ULTRASMALL IRON OXIDE NANOPARTICLES CISPLATIN (IV) PRODRUG NANOCONJUGATES: ICP-MS BASED STRATEGIES TO EVALUATE THE FORMATION AND DRUG DELIVERY CAPABILITIES IN SINGLE CELLS.

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ABSTRACT

Ultrasmall iron oxide nanoparticles (<10 nm) were explored here as nanotransporters of *cis*-diamminetetrachloroplatinum (IV) (a cisplatin prodrug) in cellular models. The coating of the particles containing reactive carboxylic acid groups enabled the formation of a stable conjugate between the prodrug and the nanoparticles using one pot reaction. The nanoconjugate was characterized by different techniques exhibiting diameters of about 6.6 $\pm$ 1.0 nm. The use of a hyphenated strategy based on high pressure liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) and permitted the quantitative evaluation of Fe and Pt in the nanoconjugate. Furthermore, the cellular uptake of the synthetic nanoconjugate was explored by single cell ICP-MS (SC-ICP-MS) which was used for the first time in this type of studies. The experiments in A2780 and A2780cis, sensitive and resistant ovarian cancer cell models respectively, revealed intracellular platinum concentrations of 12 fg/cell and 4 fg/cell, respectively which were 4-fold higher with respect to the uptake of cisplatin in both

models. Intracellular drug release from the nanoconjugate was proved by measuring DNA platination in the same cells. In this case, levels of about 250 ng Pt/mg DNA were observed, about 5-fold higher when the nanoconjugate was used in comparison to cisplatin. Furthermore, the differences between the two lines turned to be significantly smaller than in the case of using cisplatin. The quantitative analytical tools developed here provided essential information required to fully characterize the developed nanoplatforms particularly important to overcome drug resistance.

Key-words: iron oxide nanoparticles, cisplatin(IV) prodrug, nano-conjugate, single cell ICP-MS.

1. Introduction

The use of nanocarriers to improve the transport of chemotherapeutic compounds into cells represents an active area of research [1]. In fact, the significant challenge of latest studies in this field is to create new types of intelligent nanocarriers capable of responding selectively to cancer-specific condition and fast release of drugs in target cells [2]. Among them, iron oxide nanostructures (e.g. nanoparticles, nanotubes and nanowires) that show magnetic and biocompatible properties are interesting systems [3,4]. In particular, iron oxide nanoparticles with magnetite or maghemite core [5] fulfill the requirements to be classified as ‘‘smart’’ multifunctional nanoplatforms that can demonstrate: 1) prolonged circulation in the blood; 2) effective intracellular incorporation; 3) responsiveness to local physiological stimuli, such as changes in pH, which may result in an improved and localized drug release mechanism [6]. The synthesis of ultrasmall iron oxide nanoparticles (<10 nm) coated with acid-based coupling agents with polar end groups (-COOH) like tartaric and adipic acids has been explored for oral management of anemia [7,8]. Although the crystal structure of these particles has not been completely established, it seems that the metallic core mimics the well-absorbed ferritin core containing Fe in the form of ferrihydryte [9]. They have shown to be biocompatible in cell cultures and animal models with minimum toxic effects and elevated cellular uptake [10,11]. In addition, the polar groups present at the surface of these nanoparticles do not only provide hydrophilic structures and stability with respect to aggregation, but also confers adequate functionality on the surface to facilitate bioconjugation. Such an attractive combination has triggered the possibility to use this nanoplatform for drug delivery and in particular, conjugation with metallodrugs like cisplatin [12].

Cisplatin (*cis*-diamminedichloroplatinum (II)) is a widely used cancer chemotherapeutic treatment that has proved to be efficient for testicular, ovarian, and breast cancer among others [13]. Its intravenous administration is, however, hampered by important limitations like unspecific drug interactions with plasma proteins which generates drug inactivation and clearance from the circulatory system [14]. In addition, the limited drug uptake by tumor cells (between 1-2%) is reduced even further in the case of resistant cell models. To overcome such limitations the use of cisplatin (IV) prodrugs has been one of the proposed alternatives [15–17]. Octahedral platinum(IV) complexes containing two leaving groups in axial positions are more resistant to ligand substitution reactions than platinum(II) centers, minimizing unwanted side reactions with biomolecules prior to deoxyribonucleic acid (DNA) binding [18]. Once in the cell cytosol, further reduction of the platinum(IV) center to platinum(II) by small biomolecules like glutathione or ascorbate and subsequent loss of two ligands is required for the anticancer activity of these agents [19]. The use of platinum(IV) prodrugs in combination with different molecules, including proteins like albumin [20] or other bioactive structures that help to increase cell uptake has been explored by a number of authors with promising results (Phase II clinical trials) [21].

Different analytical strategies have been proposed for the evaluation of the antitumor activity of metallodrugs and, in particular cisplatin. Thanks to the presence of platinum as the central metal of this drug, ICP-MS is an excellent tool to analytically address the fate of this drug within the cells. Due to its mechanism of action, which is mainly based on the adduct formation with DNA, the cellular uptake of this drug is directly related to its toxicity. Most common approaches to quantify the uptake of platinum metallodrugs by ICP-MS are based on bulk analysis [22] of Pt in a cell

population and averaging the results by the cell number concentration. However, in the last years, the need to understand the behavior of the individual cells within a certain population has become a priority. Single cell analysis is crucial because it is now known that every cell can behave differently, also regarding cisplatin uptake, depending on many biological variables, such as cell type, cell cycle status, hypoxia, redox changes, etc. In particular, differential cisplatin [23,24] and nanoparticles uptake [25,26] at the single cell level, has been successfully evaluated using single cell ICP-MS (SC-ICP-MS).

In the present work, we explore the capabilities of the previously synthesized biocompatible ultrasmall iron oxide nanoparticles coated by tartaric and adipic acid, to be directly conjugated to the cisplatin (IV) prodrug *cis*-diamminetetrachloroplatinum (IV). The possibility of having a direct reaction between the two species would dramatically simplify the synthetic route. However, adequate analytical strategies that permit to quantitatively address the level of conjugation and release of the prodrug have to be developed to study the formation of these species. Here, the use of dodecyl sodium sulfate or (SDS)-based reversed-phase chromatography coupled to ICP-MS detection of Fe and Pt was evaluated and complemented with microscopy and light scattering experiments. Additionally, cellular uptake in sensitive and resistant A2780 cell models was addressed at the individual cell level using adequate single cell-ICP-MS strategies. Although ICP-MS is a well-established technique [27], its use for single-cell analysis is a relatively new and fast growing research field [28], and to the best of our knowledge is the first time thpeeat this strategy is used to chase metallodrug-nanoparticles bioconjugates.

2. EXPERIMENTAL

2.1. Materials

Iron (III) chloride hexahydrate (98%, Sigma-Aldrich, Madrid, Spain) was used as nanoparticle precursor. Sodium tartrate dehydrated (99-101%, Sigma-Aldrich) and adipic acid (99%, Sigma-Aldrich) were solubilized in 0.9% potassium chloride (Merck, Darmstadt, Germany) solution and used as coating agents. Ammonium acetate (>98%, Sigma-Aldrich) was used for the synthesis buffer and 5 mol L^{-1} sodium hydroxide (Merck) was prepared for nanoparticle precipitation. All working standard solutions were prepared using 18 MΩ·cm de-ionized water obtained from a PURELAB flex 3 (ELGA VEOLIA, Lane End, UK). Cis-diamminetetrachloroplatinum(IV) (99.9%) was obtained from Sigma-Aldrich. The customized oligonucleotide with the sequence 5'-TTA CTA TTC TGT TAC GAT TAC GGT TAC TCC-3' was produced by Invitrogen (Barcelona, Spain).

Sodium dodecyl sulfate (SDS, 98.5%, Sigma-Aldrich) and ammonium acetate (>98%, Sigma-Aldrich) were used in the mobile phases for the chromatographic separations. L-ascorbic acid (99%, Sigma-Aldrich) and L-glutathione reduced (≥98%, Sigma-Aldrich) were employed for *in vitro* drug release experiments.

2.2. Instruments

All ICP-MS experiments during this study were performed using the triple quadrupole instrument iCAP TQ ICP-MS (Thermo Fisher Scientific, Bremen, Germany) using the single quadrupole (SQ)-hydrogen mode (collision cell mode to eliminate $^{40}Ar^{16}O^{+}$ and $^{40}Ar^{16}O^{1}H^{+}$ polyatomic interferences) for the measurement of $^{56}Fe^{+}$ and SQ-mode (single quadrupole-mode) for $^{195}Pt^{+}$ monitoring. For phosphorous measurements, the formation of $^{31}P^{16}O^{+}$ is achieved by pressurizing the cell with O_2. For the chromatography

experiments as well as for the Pt/Fe release experiments, the ICP-MS instrument was fitted with a cyclonic spray chamber and a conventional concentric nebulizer. For single cell analysis, the ICP-MS instrument was fitted with a high performance concentric nebulizer (HPCN) and a total consumption single cell spray chamber from Glass Expansion (Glass Expansion, Australia) using a previously optimized system [29]. The cells were pumped using a microflow syringe pump SP101i (Florida, USA) fitted with a 1 mL Hamilton syringe (Nevada, USA) at 10 µL min^{-1}. The data were recorded in time-resolved analysis mode during 3 min per analysis using a dwell time of 5 ms. The instrumental parameters of the ICP-MS instrument are summarized in Table S1.

For the measurement of the dynamic light scattering (DLS) and ζ-potential of the nanoparticles, experiments were carried out in a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd. Malvern, UK). Original samples were approximately 100-fold diluted with ultrapure water. Low Resolution Transmission Electron Microscopy images were taken in a MET JEOL-2000 2100F (Tokyo, Japan) and were analysed in order to obtain particle average size, shape and aggregation. FTIR was also measured for the synthetic iron oxide NPs (see Figure S1).

The chromatographic separation of the particles was conducted using an HPLC system Agilent 1260 (Agilent Technologies, Tokyo, Japan). The column was a Nucleosil C_{18} separation column (7 µm particle size, 250 x 4.6 mm i.d., pore size 1000 Å, Phenomenex, Aschaffenburg, Germany), using as mobile phase a solution of 10 mmol L^{-1} SDS in water at 0.5 mL min^{-1}. For the separation of the oligonucleotide, a size exclusion chromatographic column Superdex Peptide 10/300 GL (30 cm x 10 mm i.d.) with fractionation range from 100 to 7000 Da (GE Healthcare, PA, USA) and a mobile phase

of 10 mmol·L^{-1} ammonium acetate was used at 0.7 mL min^{-1}. Detection was performed on-line with the iCAP TQ ICP-MS. The flow from the HPLC was introduced into the ICP-MS instrument via a 15 cm long polyether ether ketone (PEEK®) tube, which was connected to the polytetrafluoroethylene (PTFE) sample tube of the nebulizer.

2.3. Synthesis of the cisplatin (IV) prodrug-containing nanoparticles

Iron oxide nanoparticles were synthesized following a slightly modified protocol from Pereira et al. for the Fe-tartrate modified nanoparticles [7]. This method is based on the precipitation of Fe^{3+} in presence of highly basic medium (5 mol L^{-1} NaOH solution) with the addition of tartrate and adipic acid solution for the iron core coating as described somewhere else [10]. The molar ratio tartaric: adipic: Fe used corresponds to 1:1:2, which has given best performance in previous experiments [30]. The three components are mixed and constantly stirred in a buffer media (ammonium acetate 50 mmol L^{-1} at pH 4). The initial pH of the mixture is increased stepwise until reaching pH 11. When mixture turns dark brown/blackish, centrifugation and ultrafiltration (30,000 Da; 3,000 Da Ultra-15 MWCO centrifugal filter units, Millipore) steps are needed to separate the microparticulate and nanoparticulate iron fractions from the supernatant and remove excess of soluble ligands and the rest of reagents. Centrifuge Biofuge Stratos Heraeus (Thermo ScientificTM) was used for these purposes. Size and shape characterization of the particles has been conducted by TEM and DLS.

For incorporation of the cisplatin (IV) prodrug, a solution of 5 mmol L^{-1} of the prodrug was prepared in MilliQ water for further incubation with the particles for 6h (best incorporation results). The excess of the prodrug was eliminated by ultracentrifugation using a 3,000 Da Ultra-15 MWCO centrifugal filter. The verification of the incorporation

of the prodrug into the particles was conducted by HPLC-ICP-MS by monitoring Pt and Fe quasi-simultaneously. Quantification of the level of incorporation of the prodrug was obtained by carrying a post-column Pt calibration curve.

2.4. Cell cultures

The A2780 and A2780cis cell lines, sensitive and resistant to cisplatin respectively, were kindly provided by Dr. J.M. Pérez Freije (Dpt. of Biochemistry and Molecular Biology, University of Oviedo), and they were authenticated at the Biotechnological and Biomedical Assay Unit from the Scientific and Technical Services (SCTs) at the University of Oviedo. Cell lines were grown in RPMI 1640 medium (Gibco); in both cases media were supplemented with 10% fetal bovine serum (FBS) (Gibco) and 5 µg mL^{-1} Plasmocin Prophylactic (InvivoGen). All cells were grown at 37°C in a 5% CO_2 atmosphere. A2780cis cells were incubated with 1 µmol L^{-1} cisplatin every 3 passages to maintain resistance. Cells were exposed to 20 µmol L^{-1} (as prodrug) of the conjugate and compared to the uptake of cisplatin at the same concentration during 24h. Results are referred at fg Pt/cell by conducting single cell experiments of the uptake according to the calculations previously established [23].

2.5. Drug release and interaction with DNA

Isolation of DNA from A2780 and A2780cis was conducted as described in reference [22] using the silica-based column DNA purification kit PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, USA). The Kit was used according to the manufacturer's instructions with the inclusion of RNAse A treatment to generate RNA-free genomic DNA. The extracted DNA was eluted using 100 µL of the elution buffer (10 mM Tris-HCl, pH 9.0, 0.1 mM EDTA).

DNA purity was confirmed by comparing the ratio of UV measurements at 260 and 280nm with the ratio of pure DNA standards. The concentration of isolated DNA was determined by $^{31}P^{16}O^{+}$ monitoring using ICP-MS after sample digestion using 100 µL of HNO_3 (65%) and 60 µL of H_2O_2 (30%). Sample was further diluted 1:10 for analysis. A $^{31}P^{16}O^{+}$ calibration curve was constructed by applying the same digestion procedure to commercially available calf thymus DNA. Also,^{195}Pt was measured within the same experiments. Final results of the experiment, performed by triplication, are given as ng Pt/mg DNA.

2.6. Statistical analysis

Statistical analysis was performed using Student's t-test and a P-value of <0.05 was considered as significant for Figure 3 and Figure 5. In Figure 5, F-test was performed to compare variances among data sets. For the cell line A2780, $F_{exp}<F_{cri}$ and thus, there is no significant differences among SDs. In this case Student´s-t was applied. However, $F_{exp}>F_{cri}$ for the A2780cis and in this case, instead of Student´s-t, the Welch test was applied.

RESULTS AND DISCUSSION

2.5.Synthesis and characterization of the prodrug-loaded iron oxide nanoparticles.

The conjugation reaction between the carboxylic acids present on the surface of the ultrasmall iron oxide nanoparticles and cis-diamminetetrachloroplatinum(IV), as shown in Figure 1, was optimized in terms of incubation times and reaction ratios. Different incubation times and ratios NPs/prodrug were assayed to achieve highest concentration of prodrug attached to the NP. To conduct such study, it was necessary to monitor the ratio Fe/Pt in the particles, to address the absence of free prodrug in the mixture and to

quantitatively obtain the concentration of the prodrug attached to the NPs. For this aim, the coupling of liquid chromatography using a SDS-containing mobile phase to ICP-MS was evaluated. Such technique could be successfully used to separate monodispersed nanoparticles from aggregates and free ions extracted from the cytosol of cells exposed to the particles [10]. In addition, this chromatography has proved to provide satisfactory recoveries in the elution of Au, Ag and iron oxide nanoparticles [31]. Therefore, it was also tested to address the formation of the nanoconjugate. The calibration of the column with different sized nanoparticulate material is shown in the supplementary information (Figure S2). A typical chromatogram corresponding to the Fe and Pt signals using ICP-MS detection is presented in Figure 2A where the coelution of the two metals can be observed in a single and relatively narrow peak eluting at 5.6 ± 0.5 min. The retention time, according to the calibration of the column, could correspond to a size below 10 nm, in the range of 6-7 nm. TEM measurements, also shown in the inset of Figure 2A, revealed slightly higher diameters, in the range of 10 nm. The coelution of the Pt and Fe traces in the HPLC-ICP-MS profile of the SDS-modified chromatography to allow the elution of the nanoconjugate confirmed, even further, the formation of the Pt (IV) prodrug-NP conjugate and as well as its stability. The presence of the two elements (Pt and Fe) could be also observed simultaneously in the Energy-Dispersive X-Ray (EDX) spectrum (Figure 2B) of the modified nanoparticles confirming, qualitatively, the formation of the conjugate. Quantification of both Fe and Pt in the conjugate by EDX is also shown in Table S2.

Regarding the hydrodynamic diameter, the results provided by DLS (Figure 2B) showed an average diameter of 6.6 $\pm$ 1.0 nm slightly higher than this obtained for the unmodified nanoparticle which is 5.9 $\pm$ 0.9 nm. Finally, the measured ζ-potential of the

particles before attachment of the prodrug was approximately -2.5 mV, probably due to the negatively charged carboxylic acid groups coating the metallic surface. After incubation with the prodrug, the Z-potential turned to 1.08 mV, confirming the neutralization of the negative charges on the surface by the prodrug groups attached. All these results are summarized in Table 1.

Quantitatively, the chromatography of the modified particles shown in Fig. 2A also revealed the absence of the free prodrug (eluting at about 7 min, see Figure S4) that has a recovery through the column of about 87%. It has to be remarked that the optimization of the incubation between the prodrug and the NPs has been done by varying the amount of nanopartickes (molar excess) and maintaining the prodrug concentration constant (415 µmol L^{-1}). Mixtures containing theoretical molar ratios Fe:Pt of approximately 3:1 and 9:1 respectively were incubated for 1 and 6 hours. Highest intensities of Fe and Pt were obtained after 6 hours of incubation using an Fe:Pt ratio of 9:1 (shown in Figure 2A). The quantification of the Pt and Fe in the chromatographic peak was done by using a calibration curve obtained by flow injection ICP-MS (see Figure S5). The obtained results for the different batches (n=7 synthesis carried out in different days) revealed Fe:Pt molar ratios of (16±4):1, slightly higher than the theoretical one (9:1). These results show that part of the prodrug is not reacting with the NPs, even when using them in a molar excess. Additionally, the reaction is highly reproducible (RSD 22%, n=7) under the assayed conditions and the modified particles have proved to be stable for more than 2 weeks at 4ºC (see Figure S3). These results taken altogether reveal the reproducible attachment of the prodrug to the nanoparticles using the assayed conditions described previously. Adequate dilutions of the modified particles prepared in this way were further conducted to adjust the treatments to cell cultures.

The prodrug-NP conjugate is expected to be more stable and less toxic during circulation than cisplatin providing lesser unspecific interaction with, for instance, serum proteins [32].

2.6. In vitro drug release from the nanoparticle surface

For evaluation of the drug release from the nanoparticles, an *in vitro* investigation was conducted by incubating the NPs with the attached prodrug with different media: at physiological pH (pH 7.5 in Tris-HCl buffer), at the pH expected in the endolysome environment [33] (pH 5.5 in acetic-acetate buffer) and in the presence of reducing compounds like ascorbic acid and glutathione. After incubation, the solution was ultrafiltrated to isolate the particles from the released drug and both fractions were further quantified via ICP-MS using germanium as internal standard. Figure 3 shows the comparative drug release in the different media (Figure 3A) after 12, 24 and 48 hours of incubation respectively. At pH 7.5 which mimics the pH values in the blood circulation and normal tissue, less than 2% platinum was released even up to 48 h, while there is an increase up to 16% when incubating for 48 h at pH 5.5. Similar results can be observed when the nanoparticles are incubated with ascorbic acid and glutathione for 12 hours and even further for 24 hours, probably due to the reduction of the cisplatin (IV) prodrug to cisplatin [21]. In the case of glutathione, however, a decrease in the release is observed after 24 and 48 h, probably ascribed to a reduction/aggregation of the particles upon incubation with this reagent as can be deducted from the higher amount of nanoparticles found on the ultrafiltration membrane. These results indicate that the release behavior of the conjugate was highly dependent on the reducing agents and acid hydrolysis. As acidic environment and high concentration of GSH are present in tumor

cells, such a system is expected to release the active component rapidly during the treatment process [21,34].

In addition, the functionality of the released and ultrafiltrated drug was tested by incubating the obtained filtrate with a single stranded oligonucleotide. This species of known sequence (TTA CTA TTC TGT TAC GAT TAC **GG**T TAC TCC) contains two consecutive guanines (pair GG) which is a preferential binding site for cisplatin [35,36] . The incubation product was analyzed by size exclusion chromatography coupled to ICP-MS monitoring Pt and P. Figure 3B shows the chromatogram by SEC-ICP-MS (P and Pt traces) corresponding to the interaction of the released drug from the nanoparticles (after treatment with ascorbic acid) with the oligonucleotide (monitoring P). The peak at 15 min corresponds to the elution of the oligonucleotide (see Figure S6). In this case, the coelution of the P and Pt signals within this peak reveals the formation of the adduct between the released cisplatin from the NPs and the oligo, since the prodrug does not show any interaction with the oligo. Such interaction proves that the released drug shows complete functionality to form DNA adducts as necessary to induce cellular toxicity [37,38].

2.7.Incorporation of the nanoconjugates into cells

The previously synthesized Pt(IV) prodrug-containing nanoparticles could be endocytosed by cancer cells, naturally circumventing the cellular pathway of internalizing small molecule-based Pt drugs which adopt passive diffusion as well as copper transporter-mediated active transportation as the major internalization pathway [39]. Moreover, nanoparticle entrapment provides additional protection of the Pt drugs reducing their thiol-mediated detoxification. Thus, the incorporation of the modified

NPs containing the prodrug was assessed into cell cultures of ovarian cancer A2780 and A2780cis cells exposed to 20 μmol L^{-1} of the different compounds for 24h. The evaluation was conducted using single-cell sample introduction systems to address, simultaneously, cell uptake and cell to cell differential behavior.

For this aim, an optimized sample introduction system was employed to evaluate the incorporation of the nanoconjugate with the same cellular models studied previously for cisplatin [23]. Cells were exposed to 20 μmol L^{-1} of the prodrug in the nanoconjugate and the data collected using 5 ms dwell time. The raw results are shown in Figure S7 and the corresponding processed results for A2780 (three independent cell cultures) are shown in Figure 4A in fg per cell (open boxes) and compared with the uptake of the free prodrug (Pt(IV)) (solid boxes) and with cisplatin (black open boxes, taken from reference [23]). First thing that can be observed is that the cellular incorporation is significantly higher when prodrug is in the form of the nanoconjugate (median, represented by the line in the middle of the box, about 12 fg/cell) than exposing the cells directly to the prodrug (median 2 fg/cell) or to cisplatin (median about 3 fg/cell in this cell model). These results are in agreement with previous bulk investigations, also showing an increased uptake of nanoparticle-associated Pt(IV) prodrug at shorter times [4]. In addition, the interquartile range (given by the height of the box and representing the data between the first and third quartiles) gives an idea of the spreading of the data and for the uptake of the nanoconjugate ranges 9.4-18.2 fg/cell, considering the three independent cell cultures. These results show the potential of the ultrasmall nanoparticles as nanocarriers for cisplatin prodrug incorporation into cells and the similar behavior among cells.

Furthermore, Figure 4B shows the results for the same exposure conditions in the resistant model, A2780cis. In this case, when comparing among treatments it is possible to see that the incorporation of the nanoconjugate increases the uptake of the cisplatin prodrug about 4 times when compared to free cisplatin (4.8 fg/cell versus 1.2 fg/cell). In fact, the use of the nanoconjugate permits to incorporate similar Pt levels in the resistant model (A2780cis, Fig. 4B) as the use of cisplatin in the sensitive one (A2780, Fig. 4A). Similar uptake levels in both, the resistant and sensitive cell line, were also previously found at shorter times (1 and 4 hours) via bulk analysis [4]. When comparing among cell models, the results revealed a similar trend with a decreased uptake in the resistant cell model with respect to the sensitive one by 2-fold regardless of the treatment used. This was also observed in previous experiments using both, single cell [23] and bulk analysis of cisplatin.

This study shows that the delivery of a prodrug associated to a nanocarrier is more efficient in terms of cell incorporation. This might be especially important in the case of resistant cells that inhibit a higher uptake of the active drug, in this case cisplatin. In both cell lines, the uptake of the prodrug is more efficient when it is conjugated to the iron oxide nanoparticles. In future *in vivo* applications, this would imply that the necessary dose to achieve the same intracellular concentrations of the drug would be lower. This, together with an expected lower release of drug at the pH around 7.4 of the bloodstream, should have a positive effect in the reduction of side effects associated to the treatment with cisplatin.

2.8. Measurements of Pt in DNA from cells exposed to NPs-prodrug conjugates.

The analysis of DNA platination is an indicator first, of the in-vivo evolution of the prodrug (incorporated as attached to the NPs) within the cell cytosol and second, of the functionality of the released molecule (presumably cisplatin (II)) to get to its molecular target. The two cell lines under study (A2780 and A2780cis) were treated with 20 μM cisplatin and with the same concentration of the prodrug (cisplatin (IV)) attached to the iron oxide NPs for 24h. The obtained results, normalized to the DNA concentration, can be seen in Figure 5. Different observations can be made from these results: i) the prodrug is efficiently released from the nanoparticles within the cell cytosol, as suggested by the previous in-vitro simulation; ii) the released form of the prodrug (presumably cisplatin) can interact with DNA (as previously stated, the prodrug does not show any interaction with the custom oligonucleotide); iii) the degree of DNA platination is significantly higher when using the prodrug attached to the iron oxide nanoparticles than in the case of using cisplatin (4-fold in the sensitive line and 9-fold in the case of the resistant model). Thus, in the case of using the prodrug attached to the iron oxide nanoparticles, the difference in the level of DNA platination between the sensitive and the resistant models is about 1.5-fold for the sensitive one and such differences go up to almost 4-fold in the case of using cisplatin. Therefore, the use of the nanoparticles as transporters could be also a mean to reduce the acquired resistance by some cell models. Future experiments evaluating the effect of different NP sizes on the uptake of the prodrug would be necessary to completely address the potential of these nanocarriers.

3. CONCLUSIONS

A new and more efficient drug delivery strategy to provide functional cisplatin into tumoral cells has been designed using ultrasmall iron oxide nanoparticles conjugation to cisplatin(IV) prodrug. The size of the particles after conjugation to cisplatin(IV) prodrug is <10 nm permitting the efficient drug penetration into cells, as previously observed in the case of the unmodified nanoparticles. Comparatively to cisplatin, the prodrug-NPs conjugate shows a 4-5 times higher uptake regardless of the cell model in ovarian cancer (sensitive or resistant). Drug release from the conjugate occurs once the pH is lowered (as in the endosomes), but also in the presence of reducing agents such as ascorbic acid or glutathione, both present in the cell cytosol. The released drug shows functionality to be adducted to DNA in simulated media generating significant Pt-DNA interactions and also in DNA extracted from exposed cells from the sensitive and resistant cell lines respectively. Most importantly, DNA platination is only 1.5-fold higher in the sensitive model with respect to the resistant one, showing the potential of the developed nanotransporter to overcome cisplatin resistance. Thus, the high efficiency of conjugation and the stability of the prepared conjugate, including biocompatible ligands make the strategy very adequate for future *in vivo* application.

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Table 1. Characteristics of the synthesized nanoparticles before and after conjugation with *cis*-diamminetetrachloroplatinum (IV) including the used technique.

	Before conjugation	**After conjugation**
Size (metallic core, TEM)	3.6 ± 0.4 nm	<10 nm
Hydrodynamic diameter (DLS)	5.9 ± 0.9 nm	6.6 ± 1 nm
ζ-potential (Laser Doppler Micro-electrophoresis)	-2.5 mV	+1.1 mV
Polydispersity index (DLS)	0.3	0.4
Fe:Pt (HPLC-ICP-MS)	-	16:1