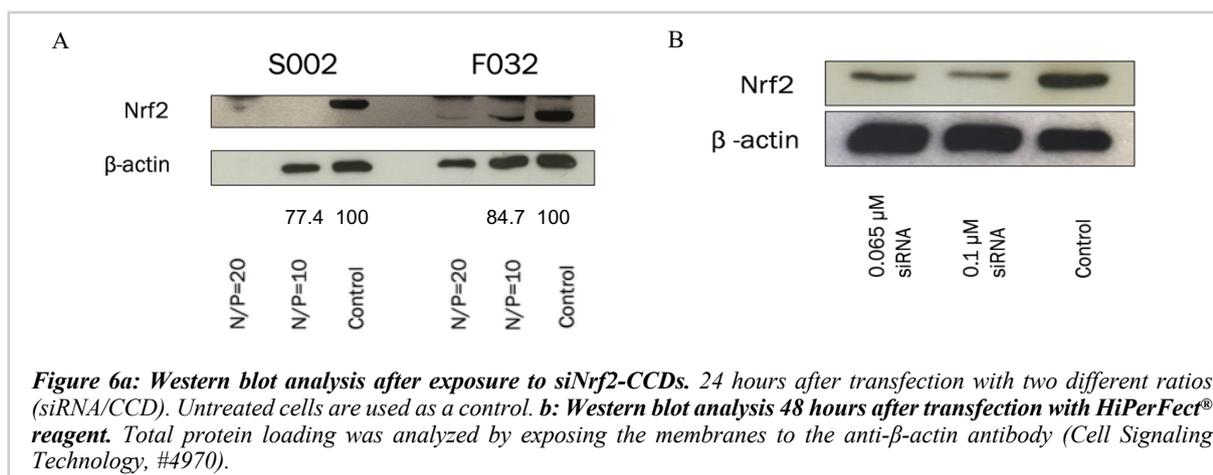
T24 cells were exposed to two types of blank CCDs (S002 and F032) in the range from 1 to 100  $\mu\text{g}/\text{m}$  for 24 and 48 hours. Results are shown in figure 5.



**Figure 5: Viability of T24 cells after exposure to two different blank CCDs.** T24 cells were incubated for 24 and 48 hours with varying concentrations (range from 1 to 100  $\mu\text{g}/\text{ml}$ ) of blank CCDs (two types – S002 and F032, diluted in HEPES-buffer = preparation 1, table 2). Data are expressed as the mean  $\pm$  standard deviation of three independent experiments, each performed in triplicate. \* =  $p \leq 0.05$  versus control (= untreated cells).

As shown in the figure above, both types of dendrimers were harmful. Exposure to a concentration of 5  $\mu\text{g}/\text{ml}$  or higher of blank S002 or, exposure to a concentration of 2.5  $\mu\text{g}/\text{ml}$  or higher of blank F032 results in a significant decrease ( $p$ -value  $\ll 0.05$ ) of the viability of T24 cells compared to the related control (control = untreated cells, 0  $\mu\text{g}/\text{ml}$  blank CCDs). This effect can be attributed to the terminal groups; after all both CCDs have positively charged groups at the surface when administered. As discussed before, positively charged compounds can interact with cell membranes and therefore cause cell death. Based on this data, S002 and F032 solubilized in HEPES-buffer were considered as toxic.

This is confirmed in figure 6a; where it was verified whether the CCDs loaded with siNrf2 were able to transfect T24 cells. Results were compared to those obtained by using a worldwide transfection method (figure 6b) with the HiPerFect<sup>®</sup> transfection reagent (see: 3.5 Cell treatments and 3.6 RNA interference). Transfection efficiency was analyzed by measuring the Nrf2 protein content through western blot analysis at 24 hours after treatment.



T24 cells were treated with both types of dendrimers carrying anti-Nrf2 siRNA in varying N/P ratios: amine groups of CCDs in moles divided by the phosphate groups of the siRNA in moles. For each nanoformulation, the following final concentrations of dendrimers and siNrf2 were obtained (corresponding with *figure 6*):

**Table 3: Tested concentrations siRNA-CCD**

Type of CCD	S002		F032	
Ratio (N/P)	20	10	20	10
[CCD] (μg/ml)	16.7	8.3	20.8	10.4
[siNrf2] (μM)	0.08	0.08	0.075	0.075

Although a decrease of the Nrf2 expression after treatment with a loaded CCD is observed, also a decrease in the β-actin signal is seen. Since β-actin is expressed in all eukaryotic cell types and the expression levels should not vary drastically after cellular treatment, its expression is used as a control for total protein loading. On the other hand, a variety in intensity of the β-actin signal may also indicate cell death. After all, in dead cells many of the proteins are degraded, this will result in a very weak signal in western blot analysis (cell death was already observed by use of the microscope). However, the toxic effect observed in *figure 6a* is less than the one already observed with the MTT test. This can be explained by the fact that the positive charge of the CCDs decreases when they are loaded with siRNA (see *table 2*, page, zeta potential). Therefore, the usage of a concentration of loaded CCD equal to a concentration of the same but unloaded CCD (as in *figure 5*, the toxicity test) will lead to a decrease in observed cytotoxicity.

Compared to the relative control, treatment with loaded S002-CCDs was the most toxic (*figure 6a*). Treatment with F032-CCD decreased the β-actin signal slightly (to 77.44% and 84.66% after respectively treatment with S002-CCD (N/P=10) and F032-CCD (N/P=10), allowing to consider the observed Nrf2 inhibition significant. Between both tested ratios (N/P=10 and N/P=20) a difference in toxicity is observed. This is possibly the result of doubling the amount of siRNA: less free and positive charged functional groups are present.

Traditional transfection of the anti-Nrf2 siRNA using HiPerFect<sup>®</sup> Transfection Reagent was performed and used as a positive control (*figure 6b*). Both siNrf2 concentrations (0.065 and 0.1 μM) were able to down-regulate the target gene at 48 hours after treatment (blot of silencing 24 hours after treatment failed). A difference in the intensity of the β-actin signal may be noticed: as told before, this signal is used to confirm

equal protein loading, incompetence regarding the used method may played a role here, since cell death was not observed.

In consideration of these results, the preparation protocol of the CCDs was modified, aiming to reduce the toxicity of the CCDs:

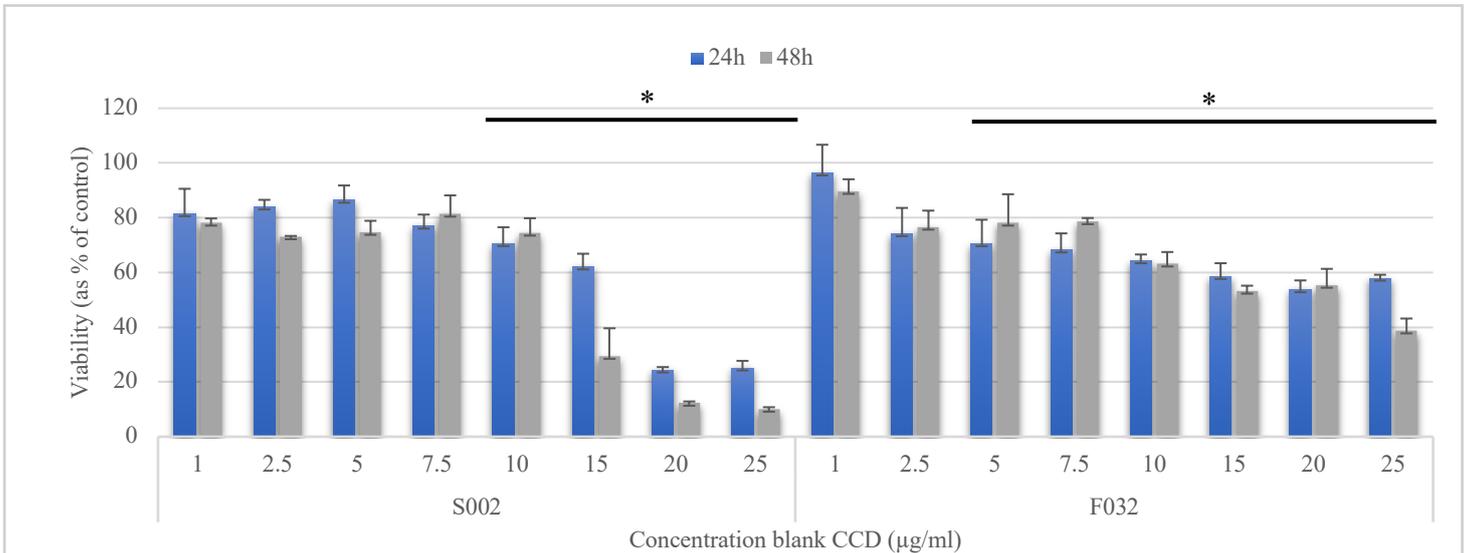
The toxicity of the CCDs is related to the positive charges of the terminal groups. Both types of dendrimers present charged groups which can be observed as a positive  $\zeta$ -potential (*table 2*). Considered is to decrease the N/P ratio (thus increase the concentration of siRNA carried by the CCD). However, a doubling in concentration of siRNA (N/P=20 to N/P=10) did not show any significant decrease ( $p \sim 0.50$  both CCDs) in the  $\zeta$ -potential (*table 2*:  $2.87 \pm 0.99$  |  $2.64 \pm 0.56$  |  $2.77 \pm 0.61$  |  $2.56 \pm 0.94$  –  $\zeta$ -potentials of loaded F032 N/P=20, F032 N/P=10, S002 N/P=20, S002 N/P=10 respectively). This, and knowing that a positive charge might enhance the uptake of nanocarriers (and its loadings) and reduces aggregation, lead to the adding of Pluronic F68<sup>®</sup> to the formulations (see 3.3.1: *Preparation of improved anti-Nrf2 siRNA-loaded dendrimers*)

Pluronic F68<sup>®</sup> is a non-ionic triblock copolymer and is built out of a central hydrophobic chain flanked by two hydrophilic chains. It is worldwide used for its protective characteristics in cell culture media, because the addition of Pluronic F68<sup>®</sup> leads to stabilization of cell membranes, protecting them from membrane shearing. Thereby, Pluronic F68<sup>®</sup> is a surfactant, which can control shear stress in suspensions.<sup>56</sup>

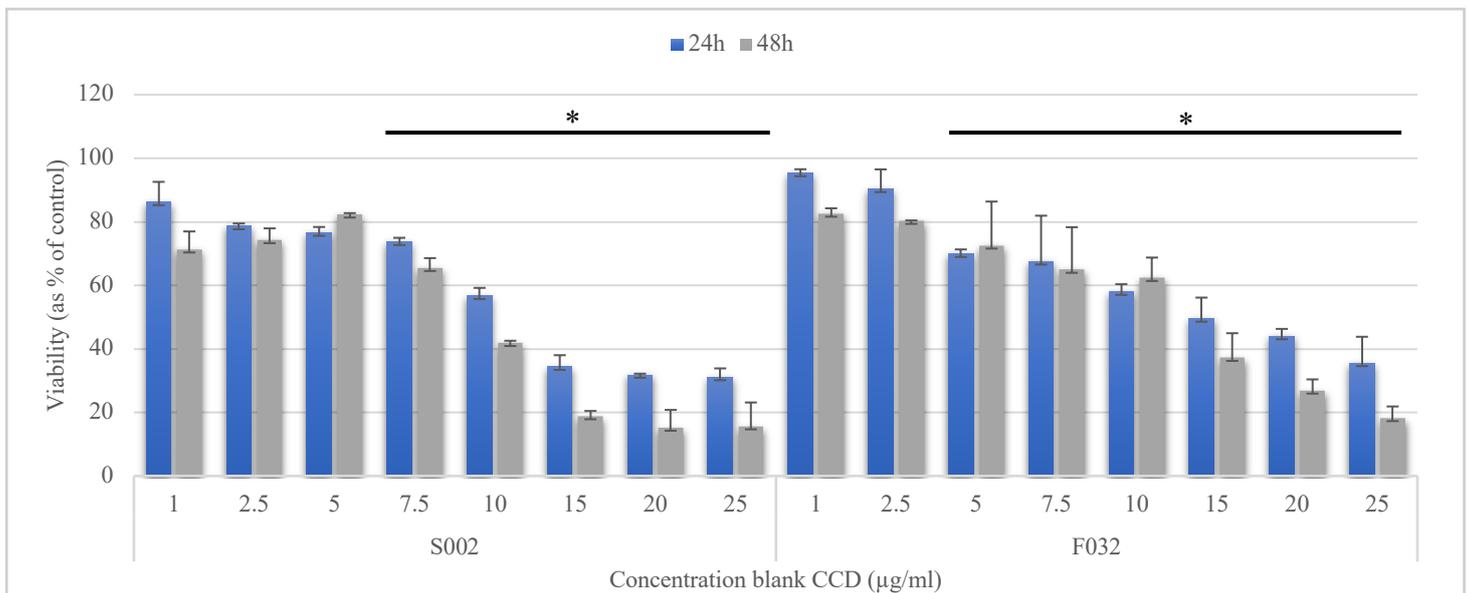
Moreover, it was decided to expose cells to a concentration of siRNA between 0.065 and 0.1  $\mu\text{M}$ . Depending on the loading capacity of the dendrimers.

#### 4.2.2. Cytotoxicity CCDs – preparation 2

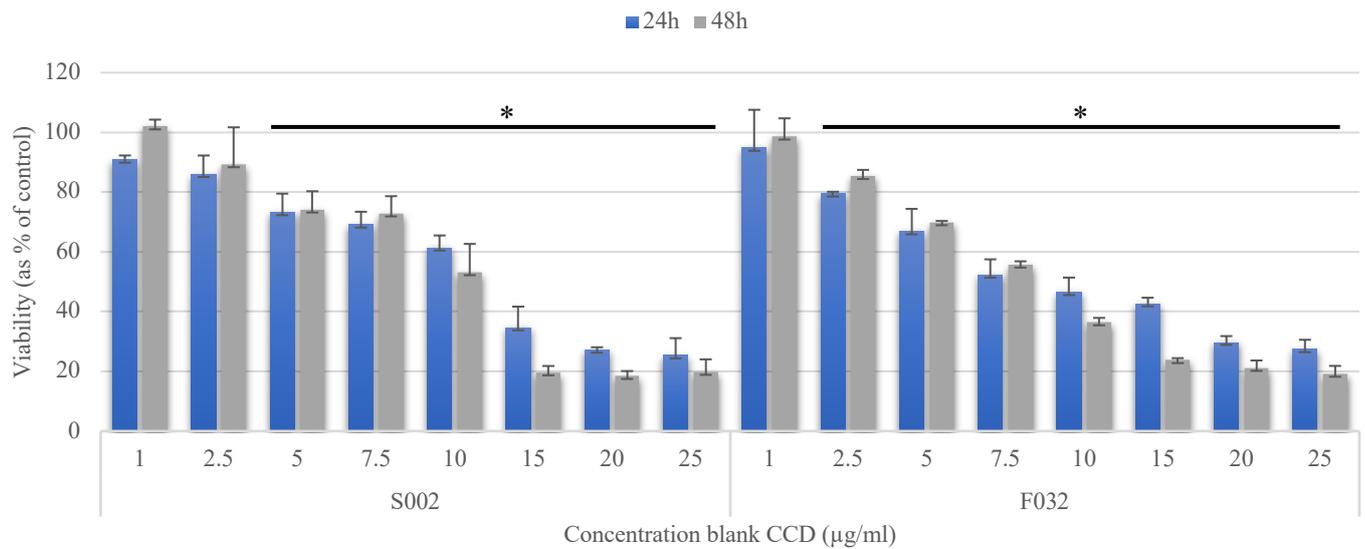
T24, B-V 253J C-r and B-V 253J cells were exposed to two types of blank CCDs (S002 and F032) with 0.1% Pluronic F68® in the range from 1 to 25 µg/ml for 24 and 48 hours. Results are shown in *figure 7* (T24), *8* (B-V 253J C-r) and *9* (B-V 253J)



**Figure 7: Viability of T24 cells after exposure to two different CCDs.** T24 cells were incubated for 24 and 48 hours with varying concentrations (range from 1 to 25 µg/ml) of blank CCDs (two types – S002 and F032, diluted in HEPES-buffer + Pluronic F68® = preparation 2, table 2). Data are expressed as mean ± standard deviation of three independent experiments, each performed in triplicate. \* =  $p \leq 0.05$  versus control (= untreated cells).



**Figure 8: Viability of B-V 253J C-r cells after exposure to two different CCDs.** Cells were incubated for 24 and 48 hours with varying concentrations (range from 1 to 25 µg/ml) of blank CCDs (two types – S002 and F032, diluted in HEPES-buffer + Pluronic F68®). Data are expressed as mean ± standard deviation of three independent experiments, each performed in triplicate. \* =  $P \leq 0.05$  versus control (= untreated cells).

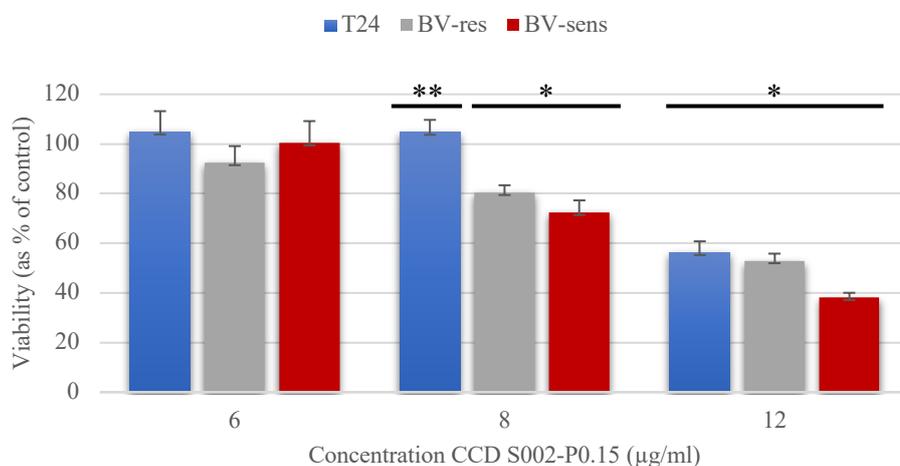


**Figure 9: Viability of B-V 253J cells after exposure to two different CCDs.** Cells were incubated for 24 and 48 hours with varying concentrations (between 1 to 25 µg/ml) of blank CCDs (two types - S002 and F032, diluted in Heps-buffer + Pluronic F68®). Data are expressed as mean ± standard deviation of three independent experiments, each performed in triplicate. \* =  $p \leq 0.05$  versus control (= untreated cells).

As expected, the second formulation of CCDs resulted in an increase of the viability of T24 cells (figure 7 compared with figure 5): adding Pluronic F68® leads to a, in case of the S002 type significant (viability after exposure 7,5 µg/ml blank CCD increased with 60%), less harmful delivery system. Addition of Pluronic F68® to CCD-F032 did not result in a significant increase nor decrease of the viability of T24 cells. However, when tested in two other cell lines, B-V 253 J C-r and B-V 253 J (resp. figure 8 and 9), even this formation with Pluronic F68® appears to be hazardous to the cells, F032 more than S002. Since a toxic delivery system is undesirable – cell death must be the result of the trappings instead of the carrier – preparation of CCDs was again optimized. In addition, it was decided to continue the study with only CCD type S002.

#### 4.2.3. Cytotoxicity S002-CCD – preparation 3

T24, B-V 253J C-r and B-V 253J cells were exposed to blank S002 CCD in three different concentrations (6, 8 and 12 µg/ml) with 0.15% Pluronic F68® (0.15% P) for 24 and 48 hours. Results are shown in figure 10.



**Figure 10: Viability of T24, B-V 253J C-r and B-V 253J cells after exposure to three different concentrations of S002-CCD.** Cells were incubated for 24 hours with blank S002 CCD diluted in Heps-buffer and with Pluronic F68® (final concentration = 0.15%). Data are expressed as mean ± standard deviation of one experiment performed in triplicate. \* =  $p \leq 0.05$  versus relative control (= untreated cells). \*\* =  $p \leq 0.05$  versus formulation with 7.5 µg/ml blank S002 CCD 0.1% Pluronic F68®.

As expected, the viability is higher in all three cell lines after treatment with S002 with a higher concentration of Pluronic F68<sup>®</sup>. The increase in viability is only significant in T24 cells (even when compared with a slightly lower dose of S002-0.1%P): 100% (8 µg/ml S002+0.15%P, *figure 10*) vs 80% (7.5 µg/ml S002+0.1%P, *figure 7*). In the other two cell lines, the ‘improved’ delivery system is less toxic, but an exposure to a concentration of 8 µg/ml S002+0.15%P or higher will lead to a significant decrease of the viability (versus relative control = untreated cells). Although a decrease in viability is undesired, if it did not exceed 20 – 25% compared to the relative control, it was considered as acceptable. Moreover, exposure to loaded CCDs lead to less cell death than exposure to unloaded CCDs (*figure 6a* versus *figure 5*). The expectation is that after loading the CCD with anti-Nrf2 siRNA the observed toxicity will be reduced. Thus, this product: S002 + Pluronic F68<sup>®</sup> 0.15% was considered as the optimal delivery system for siRNA and is therefore used as carrier to study biological effects of siNrf2 treatment.

### 4.3 Biological effect of siRNA-loaded CCDs

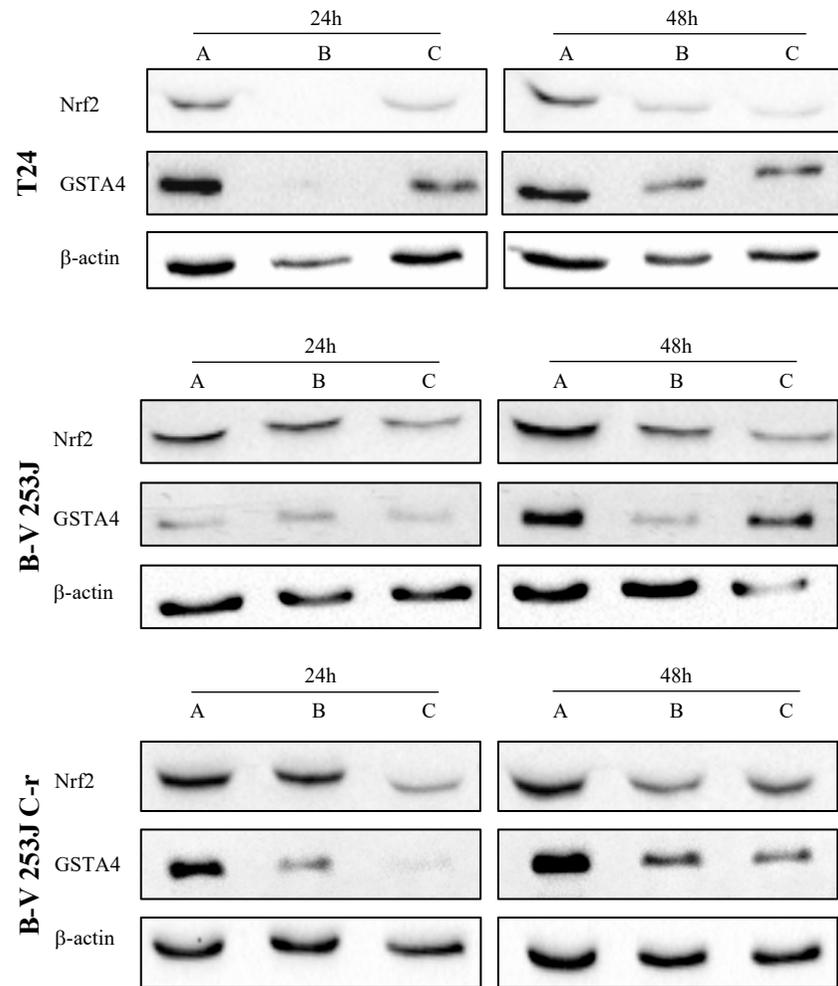
#### 4.3.1. Transfection efficiency of loaded CCD

The ability of the siNrf2-S002-P0.15% to enhance the effect of the nucleic acid was tested by western blot analysis. The expression of Nrf2 24 and 48 hours after treatment was analyzed in CDDP resistant (T24 and B-V 253J C-r) and CDDP sensitive bladder cancer cells (B-V 253J). Results demonstrated that the Nrf2 expression was inhibited until 48 hours (maximal reduction was obtained 24 hours after treatment). The reduction of Nrf2 protein was confirmed by the contemporary reduction of GSTA4, one of the effectors of Nrf2. Transfection with the HiPerFect<sup>®</sup> reagent was used as a positive control.

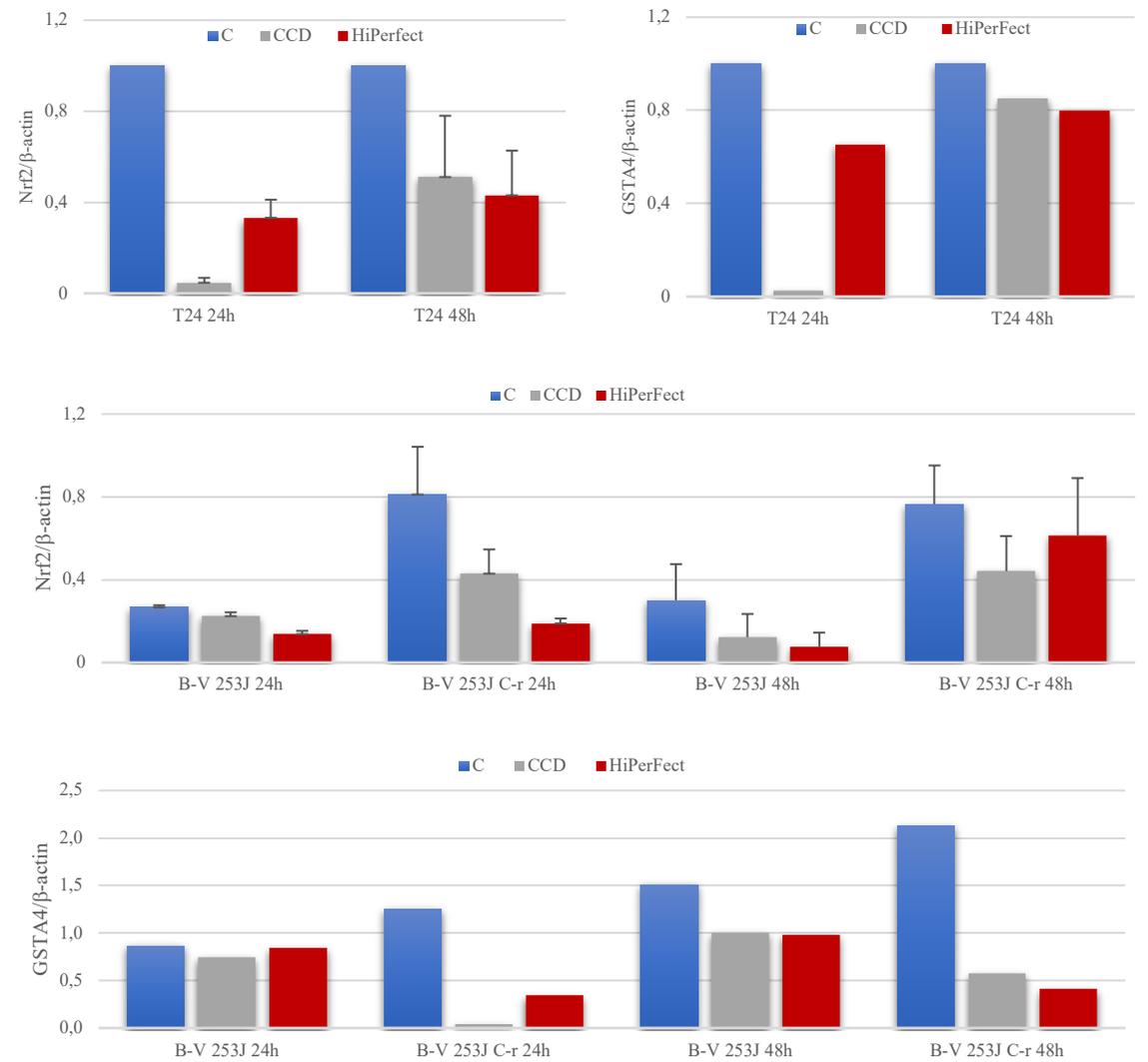
A closer look to *figure 11* learns that transfection with the S002-CCD is results in lower Nrf2 levels compared to transfection with the transfection reagent. The increased effect is probably a result of the nanocarrier: the results suggest that a higher intracellular concentration of anti-Nrf2 siRNA is reached in 24 hours when transfected with a CCD compared to transfection using the transfection reagent. 24 hours after treatment the maximum effect is seen, in the following 24 hours the Nrf2 level is rising again. A possible explanation for this rise can be found in cell division: if cells have a doubling time less than 24 hours (in case of T24<sup>57</sup>), the total amount of Nrf2 protein will rise. This while the level of activated RISC (thus the fraction of degraded Nrf2 mRNA) will remain the same, since treatment was only applied once during experiment. Therefore, the amount of Nrf2 appears to be larger at 48 hours compared to the one at 24 hours. B-V 253 J (C-r) has a doubling time of circa 49 hours.<sup>58</sup> Thus, the total Nrf2 expression remains the same. Activated RISC will still cleave the same fraction of Nrf2 resulting in a comparable Nrf2 expression at 24 and 48 hours (shown in *figure 11b*).

*Figure 11b* also demonstrates that the initial level of Nrf2 is higher in CDDP resistant cell line than in a sensitive one (Nrf2/ $\beta$ -actin: B-V 253J C-r compared to B-V 253J). This confirms that Nrf2 expression is involved in CDDP resistance.

A.



B.



**Figure 11A:** Western blot analysis of Nrf2 and GSTA4 expression in T24, B-V 253 J and B-V 253 J C-r. Cells were harvested 24 or 48 hours after treatment with siNrf2 loaded S002-P0.15% (B) or transfection by HiPerFect<sup>®</sup> reagent (C). Final concentration of siRNA: 0.08  $\mu$ M. Untreated cells acted as a control (A). Equal protein loading was confirmed by exposure the membranes to anti- $\beta$ -actin antibody. **B: Quantification of protein products.** Quantification of protein products (Nrf2 or GSTA4) was performed by densitometric scanning. Data was normalized using the  $\beta$ -actin signal and are presented as the mean  $\pm$  standard deviation of two independent experiments (Nrf2). GSTA4 data is based on one experiment.

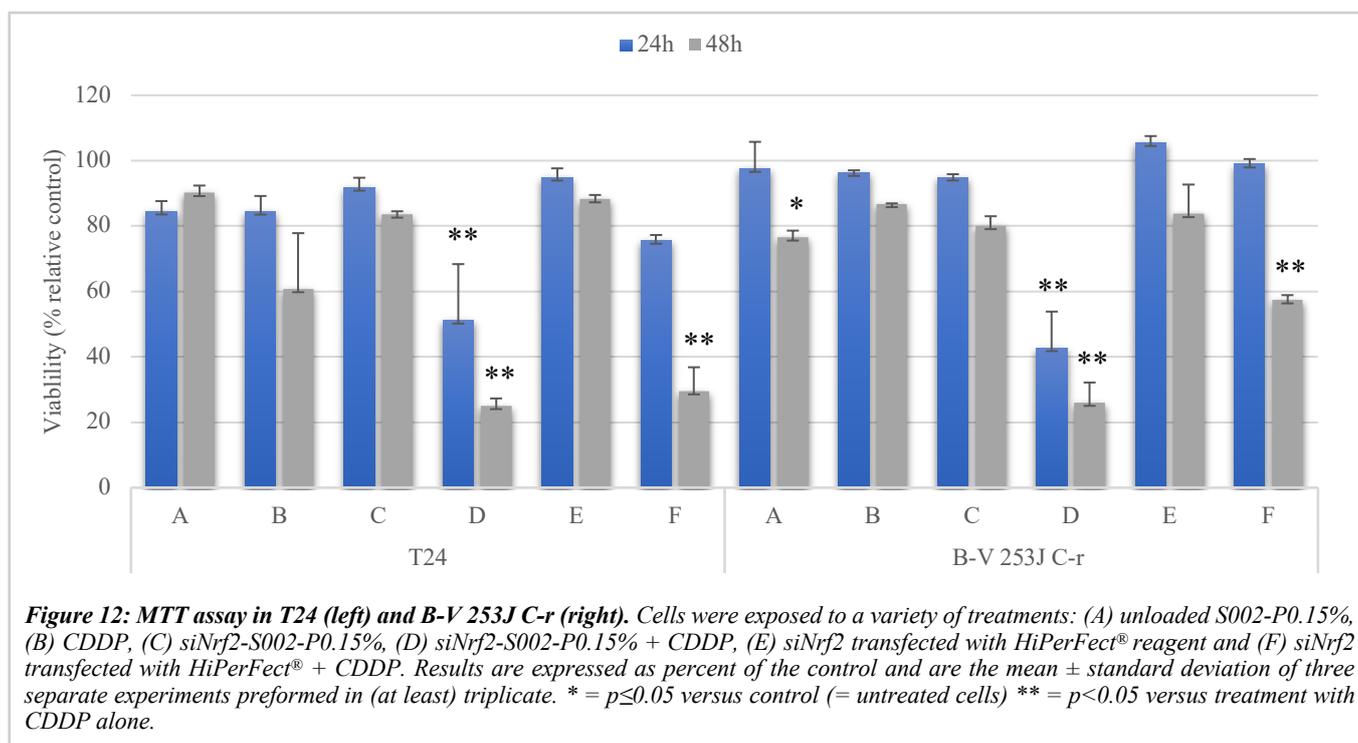
#### 4.3.2 Effect of Nrf2 silencing, alone or with CDDP, on viability

To verify if treatment with siNrf2-S002-P0.15% can sensitize CDDP resistant cells, an MTT assay was performed. T24 and B-V 253 J C-r were plated as mentioned in *table 1* and treated with loaded S002-P0.15% (0.08  $\mu$ M siRNA, 8  $\mu$ g/ml CCD) in combination with 2.5  $\mu$ g/ml CDDP. Results were compared with those obtained by treating cells with unloaded S002-P0.15%, loaded S002-P0.15% alone, and CDDP alone. Moreover, cells were transfected with anti-Nrf2 siRNA with the use of HiPerFect<sup>®</sup> transfection reagent (0.08  $\mu$ M siRNA) with or without treatment with CDDP. Results are shown in *figure 12*.

It is demonstrated that siNrf2-S002-P0.15% has the ability to sensitize T24 and B-V 253J C-r to CDDP treatment at 24 and 48 hours. As expected, treatment with CDDP alone did not affect the viability of the resistant cells, while the combined treatment (siNrf2-S002-P0.15% and CDDP) lead to decrease in the viability 24 hours after treatment (viability of T24: CDDP=80% versus combination=55% | Viability of B-V 253J C-r: CDDP=95% versus combination=40%). 48 hours after treatment this decrease in viability is even stronger: in both cell lines a decrease of 75% in viability is observed.

Transfection with the HiPerfect<sup>®</sup> reagent did not sensitize the cells (of both cell lines) to the CDDP treatment at 24 hours. *Figure 11a* showed a decrease in level of Nrf2 expression at 24 hours after the treatment, but not in the GSTA4 content. GSTA4 is, as mentioned before, one of the effectors of Nrf2 and involved in the antioxidant system and could be responsible for defusing the toxic effects of CDDP. 48 hours after treatment a decrease in the viability of both cell lines is observed. Possible is that a prolonged exposure to Nrf2+HiPerfect<sup>®</sup> will lead to a prolonged effect as well, since this method seems to be able to silence Nrf2 for a longer period (see *figure 11*).

The toxic effect of unloaded CCDs (the viability of B-V 253 J cells was affected significantly) observed in *figure 12*, as mentioned before a decrease in viability of ~20% was considered as acceptable.



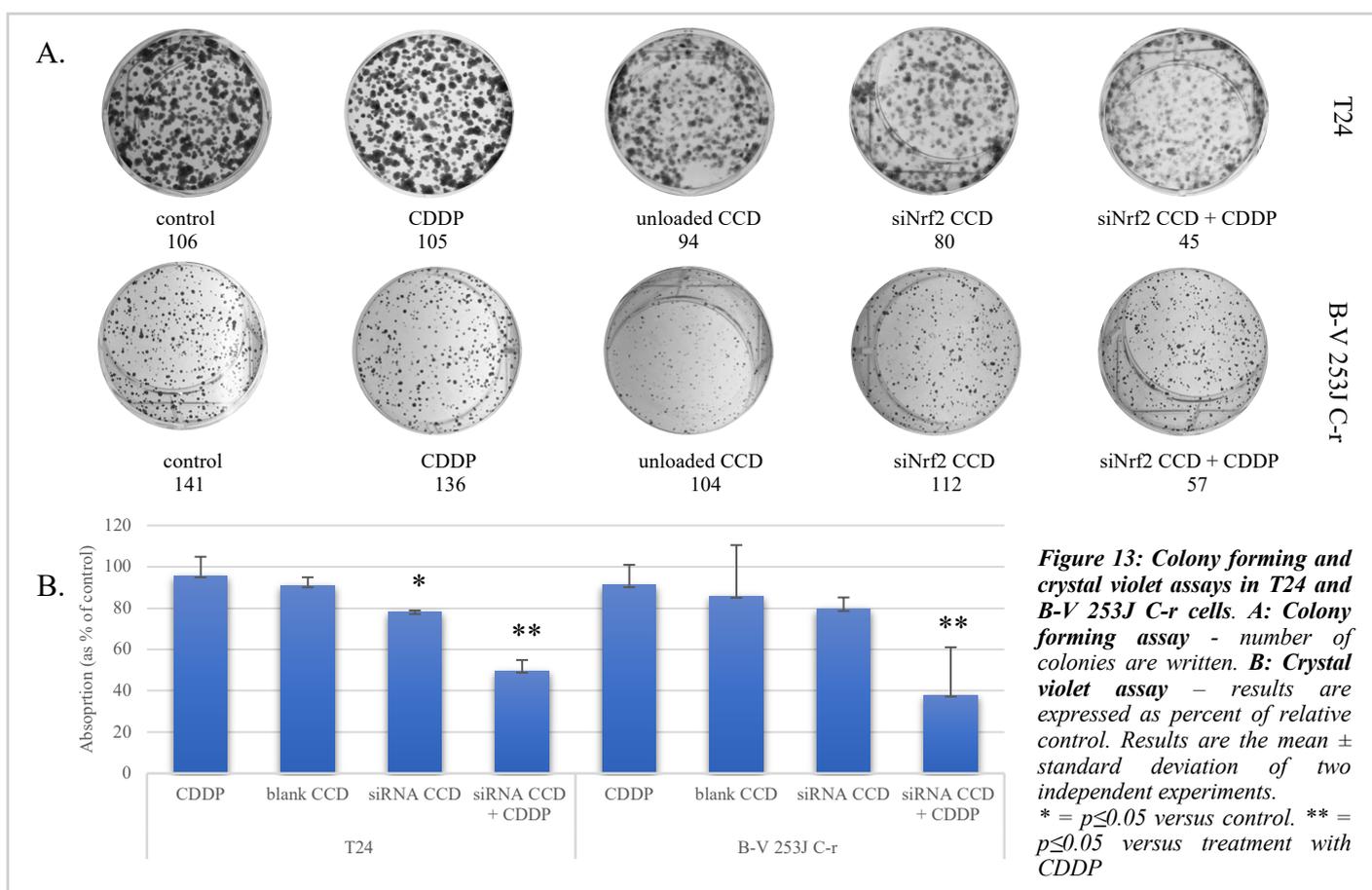
**Figure 12: MTT assay in T24 (left) and B-V 253J C-r (right).** Cells were exposed to a variety of treatments: (A) unloaded S002-P0.15%, (B) CDDP, (C) siNrf2-S002-P0.15%, (D) siNrf2-S002-P0.15% + CDDP, (E) siNrf2 transfected with HiPerFect<sup>®</sup> reagent and (F) siNrf2 transfected with HiPerFect<sup>®</sup> + CDDP. Results are expressed as percent of the control and are the mean  $\pm$  standard deviation of three separate experiments performed in (at least) triplicate. \* =  $p \leq 0.05$  versus control (= untreated cells) \*\* =  $p < 0.05$  versus treatment with CDDP alone.

### 4.3.3 Effect of Nrf2 silencing on cell growth and colony forming

To analyze if silencing of the Nrf2 gene could affect cell growth and colony forming of CDDP resistant bladder cancer cells, B-V 253J C-r and T24 were exposed to loaded and unloaded S002-P0.15% CCD, CDDP and a combination of the neoplastic agent and loaded CCDs (final concentrations: 0.04  $\mu$ M siRNA, 0.1  $\mu$ g/ml CDDP). Results demonstrated that Nrf2 silencing affects the cell growth and colony forming (*figure 13a*, number = number of colonies) negatively in both cell lines (control versus siNrf2 CCD). Moreover, these obtained results confirmed that silencing of Nrf2 leads to an increase of the sensitivity to CDDP (siNrf2 CCD + CDDP versus CDDP alone). In both cell lines the number of colonies was halved. It was noticed that exposure to CCDs (loaded and unloaded) resulted in less dense colonies. Moreover, the toxic effect of blank CCDs on the viability is demonstrated, especially in B-V 253J C-r cells: as shown in *figure 12* the viability of B-V 253J C-r cells is affected significantly. It is expected that the longer exposure to blank CCDs the more the viability is affected and thus the capacity of proliferation.

By dissolving the staining in acetic acid (*3.10 Crystal violet assay*) the colonies could be quantified by absorption. Hypothetically, the staining (and thus absorption) should be less if the colony formation is affected. This because the crystal violet dye binds to proteins and DNA. *Figure 13b* underlines that a decrease in absorption follows after treatment with anti-Nrf2 siRNA results (siNrf2 CCD versus control). Only in T24 was this decrease significant ( $p < 0.05$ ), in the other cell line it was hard to remove all the dye in between the colonies (those were very small). Therefore, the measured absorption is higher than it should be, which resulted in more variation between the wells/experiments and a not significant decrease.

Also, the observed decrease in numbers of colonies of cells treated with CDDP in combination with siNrf2 loaded CCDs appears to be significant (*figure 13b*: siNrf2 CCD+CDDP versus CDDP and/or versus control). CDDP alone did not affect the proliferation of both cell lines.



#### 4.3.4 Effects of Nrf2-silencing, alone or with CDDP, on apoptosis

After demonstrating that transfection of the anti-Nrf2 siRNA resulted in a reduction of the Nrf2 (and GSTA4) protein levels, in an inhibition of cell growth and increased the sensitivity to CDDP treatment, the consequences of Nrf2 silencing on going into apoptosis after CDDP treatment were analyzed. Nrf2 was silenced in B-V 253J C-r and T24 using the loaded and optimized S002 CCD.

Apoptosis is characterized by morphological and biochemical changes in the cell, which can be used for detection. A number of techniques have been used for determining the morphological changes in the cell and thus the fraction of apoptotic cells. One of them is based on fluorescent coloring and allows detection by flow cytometry. The coloring is obtained with the use of two agents: Annexin V and propidium iodide (PI), which will result in four distinct cell populations.<sup>59</sup>

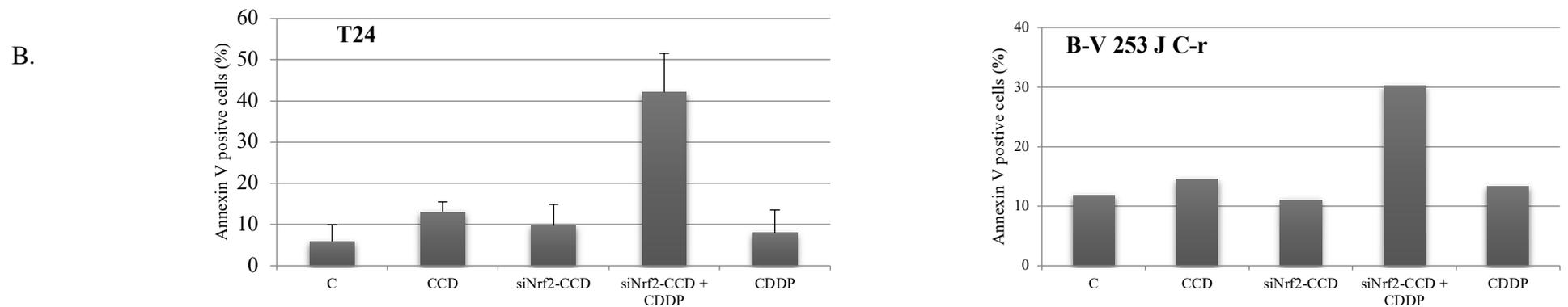
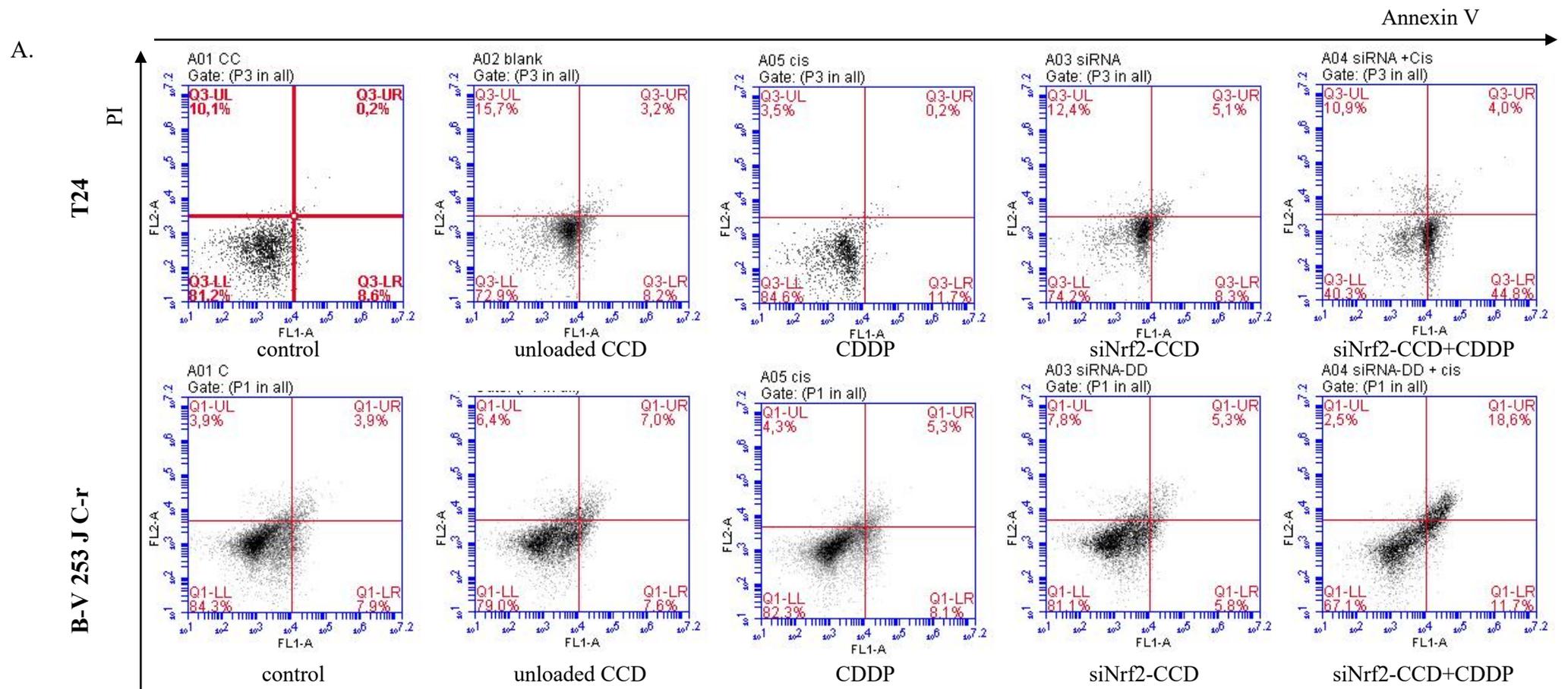
Annexin V is a fluorescent labeled protein and binds to phosphatidylserine. In case of apoptosis, cells present phosphatidylserine on the outer leaflet of the plasma membrane as a result of a loss of membrane phospholipid asymmetry. PI is a fluorescent agent that stains DNA. In case of necrosis the cell membrane will disrupt whereby the content of the cell is released.<sup>59</sup>

Therefore, you may see four cell populations: Annexin V+/PI- = early apoptotic cells | Annexin V+/PI+ = late apoptotic cells | Annexin V-/PI+ = necrotic cells || Annexin V-/PI- = live cells.

The level of apoptotic cells was measured as Annexin V positive cells (*figure 14*). It was seen that after silencing of Nrf2 in both cell lines, their sensitivity to CDDP increased (*figure 13*). This is confirmed in the following figure; an increase in the number of apoptotic cells is seen Nrf2 is silenced compared to treatment with CDDP alone (*figure 13b*). Although this increase is not significant a trend is seen ( $p=0.066$  versus control /  $p=0.051$  versus CDDP (T24)). There is no (statistic) difference between untreated and CDDP treated cells ( $p=0.32$  (T24)).

Moreover, an increase in apoptotic cells is noticed when cells are only exposed to siNrf2-CCD. Silencing of Nrf2 make cells more prone to apoptosis because the transcription factor does not only regulate the level of detoxifying compounds but also regulates the transcription of Bcl-2 and Bcl-xL (two anti- apoptotic genes).<sup>60,61</sup>

The difference between the fractions of apoptotic cells is lower than expected: microscopic observation learned that all cells were affected by CDDP treatment when cells were exposed to anti-Nrf2 siRNA compared to untreated cells (control) and treated with CDDP. It is possible that the cells were completely defragmented and therefore particles were too small to detect. Centrifugation at 3000 or 1000 rpm allows only the sedimentation of bigger fragments. Possibly, a part of the apoptotic/necrotic cells was not present in the formed pellet but stayed behind in the supernatant. It could be that therefore the fraction of apoptotic cells appeared to be smaller than expected. One could consider performing centrifugation at a higher speed, however this could cause damage to cell which are intact and allowing staining of these cells by the fluorescent compound leading to a false positive result. Another option could be to perform the assay at an earlier timepoint preventing complete disruption after treatment and which could result in measuring the complete fraction of cells going into apoptosis.



**Figure 14: Cytofluorimetric analysis.** A: analysis of apoptosis in T24 and B-V 253 J C-r cells 24 hours (B-V 253 J C-r) and 48 hours (T24) after treatment. B. Percentage of Annexin V positive cells, indicating fraction of apoptotic cells. C = control, untreated cells. Results are presented as mean  $\pm$  standard deviation of two independent experiments (T24). Apoptosis assay with B-V 253 J C-r was carried out once.

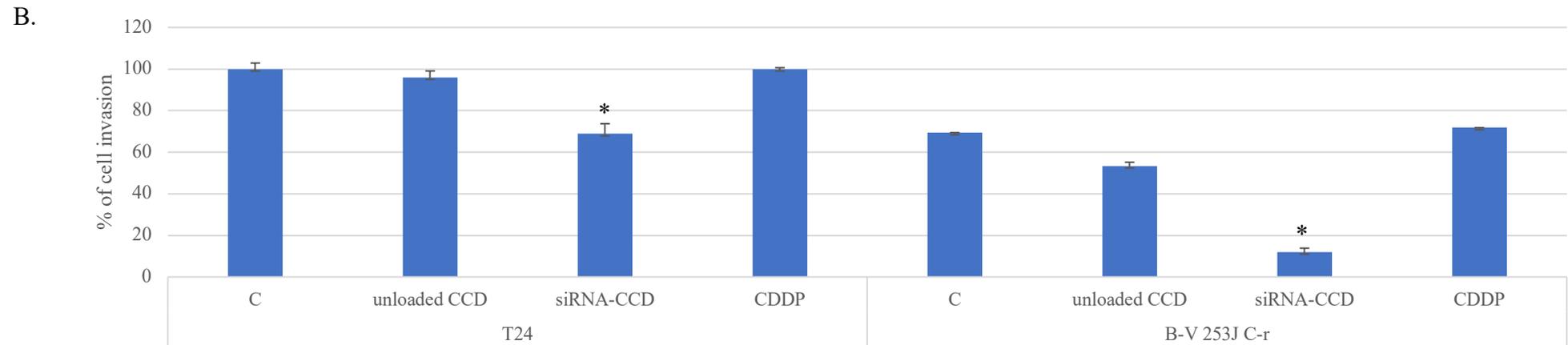
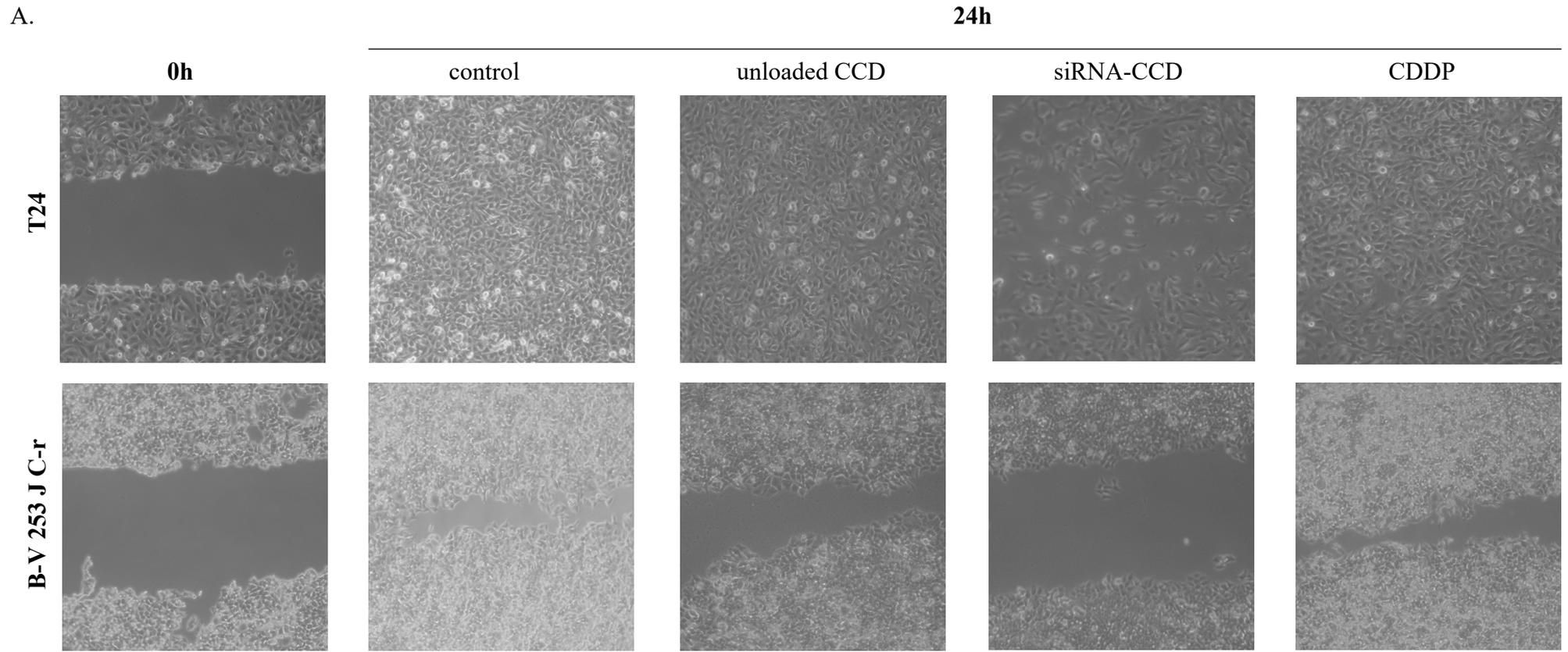
#### 4.3.5 The effect of Nrf2 silencing on migration of bladder cancer cells

Nrf2 does not only regulate cell growth and the antioxidant capacity, but also the migration of cancer cells.<sup>27,62</sup> This, of course, contributes to the metastatic properties of cancers who bring a high level of Nrf2 to expression. Since a downregulation of Nrf2 expression was obtained after exposing cells to loaded CCDs, it was hypothesized that exposure of cells to anti-Nrf2 siRNA could reduce cell migration.

Cell motility was assessed using a wound healing assay (3.10 *Wound healing assay*), evaluating random cell migration in the presence of anti-Nrf2 siRNA loaded S002-P0.15%.

Results, presented in *figure 15*, shows that silencing of Nrf2 inhibited the cell migration of T24 and B-V 253J C-r significantly. Treatment with CDDP or unloaded CCD resulted in migration of cells comparable to the control ( $p \gg 0.05$  in both cell lines for both cases).

These results are similar to those obtained with a different Nrf2 expression inhibitor: Ailanthone.<sup>63</sup> Interestingly with this compound, migration was more affected in the CDDP resistance cell line (B-V 235J C-r) than in the sensitive cell line (B-V 253J).<sup>63</sup> This can possibly be attributed to the involvement of Nrf2 in cell migration. As shown before (*figure 10b*), the content of Nrf2 is twice as high in resistant cells than in sensitive cells, which explains the difference in cell migration into the wound: cells which bring a higher level of Nrf2 to expression will be more affected than cells without or with lower content of Nrf2.



**Figure 14a: Wound healing assay at 0 and 24 hours in T24 and B-V 253 J C-r cells.** Cells were exposed to unloaded and with anti-Nrf2 siRNA loaded CCD and CDDP. Untreated cells were used as a control. **B: Quantification of wound healing assay.** The endpoint of the assay was measured by calculating the reduction in the width of the wound after 24 hours and compared to the width direct after application (width at 0h = 100%). The data is expressed as a mean  $\pm$  standard deviation of two independent experiments each performed in triplicate. \* =  $p \leq 0.05$  versus control and CDDP.

## 5. SUMMERIZING DISCUSSION AND CONCLUSION

Bladder cancer is one of the top five frequently diagnosed cancers in the European Union (top 10 worldwide), with an incidence of approximately 430.000 cases worldwide.<sup>64</sup> Treatment for urothelial carcinoma is CDDP-based and used to provide a survival advantage in bladder cancer.<sup>22-24</sup> However, relapses occur regularly and often the cells gained resistance to the previous used chemotherapy, which results a poor prognosis.<sup>64</sup> Therefore, the identification of an agent which can reduce resistance is of great interest.

In some cancers, among them bladder cancer, the gained resistance is determined by the increase of the antioxidant capacity and redox adaptation.<sup>65</sup> It has been reported that the transcription factor Nrf2 induces the transcriptional activation of more than 100 detoxification and cytoprotective genes.<sup>66</sup> Therefore, Nrf2 is an interesting target to combat resistance. This study was focused on the inhibition of Nrf2 with a specific siRNA (anti-Nrf2 siRNA or siNrf2). In order to transfect the bladder cancer cells with the siNrf2 a nanocarrier was used. With the opportunity to modify two different types, a harmless carrier for the siNrf2 was found.

Dendrimer (provided by Professor Gomez, Spain) BDLS002 was considered as a suitable carrier for anti-Nrf2 siRNA. The negatively charged siRNA can be bound by electrostatic interactions to positively charged terminal groups. This nanocomplex is formed spontaneously when both compounds are brought into the same environment. Unfortunately, this way of preparation resulted in a toxic complex because of the net positive charge of the formed complex. Compounds with a positive charge will interact with the cell membrane, which cause disruption and results in cell death. Therefore, Pluronic F68<sup>®</sup> was added to the nanocomplex suspension. It is a worldwide used surfactant in suspensions and in cell culturing it can stabilize cell membranes, protecting them from membrane shearing.<sup>56</sup> Addition of 0.15% (v/v) Pluronic F68<sup>®</sup> resulted in a harmless delivery system.

It was hypothesized that incorporating the siRNA against Nrf2 within the optimized nanocarrier would enhance the uptake of the nucleic acid. Therefore, a downregulation of the expression of Nrf2 and its effectors was expected in tumor cells which possess a high level of Nrf2 and thus resulting in a reduction of CDDP resistance and a repression of the tumor growth.

To verify the ability of the optimized delivery system to transfect bladder cancer cells, the Nrf2 expression in CDDP resistant cells (T24 and B-V 253J C-r) and CDDP sensitive cells (B-V 253J) was analyzed. A lower expression level of Nrf2 was found when cells were exposed to the siNrf2 loaded dendrimer. Thus, the used delivery system enhances the effect of siNrf2. This effect is probably a result of the nanocarrier: the results suggest that a higher intracellular concentration of anti-Nrf2 siRNA is reached in a shorter period of time than when transfection occurred after treatment with the HiPerFect<sup>®</sup> Transfection Reagent. Moreover, results show, as expected, an increase in Nrf2 expression after induction of CDDP resistance, indicating the involvement of Nrf2 in chemotherapy resistance.

Earlier results<sup>20,63</sup> demonstrated that Nrf2 is involved in maintaining the antioxidant potential (and thus in resistance to CDDP-based therapeutic agents) and metastatic properties of bladder cancer cells, and that Nrf2 is a potential target in patients diagnosed with chemotherapy resistant bladder cancer. This study underlines those findings: downregulation of the Nrf2 expression by the use of anti-Nrf2 siRNA loaded CCDs resulted

in in a significant increase in CDDP sensitivity, in an increase in apoptosis induction and a significant decrease in cell proliferation, growth and cell migration in chemotherapy resistant bladder cells.

It should be noted that all experiments are carried out in tumor cells. Therefore, an overall control is necessary; the nanocomplex cannot be used if it is harmful for healthy tissue. Beside testing the toxicity of complex in healthy cells, next steps should include a fluorescence assay: it is assumed that the carrier enhanced the uptake of siNrf2, resulting in high intracellular concentrations of the nucleic acid and therefore, the effect of siNrf2 is seen. Attachment of a fluorescent compound may reveal important information about the uptake of siNrf2.

*In vivo* studies seem to follow, there is a need to study the safety profiles with focus on toxicity. Furthermore, the carrier-siNrf2-complex should not elicit an immune response.

Another point of interest can be found in the modification of the dendrimer. Since dendrimers have a branched structure, which results in multiple functional terminal groups, they can be modified easily. Adding functional groups (such as: anti-bodies, ligands or small molecules<sup>4</sup>) by which tumor cells can be targeted could be a targeting strategy in order to reduce side effects. Moreover, if the siRNA could be attached to the dendrimer via a variety of linkers, controlled release of the siRNA could be possible, with a prolonged activity (and reducing the chance on side effects) as result. To be able to sensitize cancer cells, while making use of the advantages of nanocarriers for therapeutic agents, both the siRNA and drug must be delivered in the same device.

In conclusion, we suggest that the BDLS002 carbosilane cationic dendrimer loaded with siRNA is a promising drug delivery system for gene therapy to be used *in vivo*. Moreover, it may represent an important tool in the therapy of protein-dependent resistant cancer.

## AKNOWLEDGEMENT

It is done! I must confess I struggled writing this thesis. It was not the writing itself, but finding the time and focus, but then again: it is done. First of all, I would like to thank my daily supervisor Prof. Stefania Pizzimenti: you welcomed me in Turin and whenever I did not know what happened, why it happened and how to continue, you knew. Thank you for your trust in me and letting me make mistakes, so that I could learn from it. Prof. Giuseppina Barrera, I am grateful for the time and effort that you put into my (our) research.

Dear Martina, Martina piccola, Serena, Angele, Marge and Elisa: you guys made the lab feel like home. Thank you for letting me be a part of it. I enjoyed our chats, the lunches in the park and learning Italian from you; I will never forget the word imbuto.

At last, I would like to thank dr. ir. Inge de Graaf: thank you for giving me the opportunity to go to Turin for this project and for the valuable comments on this thesis.

Un abbraccio,

L. (Leanne) Ambrosio  
Groningen, May 2019

## REFERENCES

1. World Health Organization. Cancer – World Cancer Day. <http://www.who.int/cancer/world-cancer-day/2018/en/>. Accessed at 9/9/2018.
2. Rakesh. K Tekade, Tathagata Dutta, Abhishek Tyagi, Alok Chandra Bharti, Bhudev C. Das, Narendra K. Jain: Surface-engineered dendrimers for dual drug delivery: A receptor up-regulation and enhanced cancer targeting strategy. *Journal of Drug Targeting* 16:10 (2008) 758-772
3. Nishant S. Gandhi, Rakesh K. Tekade, Mahavir B. Chougule: Nanocarrier mediated delivery of siRNA/miRNA in combination with chemotherapeutic agents for cancer therapy: Current progress and advances. *Journal of Controlled Release* 194 (2014) 238-256
4. Mar Creixell, Nicholas A. Peppas: Co-delivery of siRNA and therapeutic agents using nanocarriers to overcome cancer resistance. *Nano Today* (2012) 7, 367-379
5. E. Ortiz-Tudela, A. Mteyrek, A. Ballesta, P.F. Innomiato, F. Lévi: Cancer Chronotherapeutics: Experimental, Theoretical, and Clinical Aspects. In: Kramer A., Merrow M. (eds) *Circadian Clocks. Handbook of Experimental Pharmacology*, volume 217. Springer, Berlin, Heidelberg
6. Jack H. Che-Ming, Zhang Liangfang: Nanoparticle-based combination therapy toward overcoming drug resistance in cancer. *Biochemical Pharmacology* 83:8 (2012) 1104-1111
7. Margaret A. Phillips, Martin L. Gran, Nicholas A. Peppas: Targeted nanodelivery of drugs and diagnostics. *Nanotoday* (2010) 5:2; 143-159
8. J.M. Adams, S. Cory: Bcl-2-regulated apoptosis: mechanism and therapeutic potential. *Current Opinion in Immunology* (2007), 19: 488-496
9. Nika N. Danial, Stanley J. Korsmeyer: Cell Death: Critical Control Points. *Cell*, 116:2 (2004) 205-219
10. Elaine M. Leslie, Roger G. Deeley, Susan P.C. Cole: Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicology and Applied Pharmacology*. 204:3 (2005) 216-237
11. Michael M. Gottesman: Mechanisms of Cancer Drug Resistance. *Annu. Rev. Med.* (2002) 53: 615-627
12. Shaloom Dasari, Paul Berenard Tchounwou: Cisplatin in cancer therapy: molecular mechanisms of action. *Eur. J. Pharmacol.* (2014) 364-378
13. Magali Rebutti, Carine Michiels: Molecular aspects of cancer cell resistance to chemotherapy. *Biochemical Pharmacology* (2013) 85:9; 1219-1226
14. Rossella Marullo, Erica Werner, Natalya Degtyareva, Bryn Moore, Giuseppe Altavilla, Suresh S. Ramalingam, Paul W. Doetsch: Cisplatin Induces a Mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetics functions. (2013) *PLoS ONE* 8(11): e81162. DOI: 10.1371/journal.pone.0081162
15. Subash C. Gupta, David Hevia, Sridevi Patchva, Byoungduck Park, Wonil Koh, Bharat B. Aggarwal: Upsides and downsides of reactive oxygen species for cancer: the roles of reactive oxygen species in tumorigenesis, prevention, and therapy. *Anitoid Redox Signal.* (2012) 1295-1322 Doi:10.1089/ars.2011.4414
16. Sahdeo Prasad, Subash C. Gupta, Amit K. Tyagi: Reactive oxygen species (ROS) and cancer: Role of antioxidative nutraceuticals. *Cancer Letters* (2017) 387: 95-105
17. Winterbourn CC: Reconciling the chemistry and biology of reactive oxygen species. *Nat Chem Biol.* (2008) 278-286
18. Helene Pelicano, Dennis Carney, Peng Huang: ROS stress in cancer cells and therapeutic implications. *Drug Resistance Updates* (2004) 7:2; 97-110
19. Zhengzhi Zou, Haocai Chang, Haolong Li, Songmao Wang: Induction of reactive oxygen species: an emerging approach for cancer therapy. *Apoptosis* (2017) 22:1321-1335
20. Eric Ciamporocero, Martina Daga, Stefania Pizzimenti, Antonella Roetto, Chiara Dianzani, Alessandra Compagnone, Antonietta Palmieri, Chiara Ullio, Luigi Cangemi, Roberto Pili, Giuseppina Barrera: Crosstalk between Nrf2 and YAP contributes to maintaining the antioxidant potential and chemoresistance in bladder cancer. *Free Radical Biology and Medicine* 115 (2018) 447-457
21. Ondrej Zitka, Sylvie Skalickova, Jaromir Gumulec, Michal Masarik, Vojtech Adam, Jaromir Hubalek, Libuse Trnkova, Jarmila Kruseova, Tomas Eckschlagger, Rene Kizek: Redox status expressed as GSH:GSSG ratio as a marker for oxidative stress in paediatric tumor patients. *Oncology Letters* (2012) 4:6; 1247-1253
22. Duniyaporn Trachootham, Jerome Alexandre, Peng Huang: Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nature Reviews Drug Discovery* (2009) 8: 579-591
23. Annette Hayden, James Douglas, Matthew Sommerlad, Lawrence Andrews, Katherine Gould, Syed Hussain, Gareth J. Thomas, Graham Packham, Simon J. Crabb: The Nrf2 transcription factor contributes to resistance to cisplatin in bladder cancer. *Urologic Oncology* (2014) 32:6; 806-814
24. Martin C.S. Wong, Franklin D.H. Fung, Colette Leung, Wilson W.L. Cheung, William B. Goggins, C.F. Ng: The global epidemiology of bladder cancer: a joinpoint in regression analysis of its incidence and mortality trends and projection. *Scientific Reports* (2018) 8:1129 DOI: 10.1038/s41598-018-19199-z
25. Andrea Mari, David D'Andrea, Mohammad Abufaraj, Beat Foerster, Shoji Kimura, Shahrokh F. Shariat: Genetic determinants for chemo- and radiotherapy resistance in bladder cancer. *Translational Andrology and Urology* (2017) 6:6; 1081-1089
26. Martina Daga, Chiara Ullio, Monica Argenziano, Chiara Dianzani, Roberta Cavalli, Francesco Trotta, Carlo Ferretti, Gian Paolo Zara, Casimiro L. Gigliotti, Eric, S. Ciamporocero, Piergiorgio Pettazzoni, Denise Corti, Stefania Pizzimenti, Giuseppina Barrera: GSH-targeted nanosponges increase doxorubicin-induced toxicity “in vitro” and “in vivo” in cancer cells with

- high antioxidant defenses. *Free radical biology and medicine* (2016) 97: 24-37
27. Montserrat Rojo de la Vega, Eli Chapman, Donna D. Zhang: NRF2 and the Hallmarks of Cancer. *Cancer Cell*, 34 (2018) 21-43
  28. Lauren E. Tebay, Holly Robertson, Stephen T. Durant, Steven R. Vitale, Trevor M. Penning, Albenia T. Dinkova-Kostova, John D. Hayes: Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues and energy status and the pathways through which it attenuates degenerative disease. *Free Radic Biol Med* (2015) 108-146
  29. Keiko Taguchi, Masayuki Yamamoto: The KEAP1-NRF2 System in Cancer. *Front. Oncol.* (2017), DOI: 10.3389/fonc.2017.00085
  30. Michael John Kerins, Aikseng Ooi: A catalogue of somatic NRF2 gain-of-function mutations in cancer. *Scientific Rep.* (2018) 8:12846 DOI: 10.1038/s41598-018-31281-0
  31. Jiayu Zhu, Hui Hui Wang, Feng Chen, Jingqi Fu, Yuanyan Xu, Yongyong Hou, Henry H. Kou, Cheng Zhai, M. Bud Nelson, Qiang Zhang, Melvin E. Andersen, Jingbo Pi: An overview of chemical inhibitors of the Nrf2-ARE signaling pathway and their potential applications in cancer therapy. *Free Radical Biology and Medicine* (2016) 99: 544-556
  32. Jong-Lyel Roh, Eun Hye Kim, Hyejin Jang, Daiha Shin: Nrf2 inhibition reverses the resistance of cisplatin-resistant head and neck cancer cells to artesunate-induced ferroptosis. *Redox Biology* (2017) 11: 254-262
  33. Jeong-Min Cho, Sarala Manandhar, Hyang-Rim Lee, Hyun-Min Park, Mi-Kyoung Kwak: Role of the Nrf2-antioxidant system in cytotoxicity mediated by anticancer cisplatin: Implication to cancer cell resistance. *Cancer Letters* (2008) 260;1-2: 96-108
  34. Haopeng Sun, Jie Zhu, Hongzhi Lin, Kai Gu, Feng Feng: Recent progress in the development of small molecule Nrf2 modulators: a patent review (2012-2016). *Expert Opinion on Therapeutic Patents* (2017) 27:7; 763-785.
  35. Peng-Cheng Li, Mei – Juan Tu, Pui Yan Ho, Joseph L. Jilek, Zhijian Duan, Qian-Yu Zhang, Ai-Xi Yu, Ai-Ming Yu: Bioengineered NRF2-siRNA is effective to interfere with NRF2 pathways and improve chemosensitivity of Human Cancer Cells. *Drug Metabolism and Disposition.* (2018) 46: 2-10
  36. Sang Hun Chun, Ji Soo Yuk, Soong Ho Um: Regulation of cellular gene expression by nanomaterials. *Nano Convergence* (2018) 5:34. DOI: <https://doi.org/10.1186/s40580-018-0166-x>
  37. Ernie Hood: RNAi: What's all the noise about gene silencing? *Environmental Health Perspectives.* (2004) 112:4; A224-A229.
  38. Jenny K.W. Lam, Michael Y.T. Chow, Yu Zhang, Susan W.S. Leung: siRNA versus miRNA as therapeutics for gene silencing. *Molecular Therapy Nucleic Acids* (2015) 4, e252; DOI: 10.1038/mtna.2015.23
  39. Michael P. Gantier, Bryan R.G. Williams: The response of mammalian cells to double-stranded RNA. *Cytokine Growth Factor Rev.* (2007) 18:5-6; 363-371
  40. Hao Yin, Rosemary L. Kanasty, Ahmed A. Eltoukhy, Arturo J. Vegas, J. Robert Dorkin, Daniel G. Anderson: Non-viral vectors for gene-based therapy. *Nature Reviews Genetics* (2014) 15: 541-555
  41. Dinesh Kumar Mishra, Neelam Balekar, Pradyumna Kumar, Mishra: Nanoengineered strategies for siRNA delivery: from target assessment to cancer therapeutic efficacy. *Drug Delivery and Translational Research* (2017) 7:2; 346-358
  42. Roberta Cavalli, Luca Primo, Roberto Sessa, Giulia Chiaverina, Laura di Blasio, Jenny Alongi, Amedea Manfredi, Elisabetta Ranucci, Paolo Ferruti: The AGMA1 polyamidoamine mediates the efficient delivery of siRNA. *Journal of Drug Targeting* (2017) 25:9-10; 891-898
  43. Javier Sánchez-Nieves, Peter Fransen, Daniel Pulido, Raquel Lorente, M. Ángeles Muñoz-Fernández, Fernando Albericio, Miriam Royo, Rafael Gómez, F. Javier de la Mata: Amphiphilic cationic carbosilane-PEG dendrimers: synthesis and application in gene therapy. *European Journal of Medicinal Chemistry* (2014) 76: 43-52
  44. Fakhar ud Din, Waqar Aman, Izhar Ullah, Omer Salman Qureshi, Omer Mustapha, Sumaila Shafique, Alam Zeb: Effective use of nanocarriers as drug delivery systems for the treatment of selected tumors. *International Journal of Nanomedicine* (2017) 12: 7291-7309
  45. Edgar Pérez-Herrero, Alberto Fernández-Medarde: Advanced targeted therapies in cancer: Drug nanocarriers, the future of chemotherapy. *European Journal of Pharmaceutics and Biopharmaceutics* (2015) 93: 52-79
  46. Hongtao Lv, Shubiao Zhang, Bing Wang, Shaohui Cui, Jie Yan: Toxicity of cationic lipids and cationic polymers in gene delivery. *Journal of Controlled Release* (2006) 114: 100–109.
  47. Yiyun Cheng, Zhenhai Xu, Minglu Ma, Tongwen Xu: Dendrimers as drug carriers: Applications in different routes of drug administration. *Journal of Pharmaceutical Sciences* (2008) 97:1; 123-143
  48. Nick Weber, Paula Ortega, Mairia Isabel Clemente, Dzmitry Shcharbin, Maria Bryszewska, F. Javier de la Mata, Rafael Gómez, M. Angeles Muñoz-Fernández: Characterization of arbosilane dendrimers as effective carriers of siRNA to HIV-infected lymphocytes. *Journal of Controlled Release* (2008) 132:1; 55-64
  49. Irene Heredero-Bermejo, José M. Hernández-Ros, Leticia Sánchez-García, Marek Maly, Cristina Verdú-Expósito, Juan Soliveri, F. Javier de la Mata, José L. Copa-Patiño, Jorge Pérez-Serrano, Javier Sánchez-Nieves, Rafael Gómez: Ammonium and guanidine carbosilane dendrimers and dendrons as microbicides. *European Polymer Journal* (2018) 101: 159-168
  50. Roberta Cavalli, Agnese Bisazza, Michele Trotta, Monica Argenziano, Andrea Civra, Manuela Donalizio, David Lembo: New chitosan nanobubbles for ultrasound-mediated gene delivery: preparation

- and in vitro characterization. *International Journal of Nanomedicine* (2012) 7: 3309-3318
51. Juan C. Stockert, Alfonso Blázquez-Castro, Magdalena Cañete, Richard W. Horobin, Ángeles Villanueva: MTT assay for cell viability: Intracellular localization of the formazan products is in lipid droplets. *Acta Histochemica* (2012) 114:8; 785-796
  52. Paul W. Sylvester: Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. *Drug Design and Discovery* (2011) 716; 157-168
  53. Nicolaas A.P. Franken, Hans M. Rodermond, Jan Stap, Jaap Haveman, Chris van Bree: Clonogenic assay of cells in vitro. *Nature Protocols* (2006) 1: 2315-2319
  54. Rodriguez L.G., Wu X., Guan JL. (2005) Wound-Healing Assay. In: Guan JL. (eds) *Cell Migration. Methods in Molecular Biology™*, vol 294. Humana Press
  55. Laura Koontz: Chapter Four – Agarose Gel Electrophoresis. In: *Methods in Enzymology*, volume 529 (2013) 35-45
  56. Elena V. Batrakova, Alexander V. Kabanov: Pluronic Block Copolymers, Evolution of Drug Delivery Concept from Inert Nanocarriers to Biological Response Modifiers. *J. Control. Release* (2018) 98-106
  57. John R. W. Masters, Peter J. Hepburn, Lawrence Walker, Wilm J. Highman, Ludwik K. Treidosiewicz, Susan Povey, Mohamed Parkar, Bridget T. Hill, Peter R. Riddle, Leonard M. Franks: Tissue Culture Model of Transitional Cell Carcinoma: Characterization of Twenty-two Human Urothelial Cell Lines. *Cancer Research* (1986) 46: 3630-3636
  58. Colin P. N. Dinney, Randi Fishbeck, Rakesh K. Singh, Beryl Eve, Sen Pathak, Nicholas Brown, Bei Xei, Domic Fan, Corazon D. Bucana, Isaiah J. Fidler, Jerald J. Killion: Isolation and characterization of metastatic variants from human transitional cell carcinoma passaged by orthotopic implantation in athymic nude mice. *The Journal of Urology* (1995) 154:4;1532-1538
  59. Pavlína Majtnerová, Tomáš Roušar: An overview of apoptosis assays detecting DNA fragmentation. *Molecular Biology Reports* (2018) 45:5; 1469-1478
  60. Suryakant K. Nitrué, Anil K. Jaiswal: Nrf2-induced antiapoptotic Bcl-xL protein enhances cell survival and drug resistance. *Free Radical Biology and Medicine* (2013) 57: 119-131
  61. Suryakant K. Nitrué, Anil K. Jaiswal: Nrf2 protein upregulates antiapoptotic protein Bcl-2 and prevents cellular apoptosis. *J. Biol. Chem.* (2012) 287: 9873-9886
  62. Jonathan W. Haskins, Don X. Nguyen, David F. Stern: Neuregulin 1-activated ERBB4 interacts with YAP to induce Hippo pathway targetgenes and promote cell migration. *Sci Signal* (2014) 7:355; 1-10
  63. Martina Daga, Stefania Pizzimenti, Chiara Dianzani, Marie Angele Cucci, Roberta Cavalli, Margherita Grattarola, Benedetta Ferrara, Valentin Scariot, Francesco Trotta, Giuseppina Barrera: Ailanthone inhibits cell growth and migration of cisplatin resistant bladder cancer cells through down-regulation of Nrf2, Yap, and c-Myc expression. *Phytomedicine* (2019) 56: 156-164.
  64. Leonardo L. Monteiro, J. Alfred Witjes, Piysh K. Agarwal, Christopher B. Anderson, Trinity J. Byvalacqua, Bernard H. Bochner, Joost L. Boormans, Sam S. Chang, Jose L. Domínguez-Escrig, James M. McKiernan, Colin Dinney, Guilherm Godoy, Girish S. Kulkarni, Paramanathan Mariappan, Michael A. O'Donnell, Cyril A. Rentsch, Jay B. Shah, Robert S. Svatek, Antoin G. van der Heijden, F. Johannes P. van Valenberg, Wassim Kassou: ICUD-SIU International consultation on bladder cancer 2017: management of non-muscle invasive bladder cancer. *World Journal of Urology* (2019) 37:1; 51-60
  65. Matteo Landriscina, Francesca Maddalena, Gabriella Laudiero, Franca Esposito: Adaptation to oxidative stress, chemoresistance, and cell survival. *Antioxidants & Redox Signaling* (2009) 11: 2701-2716
  66. Chiara Gorrini, Isaac S. Harris, Tak W. Mak: Modulation of oxidative stress as an anticancer strategy. *Nature Reviews Drug Discovery* (2013) 12: 931-947.

**APPENDIX 1: Instructions HiPerFect® Transfection Reagent, Qiagen**