

# Comparison of therapeutic efficacy of quadrupole stimuli-targeted nanocontainers loaded with doxorubicin (Nano4Dox platform) and cisplatin (Nano4Cis platform) to Doxil and Lipoplatin, respectively

George Kordas<sup>1</sup> and Eleni Efthimiadou

Sol-Gel Laboratory Institute of Nanoscience and Nanotechnology, National Center of Scientific Research "Demokritos", Paraskevi Attikis, Greece

**ABSTRACT** We developed polymeric nanocontainers consisting of a shell with pH, T, and redox sensitivity grafted with targeting groups such as folic acid, leuprolide, and so on to target breast and prostate cancers. The quadrupole stimuli-targeted nanocontainers were loaded with doxorubicin and cisplatin called Nano4Dox and Nano4Cis platforms, respectively. In this study, we report the therapeutic efficacy of these platforms and compare with the corresponding liposomal analogs such as Doxil and Lipoplatin. Our Nano4XX (XX = Dox, Cis, etc.) platforms are extremely stable in the blood system. Our Nano4XX technology presents a significant improvement over the state-of-the-art accounting for the first time to include four stimuli (pH, temperature, reducing environment, and external magnetic field) those matching the values of cancer. The active targeting capabilities of our nanocontainers result from the surface attachment of certain ligands that bind to proteins overexpressed on tumor cells called receptors. The targeting groups make the difference in the efficiency of the nanocontainers to target specific cancer and promote therapeutic activity. Due to this strategy, 5% of the nanocontainers reaches cancer, and considering 98% loading of drugs, a significant number of drugs reaches cancer a great improvement over the free compound, namely outsourcing the traditional chemotherapy. In mice experiments, our leading drug candidate Nano4Dox has proven significantly more safe and effective *in vivo* than the current gold standard Doxil (liposomal doxorubicin), an absolute blockbuster nanomedicine in oncology. The Nano4Cis platform performs also better than the Lipoplatin. Our Nano4XX platforms can be loaded with any anticancer compound, thereby improving pharmacotherapeutic efficacy and reducing side effects, resulting in a "best-in-class" clinical profile.

## KEYWORDS

Nanocontainers,  
Cisplatin,  
Liposomal  
doxorubicin

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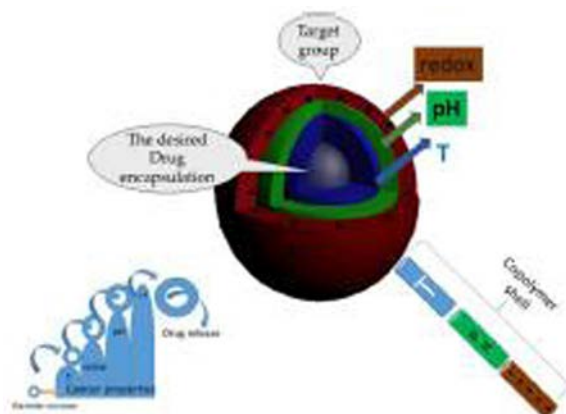
<sup>1</sup>Corresponding author: Sol-Gel Laboratory Institute of Nanoscience and Nanotechnology, National Center of Scientific Research

"Demokritos" 153 10 A. Paraskevi Attikis, Greece

E-mail: g.kordas@inn.demokritos.gr

## INTRODUCTION

When a person is diagnosed as having cancer, the physicians recommend surgery, chemotherapy, and irradiation therapy for the tumor. All these options have severe disadvantages and result in discomfort for the patient. Side effects of chemotherapy are visible, like hair loss, but other side effects are invisible occurring inside the body in crucial organs. The chemotherapy drugs lack specificity accompanied with drug resistance leading to the failure of the therapy. Nanomedicine has been employed to solve these problems. Nanomedicine presents a promising alternative to traditional chemotherapy aiming to cure cancer without side effects of the traditional chemotherapy. These goals were accomplished by the development of several nanovehicles such as nanospheres,<sup>1</sup> liposomes,<sup>2</sup> dendritic polymers,<sup>3</sup> polymeric hydrogels,<sup>4</sup> micelles,<sup>5</sup> and so on. These nanocarriers have a number of disadvantages such as uncontrolled release of cargo *in vivo*, incompatibility with various organs, limited loading of pharmaceuticals, oxidation of liposomal phospholipids, and low stability in the body. Furthermore, these nanocarriers neglect the tumor characteristics, such as the increased temperature, the lower pH, and the redox environment of cancer with respect to the healthy tissue. This study considers the cancer local environment via the development of a pH, T, and redox-sensitive shell leading to the so-called Nano4XX platforms grafted with targeting groups such as folic acid (FA) and leuprolide for breast and prostate cancers, respectively. Figure 1 shows the Nano4XX platform. In a previous article,<sup>6</sup> we reported the synthesis of our platforms as well as a complete loading and release, *in vitro* cytotoxicity, *in vivo* pharmacokinetic, and radiolabeling study. Here, we report for the first time the therapeutic efficacy of the Nano4Dox and Nano4Cis platforms and compare their performance to the liposomal analogues, namely Doxil and Lipoplatin. Doxil (liposomal doxorubicin) presents, the current gold standard, an absolute blockbuster nanomedicine in oncology.



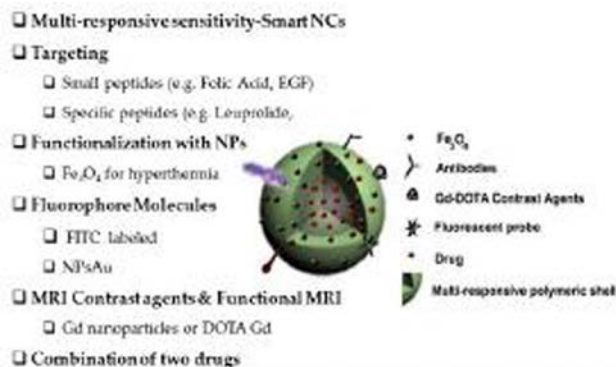
**Figure 1** Quadruple stimuli targeted nanocontainers.

Our work is driven by the strong clinical need and industrial interest in nanomedicines in the field of oncology. The first two nanomedicines for cancer treatment, liposomal Doxil and albumin-bound nanoparticle (Abraxane), exhibited commercial worldwide sales of approximately \$814.6 million and \$950 million in 2015, respectively.<sup>7,8</sup> The different needs of the pharmaceutical industry, medical professionals, patients, healthcare insurance companies, and policy makers all play a part in driving the market for improved drug delivery of anticancer pharmaceuticals. Nano4XX platform aims to

establish the new standard nanomedicine platform in this rapidly growing market.

## SAMPLE PREPARATION

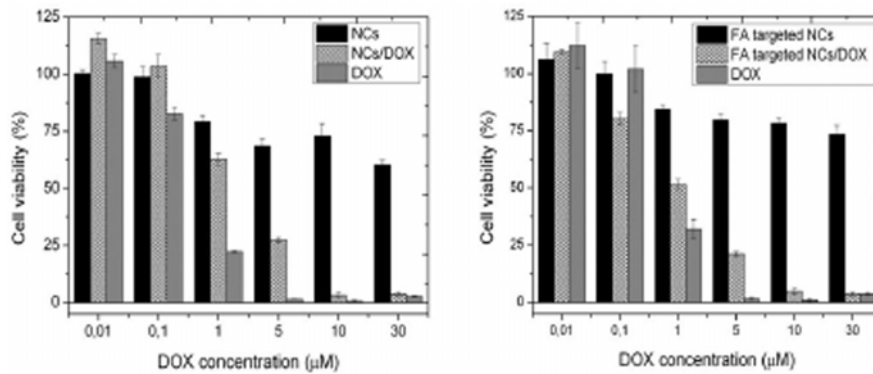
The production of the Nano4Dox and Nano4Cis platforms involves several steps as described entirely in a recent paper.<sup>6</sup> Here, we only sketch the procedures employed leading to the production of nanocontainers as shown in Figure 2. In the first step, the polymeric core was fabricated. In the second step, the cross-linked shell was produced aiming at introducing pH, Thermo, and redox sensitivity with simultaneous removal of the core. Iron NPs were doped on the platform surface for hyperthermia application.<sup>9</sup> Finally, doxorubicin and cisplatin were loaded into the nanocontainers using equal amounts of the nanocontainers and drug treated with PBS buffer solution under pH of 7.4.<sup>6</sup> The mixture was stirred at room temperature for 3 days in the dark. Then, the solution was centrifuged three times at 10,000 for 5 minutes. PBS washed the isolated product three times. The surface of the nanocontainers was functionalized by targeting groups depending on cancer type, e.g., (FA) for breast cancer and leuprolide for prostate cancer (Figure 2).<sup>6</sup> The surfaces were also grafted by fluorophore molecules such as fluorescein isothiocyanate (FITC) with excitation and emission spectrum peak<sup>6</sup> giving it a green color<sup>6</sup> and Au nanoparticles for surface-enhanced Raman spectroscopy.<sup>10</sup> Radiolabeling of the quadrupole stimuli-responsive targeted nanocontainers was accomplished by the gamma emitting radionuclide (<sup>99m</sup>Tc) that was detected by PET.<sup>6</sup> The biodistribution profile was observed at 1 and 24 h post-injection (pi) in normal mice.<sup>6</sup> The growth inhibition of MCF-7 and HEK-293 cells for all complexes was confirmed by using the MTT assay.<sup>6,11</sup>



**Figure 2** Quadrupole stimuli-responsive nanocontainers grafted by targeting groups, iron nanoparticles, FITC, Au nanoparticles, gadolinium loaded with a number of commercial anticancer drugs. FITC, fluorescein isothiocyanate.

## CYTOTOXICITY STUDIES

Figure 3 shows the cytotoxicity profile of the Nano4Dox (left) and grafted with FA Nano4Dox (right) on HEK-293 cells between 0.1 and 100  $\mu$ M. Similar study was done in the empty platform (Figure 3, without FA grafted [left] and with FA grafted [right]) and Doxil. The effect of Dox (0.1, 1, 10, and 30  $\mu$ M), Dox-loaded NCs (containing the corresponding amount of doxorubicin), and hollow nanocontainers (with the same amount of the loaded polymers) was investigated by the MTT assay on MCF-7. One can perceive that the



**Figure 3** Cytotoxicity profile of empty Nano4Dox, Nano4Dox, and free DOX (right) and FA grafted empty Nano4Dox, FA grafted Nano4Dox, and free DOX (right) on MCF-7 cells. FA, XXX.

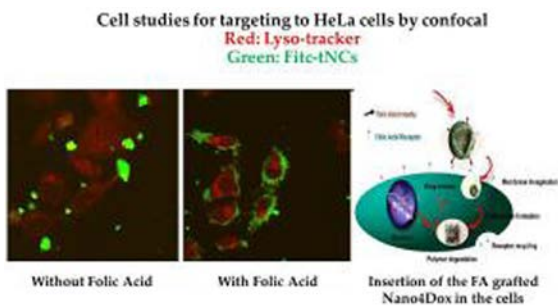
polymer is nontoxic in low concentrations (0.1–10 µM), and when the concentrations increase, the toxicity also increases. When cells are treated with the free polymer at 30, 50, and 100 µM, the cell viability is 90, 78, and 62%, respectively. Nano4Dox platform presents comparable performance with free doxorubicin in all concentrations tested, approving that the encapsulation of doxorubicin in the platform does not affect the drug's accomplishment. Similar behavior is observed when the nanocontainers are grafted with FA (Figure 3, right). This implies that grafting with FA of the nanocontainers does not disturb the cytotoxicity actions of the system.

In another study, Nano4XX cytotoxicity was also investigated against HEK-293 cells, and they were found to be nontoxic.<sup>6</sup>

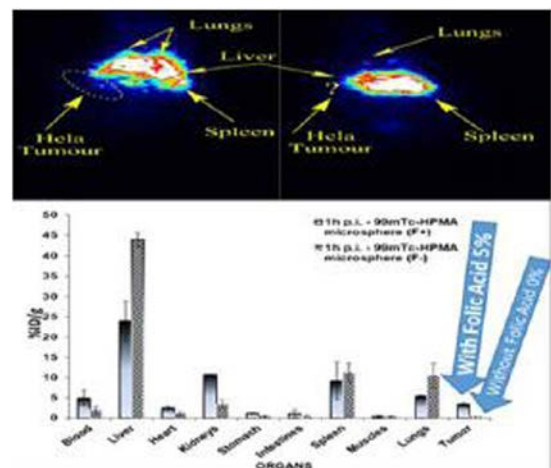
### Targeting ability

Another question to be answered in our work was whether the Nano4XX platforms when grafted by a targeting group (e.g., FA for breast cancer and leuprolide for prostate cancer) allows coupling on a receptor of a cancerous cell. This ability of the grafted platforms was demonstrated first *in vitro* as shown in Figure 4. Figure 4 displays two experiments where the Nano4Dox are grafted with (center) and without (right) FA. The Nano4Dox platform without FA agglomerate outside the cells (Figure 3, right) painting the surface of the cell green due to FITC. The grafted Nano4Dox platform inserts the cell painting the interior of the cell red color due to insertion of the Nano4Dox platform formatting lysosomes with Lyso-tracker (Figure 4, center). Figure 4 right summarizes the mechanism of insertion of the grafted Nano4Dox platform first via targeting, second via the insertion into the cell, and third via the release of the doxorubi-

cin into the cell. This mechanism of killing cancer is called “Trojan Horse” approach where the release of doxorubicin into cancer cells lead to the reduction of cancer. This reduction was demonstrated in *in vivo* study summarized in the following study. But now we show an *in vivo* study of targeting of the grafted Nano4Dox platform via positron emission tomography (PET) using SCID mice (Figure 5). Figure 5 shows the distribution of the Nano4Dox platform in various organs with (F+) and without (F-) FA as determined by PET. In tumor, the concentration of the NCs amounts to 5 and 0% of the total amount injected with and without FA, respectively. The concentration of the Nano4Dox in the heart, stomach, muscles, and intestines is negligible. The concentration in the liver, spleen, and lungs decreases after grafting the nanocontainers with FA, while it increases in the kidneys and blood with F (+). Nano4Dox are passively localized at lungs. Taking into consideration that advanced metastatic breast and prostate cancers usually reach lung and liver, there should be a method to inhibit metastatic tumor reaching these organs. The life expectations are low if these organs are infected. Our technology can be used as a method to attack metastatic cancer in early stages. These organs are of great importance. These experiments were conducted after 1 hour after injecting the Nano4Dox in the animals. The concentration of the Nano4Dox in the organs (e.g., liver) decreases with the time, indicating the removal of the NCs from the organs.



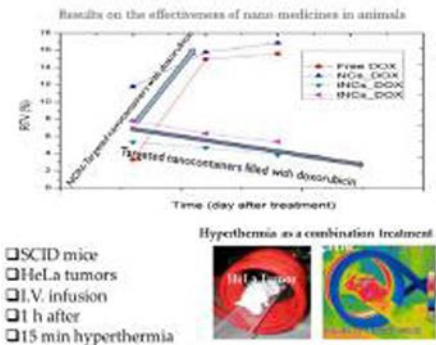
**Figure 4** Confocal microscopy study of Nano4Dox platform without FA (right) and with FA (center) grafted. Mechanism of insertion of FA grafted Nano4Dox in the cells (right).



**Figure 5** *In vivo* proof of targeting using positron emission tomography.

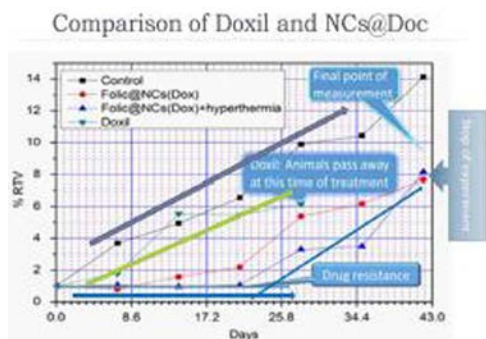
## Therapeutic efficacies of the Nano4XX platforms compared to the liposomal analogues

The therapeutic efficacy of our Nano4XX (Dox and Cis) platforms was determined by using HeLa tumor-bearing SCID mice and by monitoring the volume of cancer as a function of the time. Figure 6 shows the results of the Nano4Dox experiments. The blue triangles show the



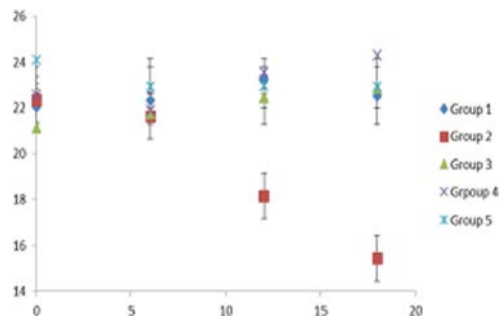
**Figure 6** Therapeutic efficacy of the Nano4Dox platform assisted by hyperthermia.

growth of cancer as a function of time in the SCID mice treated with nanocontainers loaded with DOX but not grafted with FA. The red dots correspond to the experiment in which the animals were treated with doxorubicin. One can observe from this graph that the volume of cancer increases with the increase of the time. Contrary to this experiment, the therapy outcome is different when the nanocontainers are grafted with FA and loaded with doxorubicin. We observe a 20% volume reduction in 20 days (violet triangles). The same experiment was repeated using hyperthermia (green triangles). The application of hyperthermia leads to a better outcome of the therapy (a better reduction of cancer volume as a function of the time). The main question of this study was whether the Nano4Dox platform performs better than the commercial Doxil chemotherapy drug. This question was answered by injecting Doxil and Nano4Dox into HeLa tumor-bearing SCID mice and monitoring the volume of cancer as a function of the time. The results are summarized in Figure 7. First experiment was with the control in which no drug was given to the mice. This result is presented as the black rectangles assisted with the black upward arrow to help the reader to better observe the result. The cancer volume increases with



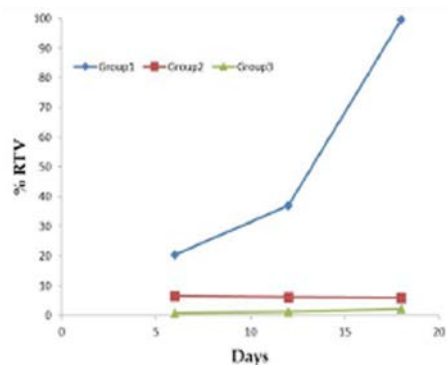
**Figure 7** Tumor efficacy experiment in different groups. Group 1: Control group injected with PBS (Black), Group 2: Nano4Dox (Red), Group 3: Nano4Dox (Blue) combined with 15 min hyperthermia treatment, Group 4: Doxil® injected (green).

the time as expected. The green triangles present the results of cancer volume as a function of time after delivering Doxil to the animal. One can observe an increase of the cancer volume where the animal passes away after 27 days of the treatment with Doxil. In contrast to these results, the insertion of FA-targeted Nano4Dox platform shows a therapeutic effect, especially after hyperthermia treatment. The blue triangles assisted by the solid blue arrow show a cancer volume reduction by 20% in 2 weeks, and then we observed an increase of cancer volume with time due to the occurrence of drug resistance. The experiment was stopped after about 40 days due to ethics reasons. Another important result of this study is the weight variation of the animal with the time delivered with Doxil and the Nano4Dox platforms. This experiment was performed again by using HeLa cervical tumor-bearing SCID mice and by monitoring the weight of the animal as a function of time in different groups as shown in Figure 8. Each group has six repetitions. The first group was the control group, the second group was treated with the gold standard Doxil, the third group with targeted Nano4Dox, fourth group was treated with the same combined with hyperthermia, and final group was treated with empty platform. One can observe a



**Figure 8** Weight variations during chemotherapy.

drastic reduction of animal weight in Doxil-treated animals in contrast to the Nano4Dox-treated animals that exhibit a constant weight with the time. The results of this study demonstrated that our Nano4Dox platform outperforms the existing carrier technology (liposomes, dendritic polymers, polymeric hydrogels, micelles, etc.) and yields an outstanding therapeutic effect. The Nano4Dox technology constitutes a significant improvement over the state of the art since it is the first to integrate four stimuli (pH, temperature, reducing environments and alternating magnetic fields) as well as proprietary targeting capabilities.<sup>12</sup> The “active targeting” aspect of our Nano4Dox platform results from the surface attachment of certain ligands that bind to proteins overexpressed on tumor cells, which has been shown to improve the target specificity and improve therapeutic activity. Due to this targeting, 5% of the compound reaches the tumor; this is absolute best in class. We succeeded to prove that the quarto-stimuli nanocontainers loaded with doxorubicin function more effectively than the free drug Dox, reducing the drug toxicity and extending the animals’ life. This *in vivo* advanced results highlight the quarto-stimuli targeted nanocontainers as the most potential carriers for drug delivery. The work conducted for Nano4Dox was repeated for the Nano4Cis platform that behaves better than the commercial lipoplatin, and the results are summarized in Figure 9. Cisplatin is used as a traditional chemotherapeutic drug but presents many side effects such as hematotoxicity and other significant side effects leading to the treatment limitations for cancer therapy. Cisplatin loaded in multistimuli-targeted nanocontainers do not exhibit the known side effects of cisplatin. In detail, the drug was loaded into our targeted Nano4Cis platform using PBS at pH of 7.4 for



**Figure 9** Tumor efficacy protocol. Group 1: Control group injected with PBS, Group 2: Lipoplantin injected, Group 3: Nano4Cis.

24 hours. The mixture was centrifuged, and free cisplatin was isolated from the supernatant. The loading capacity was determined by using the standard curve method, and calculations depict that the LC% was 82% and was EE% was 92%. We compare *in vivo* cisplatin therapeutic affection encapsulated on liposomes with our cisplatin loaded in multistimuli-targeted quadrupole stimuli nanocontainers. For this evaluation, we used tumor-bearing animals by HeLa cells for tumor efficacy investigation. We used three groups of animals. The first was the control group, the second was the Lipoplantin, and the third was Nano4Cis platform. The loaded targeted nanocontainers were injected on SCID mice. The studies measure the tumor volume as a function of the time. Figure 9 demonstrates the results of our comparable study by which we can strongly support that our Nano4Cis platform loaded with cisplatin behave better than the Lipoplantin.

## CONCLUSION

This study demonstrates that the FA-grafted Nano4XX (doxorubicin and cisplatin) exhibit excellent targeting capabilities yielding 5% bonding to cancer after 1 hour of injection. According to the confocal study, the FA-grafted Nano4XX platforms enter cancer accounting for the therapeutic effect. The therapeutic efficacy of the FA-grafted Nano4XX platforms was demonstrated *in vivo* via PET spectroscopy. % cancer volume reduction in two weeks in contrast to an increase of cancer volume when treated with Doxil. After 2 weeks of observance, we detected an increase in cancer volume with the time due to the drug resistance phenomenon. The Nano4XX platforms diminished the toxicity of the drug allowing the use of more toxic cancer therapeutic drugs to be used that are more efficient for cancer therapy. We can use also conjugated drugs for better treating cancer avoiding possibly drug resistance. These platforms open a way for better cancer treatment. The technology is patented under US 2016/0263221.<sup>13</sup>

## ACKNOWLEDGMENT

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## NOTES

1. <https://www.transparencymarketresearch.com/nanomedicine-market.html>: Registering a double-digit CAGR during the course

of the forecast period, the global nanomedicine market is slated to amount to a value of US\$177 bn by 2019.

2. <https://www.bccresearch.com/market-research/healthcare/nanotechnology-medical-applications-market-hlc069d.html>: The global nanomedical market was valued at \$134.4 billion in 2016. This market is projected to grow at a compound annual growth rate (CAGR) of 14.0% from 2017–2022, and should reach \$293.1 billion by 2022 from \$151.9 billion in 2017.
3. <https://www.grandviewresearch.com/blog/liposomal-doxorubicin-market-size-trends>: In 2015, the global liposomal doxorubicin market was valued at USD 814.6 million and is expected to grow at a CAGR of 6.4% over the forecast period.
4. <https://www.marketresearch.com/product/sample-7777835.pdf>: Global drug sales for Abraxane are forecast to increase from \$98.34m in 2012 to \$213.75m in 2022 at a Compound Annual Growth Rate (CAGR) of 8.07%.

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