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Bioanalytical strategies to evaluate cisplatin nanodelivery systems: From synthesis to incorporation in individual cells and biological response

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ABSTRACT

Cisplatin metallodrugs have been widely used in the treatment of multiple cancers over the last years. Nevertheless, its limited effectiveness, development of acquired drug resistances, and toxic effects decrease nowadays their application in clinical settings. Aiming at improving their features, investigations have been oriented towards the coupling of cisplatin to nanocarriers, like liposomes or inorganic nanoparticles. Moreover, these systems can be further developed to allow targeted co-delivery of drugs. In this review, we describe the major nanosystems and the optimal analytical strategies for their assessment. Finally, we describe the main biological effects of these metallodrug conjugates and the available approaches for their study.

1. Introduction: the use of cisplatin and its limitations

Cis-diamminedichloroplatinum (II), cisplatin, is a potent metallodrug widely used to treat numerous cancers, including ovarian, prostate, head and neck. The primary and widely accepted mechanism of action for cisplatin is the binding to cellular DNA, after activation by replacing the chloride ligands with water ligands, resulting in DNA-platinum adducts. This prevents the cell from replicating its DNA until the damage is repaired [1]. If the cell cannot repair the DNA or the damage is too severe, then the cell dies. However, although its antineoplastic effects have been mainly associated with its ability to generate such unrepairable nuclear DNA lesions, increasing evidence reveal that its mode of action also includes the alteration of both, nuclear and cytoplasmic, signaling pathways. Nevertheless, all these mechanisms rely on the drug reaching the target tumor cell. This occurs in a relatively low percentage, since according to several pharmacokinetic studies, 65–98% of cisplatin administrated intravenously is bound to blood plasma proteins, particularly albumin, in a therapeutically inactive form [2]. In addition, once transported into the cell, cisplatin has different fates. First, it can be exported from the cell using a transmembrane transporter system. Second, it can be chemically neutralized by binding sulfhydryl groups in proteins such as glutathione or metallothioneins[3]. Finally, cisplatin can react non-specifically with a variety of subcellular components: proteins, RNA, and DNA. It is estimated that just about 1% of total cisplatin yields the formation of DNA adducts [4].

Besides the limited effectivity of cisplatin as a chemotherapeutic agent, other drawbacks limit its wide therapeutic application. The first and most important refers to the inherent and acquired drug resistance, a multifactorial and still not well-characterized process [5]. The former is resistance without any prior drug exposure, while the latter is a result of drug exposure. Several mechanisms have been suggested to participate in conferring platinum-resistant properties to a tumor cell including genetic alterations in genes involved in drug uptake (and efflux), DNA repair, apoptosis and cell cycle control pathways. The decreased influx and increased efflux of cisplatin cause lower drug accumulation in the cancer cells. On the other hand, once the cisplatin is bound to DNA, cells develop molecular mechanisms to remove DNA lesions that induce cell drug resistance. For instance, the nucleotide excision repair (NER) system excises damaged nucleotides on both strands and then synthesizes DNA to reconstitute the gene integrity. Thus, cells with over-expression of NER show lower sensitivity to cisplatin [6]. Lastly, cisplatin resistance is also possible due to drug-induced dysregulation of microRNA function, which can cause problems in cell signaling, DNA methylation or cell survival resulting in increased resistance to cisplatin [7,8].

Besides drug resistance, the second limitation of the use of cisplatin is

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the associated toxicological effects that are closely related to the lack of specificity of the drug. Since the compound is mainly renal excreted, dose-limiting nephrotoxic effects have been observed in most patients [9]. The long-term effects of cisplatin on renal function have not been fully understood, but the administration of cisplatin is believed to cause a subclinical or permanent reduction in the glomerular filtration rate. In addition, ototoxicity is often observed in patients developing further hearing loss with a cumulative effect [10]. The exact mechanism of ototoxicity induced by cisplatin is not clear either. However, it has been generally accepted that the excessive generation of reactive oxygen species (ROS) in cochlear cells plays a crucial role in hearing loss. Finally, hematotoxicity characterized by anaemia, leucopenia, neutropenia, lymphopenia, and thrombocytopenia is also associated with cisplatin treatment.

Overall, despite the wide and positive use of cisplatin-based treatments in the oncology field, there are still some limitations that hamper its optimal performance in the clinical setting. Bearing this in mind, new approaches have been studied in the last years to overcome such disadvantages.

2. Preferred properties of cisplatin nanodelivery systems

In the search for alternatives to reduce the above-described cisplatin negative effects (mainly, resistance and secondary toxic effects) different alternatives have been considered. One was based on the design of new chemical structures containing various Pt ligands that reduced cell toxicity resulting in the second-generation Pt-drugs, including, carboplatin (cis-diammine (1,1-cyclobutane dicarboxylate) platinum(II)) and oxaliplatin ((1 R,2 R)− 1,2-diaminocyclohexane) [11]. Carboplatin showed no renal toxicity but produced higher myelotoxicity than cisplatin with a similar effectiveness to the parent drug. Many other structures have been designed and explored over the years. Among them, just carboplatin and oxaliplatin have been granted worldwide clinical approval, while lobaplatin, nedaplatin, and heptaplatin have only been approved in China, Japan, and Korea, respectively [12,13]. Others were discontinued in clinical trials due to severe toxicity or insufficient anticancer activity.

Alternatively, more attention was paid to the use of drug delivery techniques and particularly those using nanocarriers. The advantages of nanocarriers are, among others, prolonged cisplatin circulation minimizing the interactions with plasma proteins, tumor-targeted drug delivery, controlled cisplatin release and enhanced cisplatin cellular internalization [14]. Particularly in the case of cisplatin, it has long been observed that cisplatin-resistant cells tend to exhibit decreased levels of the drug that can be the result of two independent cellular pathways: decreased uptake or increased export. Movement of cisplatin through the cellular lipid bilayer membrane was initially thought to occur predominantly by passive diffusion. However, increasing evidence in the literature indicates that active processes using specific membrane transporters are more likely to determine the cellular uptake and efflux of cisplatin [15]. The down-regulation of these transporters and alteration in membrane protein trafficking have been observed in the resistant models and correlated with the reduction of intracellular platinum accumulation. This represents a key factor influencing the effectiveness of tumor chemotherapy [16]. To circumvent such a scenario, the use of nanodelivery systems containing biodegradable and biocompatible components that are taken up by endocytosis instead of by specific transporters is highly desirable and preventing also the drugs from being recognized by efflux pumps. This would yield a *higher intracellular cisplatin accumulation* unaffected by the deregulation of specific membrane transporters.

Regarding the previously described toxic side effects of platinum-based chemotherapeutic drugs, efforts to design targeted and controlled-release drug delivery systems are ongoing. *Temporal control of cisplatin release* from carriers aims to maintain drug concentration in blood or target tissues at an efficacious level. Nanocarriers that can deliver drugs in a temporally controlled manner can potentially enhance the therapeutic efficacy of the drugs, reduce their systemic side effects, and improve patient adherence to regimens by reducing the dose and administration frequency [17]. At a chemical level, controlled release can be achieved by installing linkers containing functional groups that are susceptible to either enzymatic or nonenzymatic cleavage between the nanotransporters and cisplatin. As it is possible to localize the stimuli that trigger cisplatin release, these carriers have been explored for *target-specific* drug delivery. For example, based on the weakly acidic pH of many solid tumors, nanocarriers with pH-sensitive linkers have been developed for tumor-specific and temporal controlled cisplatin delivery [18].

For *spatial control* of drug delivery, nanocarriers should be designed to selectively localize and accumulate in tumors by taking advantage of the abnormal vasculature structure of solid tumors, known as the enhanced permeability and retention (EPR) effect [19]. Many solid tumors are known to develop leaky capillary walls, through which cisplatin-loaded nanocarriers can extravasate and access tumors. Since nanocarriers do not readily traverse normal endothelium, the EPR effect enables the nanocarriers to reach tumors more selectively than free drugs, which translates to relatively low toxicity in normal tissues and high therapeutic efficacy in tumors. In the next section, a brief overview of the most successful nanoformulations existing for cisplatin administration will be provided with a focus on the advantages concerning the parent formulation.

3. Most successful nanocarriers for cisplatin administration

Efficacious nanodelivery of cisplatin-based drugs is influenced by multiple factors, among which the most relevant include the chemistry chosen for drug encapsulation, the selection of prodrugs, the usage of cell-specific targeting strategies and the inclusion within a co-delivery system. Next, these topics are further discussed.

3.1. Chemical structures for cisplatin encapsulation

Cisplatin incorporation into nanocarriers can be achieved by either encapsulation in a matrix or attachment to a particle surface. Among encapsulating agents, the use of organic nanostructures like liposomes and polymeric nanoparticles (NPs) (Fig. 1) has been shown as the most successful combination. Six stable nanoformulations for systemic administration including four liposomal formulations (Lipoplatin, LiPlaCis, SPI-077, and L-NDDP (Aroplatin)), an HPMA-copolymer-platinum conjugate (AP5280), and a polymer-cisplatin complex micelle (NC-6004, Nanoplatin) have already reached clinical development and showed some encouraging results [20]. Additionally, the possibility of chemically attaching cisplatin or a precursor molecule (prodrug) on the surface of an inorganic NP has also been the focus of many interesting studies (Fig. 1). The next section will cover a brief overview of the most advanced preparations that reached clinical trials.

3.1.1. Encapsulation of cisplatin using biopolymers: liposomes and polymeric NPs

Liposomes are spherical vesicles consisting of amphiphilic phospholipid bilayers. Phosphatidylcholine and phosphatidylethanolamine are the common building blocks for liposomal preparation whereas cholesterol is a frequent additive that serves to modify the rigidity of the lipid membranes. Liposomal drug loading can be accomplished either through active extrusion or passive diffusion. Lipoplatin is probably the better-known structure containing encapsulated cisplatin in a lipid bilayer vesicle using active extrusion with an aqueous interior that shows a size of about 110 nm [21]. The addition of polyethylene glycol (PEG) to the surface of liposomes resolved the problem of relatively high blood clearance and led to preferential trapping and accumulation of liposomes in the leaky tumor vasculature resulting in enhanced drug exposure at the tumor site. Lipoplatin has shown concentrations in

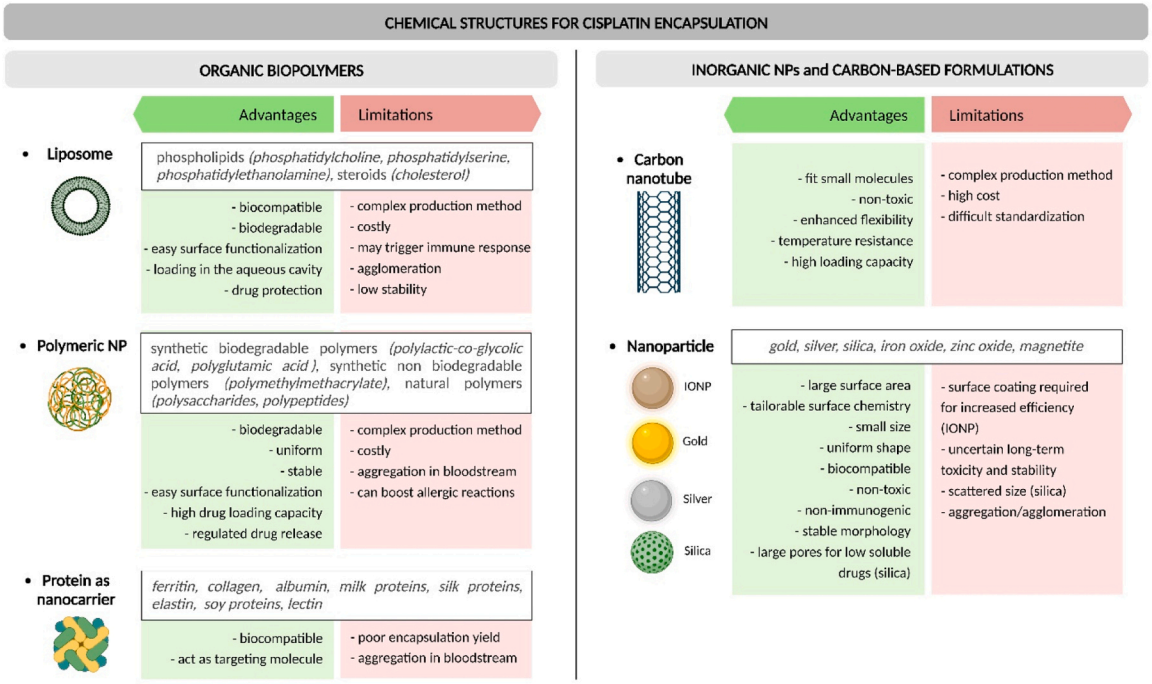

Fig. 1. Structural formulations for cisplatin encapsulation. A description of the main benefits and drawbacks together with the most often used compounds for organic biopolymers (left) and inorganic nanoparticles (right) is shown. *NP,* nanoparticle; *IONP*, iron oxide nanoparticle. Created with BioRender.com.

tumors and metastases at levels up to 50-fold higher compared to the adjacent normal tissue revealing the advantages of a targeted system. Increased entry of Lipoplatin into cells has been also observed which could be ascribed to the fusion of liposomes with the tumor cell membrane. Once inside the cytosol, cisplatin is released from the inner cavity yielding a free drug that can further interact with DNA, forming the so-called cisplatin adducts but also inducing a signaling cascade that triggers the cell apoptotic pathway. Phase I human studies of Lipoplatin albeit revealed its mild hematological and gastrointestinal toxicity and did not show most other side effects characteristic of cisplatin treatment such as nephron-, neuro- and ototoxicity, as well as hair loss. Lipoplatin as well as the other liposomal formulations (SPI-077, and L-NDDP) are nowadays in Phase II and Phase III clinical trials being tested for different types of carcinogenic processes [22].

In contrast to liposomes that carry drug cargoes in their aqueous cavities, polymeric NPs contain a solid, polymer-filled structure in which cisplatin is covalently attached. The solid structure also gives polymeric NPs higher stability, more sustained and controllable drug release profiles, and more uniform size distribution. Polymeric NPs are typically prepared through the self-assembly of amphiphilic di-block copolymers. A variety of polymers have been used to prepare polymeric NPs, including biodegradable and biocompatible synthetic polymers such as poly(lactic-co-glycolic acid) (PLGA) and polyglutamic acid and natural polymers such as polysaccharides and polypeptides. PEG-functionalized PLGA NPs are especially desirable because pegylated polymeric NPs have significantly reduced systemic clearance compared with similar particles without PEG. These NPs can release cisplatin under desired conditions, providing potential for tumor specificity. Within this type of NPs, AP5280 is currently under clinical trial Phase II [23].

In NC-6004, cisplatin is encapsulated into approximately 30 nm size polymeric micelles through the polymer-metal complex formation between PEG poly (glutamic acid) block copolymers (PEG-P(Glu)). The notable benefits of the polymer-cisplatin complex micelle (NC-6004) and polymer-platinum conjugate (AP5280) include their superior stability and small dimension (<30 nm vs >100 nm for liposomal formulations), thus facilitating tumor tissue distribution. NC-6004 can circulate in the bloodstream for longer periods because the outer PEG shell protects the micelle resulting in increased tumor-specific accumulation.

An interesting alternative to the entrapment of cisplatin into liposomes or NPs of biopolymers is the use of proteins as nanocarriers. Human ferritin nanocages compare favourably with other systems, particularly for human applications in vivo. This protein is present both inside the cells and in the blood under physiological conditions. In addition, ferritin has exhibited fascinating capabilities for encapsulating cisplatin through disassembling/reassembling process that can be carried out by adjusting the pH. Interestingly, intact ferritin without any modifications can also intrinsically target cancer cells, potentially by binding to transferrin receptors, which are often overexpressed in many cancer cells. The only limitation to its use is the relatively poor encapsulation yields achieved as well as the aggregation that is experienced during the process. However, there is still ongoing research to increase the knowledge on this possibility. A summary of the existing possibilities for cisplatin encapsulation using organic and inorganic polymers is shown in Fig. 1.

3.1.2. *Conjugation of cisplatin to carbon nanotubes and inorganic NPs*

Inorganic NPs and carbon-based nanoformulations have been also evaluated approaches for cisplatin using as the most common incorporation mechanism of the drug, the direct conjugation to the nanocarrier. Carbon nanotubes (CNTs) are of special interest in the area of drug delivery due to their numerous unique physical and chemical properties. In addition, thanks to their hollow cylindrical structure, CNTs provide internal cavities which are capable of accommodating small molecules or ions, like cisplatin. While the toxicological effects of CNTs themselves have been debated in the literature, it has been recently shown that appropriately functionalized and highly purified single-wall CNTs (SWCNTs) can be nontoxic and well-tolerated in vivo. Several reports have demonstrated that CNTs readily cross cell membranes due to their intrinsic lipophilic character and high aspect ratio (needle-like structure), and thus can transport drug molecules like cisplatin. Drug molecules can be attached onto the surface or sidewalls of the nanotubes either by specific adsorption or by covalent attachment but also, they

can be encapsulated within the interior cavity of CNTs to provide an insulating environment for drug molecules. This feature prevents degradation and leakage and other unwanted in vivo interactions before the drug reaches its target sites. Often, a cisplatin prodrug is covalently bound to the COOH group of functionalized CNTs [24].

The most popular inorganic nanomaterials for the delivery of platinum-based chemotherapeutics are iron oxide, gold, silver and silica NPs. These nanoplatforms are beneficial compared to microscale materials owing to their smaller size, larger surface area, and tailorable surface chemistry. Compared with gold and silver, superparamagnetic NPs (super MNPs) such as iron oxide are considerably economical and easy to synthesize, some of which have been approved by US Food and Drug Administration (FDA) as imaging contrast agents and medicine against hypoferric anemia. One particular feature of these nanostructures is the potential for active targeting via magnetic fields, potentially allowing circulating MNPs to be trapped and concentrated as they perfuse tumors. The magnetic properties of MNPs constrain their materials, size, and shape, all of which negatively influence their potential drug-loading capacity. The simple strategy of direct absorption of cisplatin to the surface of iron oxide NPs (IONPs) results in early drug release during circulation. Overcoating these NPs with a polymer shell helped to decrease leaching and enhanced delivery. Cisplatin binding to MNPs was increased by modifying the surface with carboxylic functionalities that chelate the platinum in place of the chloro ligands in cisplatin. Our group has also explored the possibility of using ultrasmall IONPs (< 10 nm) coated by biocompatible carboxylic acids (tartaric and adipic acids) as nanocarrier of cisplatin (IV) prodrugs using the direct conjugation on the surface of the nanotransporter. These structures revealed a high penetration into cells and the release of the active cisplatin that was able to form cisplatin-DNA adducts in a time-dependent manner.

The use of gold NPs (AuNPs) as chemotherapeutic drug delivery vehicles is attractive as it is non-toxic, non-immunogenic, and provides a highly tunable surface to which drugs can be attached. The authors tested the drug loading using small, medium, and large AuNPs (25, 55, and 90 nm, respectively), where synthesis shows good reproducibility and monodispersity. In this study, the drug loading was found to rise significantly with increasing particle size: 25 nm (about 900 drug molecules per AuNP); 55 nm (about 15 $\times 10^3$); 90 nm (up to 55 $\times 10^3$) [25]. By using both iron oxide and gold within one drug delivery vehicle, a multifaceted system can be developed which exploits the surface chemistry of the gold whilst retaining the magnetic character of the iron oxide, allowing for biologically sound drug delivery and imaging. Lin et al.[26] have demonstrated that a gold shell does not degrade the magnetic properties of IONPs. With a similar approach containing a gold coating over an iron oxide core, other authors have immobilized cisplatin using a thiolated PEG linker. These FeNPs showed little inherent cytotoxicity, whereas the gold-coated FeNPs turned out to be as active as cisplatin in the A2780 and A2780/cp70 ovarian cancer cell lines. More importantly, the cisplatin-decorated Au@FeNPs showed to be up to 110-fold more cytotoxic than cisplatin. Potentially, this technology could be used in patients to ensure drugs are targeted only to solid tumors, thereby leaving healthy tissues and organs intact and greatly reducing the side effects associated with chemotherapy [27].

3.2. Targeted delivery of cisplatin nanocarriers

Cisplatin-associated toxicity is often related to the lack of drug specificity towards tumor cells. Although engineered platinum nanotherapies can be passively targeted to tumor sites via the EPR effect, the tumour-targeting capacity may be further enhanced by decorating the cisplatin-loaded NPs with different targeting moieties. This targeting approach involves the co-immobilization of cisplatin and the target of cell surface receptors overexpressed by tumor cells to enhance the cellular uptake of the nanocarriers (see Fig. 2). Designing drug delivery systems that target specific sites with controlled release of the drug over a period is challenging. However, with surface engineering, it is possible to introduce ligands, such as peptides, antibodies, or nucleic acid aptamers that can target NPs to a cancer cell of interest. Compared to antibody-based targeted nanocarriers, peptide-conjugated ones offer various advances: for example, most of the therapeutic monoclonal antibodies (mAb) do not target tumor-specific antigens, it requires screening to select mAbs for dominant epitopes, the target must be antigenic, and it also depends on the strain of animals used. However, in the case of peptides, the target is not necessarily antigenic, and there is no requirement for prior information about the target molecule. In this case, specific methods are needed for the preparation of the modified nanocarrier including the targeting peptides [28].

Cell-penetrating peptides (CPPs), known as short peptides, have shown great capabilities in transporting various substances including NPs into the cell nucleus and cytoplasm. The underlying mechanism could be grouped into three types: 1) the CPP is targeted to cancer cells through the electrostatic effect or the hydrophobic combination; 2) the CPPs enter cells via endocytosis; or 3) CPPs cross the cell membrane by forming a new kind of membrane structure. Recently, significant attention has been taken to the field of CPPs-related drug delivery and tumor therapy using such peptides incorporated on the surface of nanotransporters. As an example, based on the overexpression of the luteinizing hormone-releasing hormone (LHRH) in many tumors, including breast (about 50%), ovarian and endometrial (about 80%),

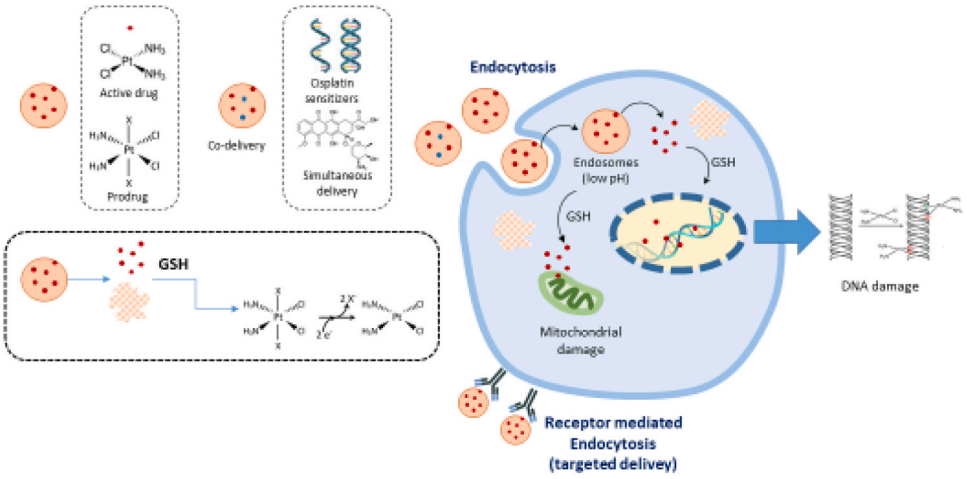

Fig. 2. Mechanistic pathways of cell entrance for cisplatin nanocarriers using either endocytic pathways or targeted cell entrance. The mechanism of Pt(IV) reduction upon entrances into the cell cytosol is also shown in the graph.

and prostate (about 90%), NPs carrying cisplatin for targeted therapy of gonadal tumors have been developed. For this aim, LHRH-peptide conjugated on dextran NPs incorporating cisplatin exhibited improved cellular uptake and promoted cytotoxicity, when compared with the non-targeted NPs. Moreover, both the non-targeted and targeted NPs significantly decreased the systemic toxicity of cisplatin and increased the maximum tolerated dose. Importantly, the targeted nanocarriers enhanced the accumulation of cisplatin in the injected primary tumor and metastasis-containing organs, and meanwhile significantly reduced its nephrotoxicity [29].

Another example includes the overexpression of transferrin receptors in metastatic and drug-resistant cancer cells in comparison to normal cells due to the increased requirement of iron molecules. Taking advantage of such overexpression, a transferrin-functionalized protein-lipid hybrid NP was designed to load both cisplatin and docetaxel for lung cancer treatment. This hybrid system exhibited a remarkable tumor cell inhibition ability, and outstanding tumor suppression capacity compared with other systems [30] for this type of malignancy. Similarly, transferrin (Tf) modified self-assembled polymeric NPs have been successfully applied to co-delivery doxorubicin and cisplatin (DDP), to achieve targeted and combined tumor therapy [31]. Also, the epidermal growth factor receptor (EGFR), overexpressed in lung carcinomas, is a promising target. Thus, EGFR-targeted lipid polymeric NPs were fabricated including a cisplatin-loaded hydrophobic polymeric core, a doxorubicin-loaded phospholipid layer, and an outer layer of EGF-PEG-DSPE ligand [32]. The same receptor was also targeted utilizing aptamers.

Aptamers are single-stranded DNA or RNA with 20–100 nucleotides in length that can specifically bind to target molecules via formed three-dimensional structures. Compared to traditional protein antibodies, aptamers have several advantages, such as small size, high binding affinity, specificity, good biocompatibility, high stability and low immunogenicity, which all contribute to their wide application in the biomedical field. Thus, EGFR aptamer-conjugated PLGA-based nanoplatforms have been used as a system to actively deliver cisplatin to triple-negative breast cancer cells [33]. Similarly, a strategy to deliver cisplatin to prostate cancer cells was achieved by constructing Pt (IV)-encapsulated prostate-specific membrane antigen (PSMA) aptamers targeted NPs. PSMA is abundantly expressed in prostate cancer, its metastatic form, and the hormone-refractory form, thus the polymeric NPs based on PLGA-PEG and containing (PEG)-functionalized polymers with PSMA (Apt) on the surface could target, specifically, prostate cancer tumor cells [34].

Regarding antibody targeting, a mAb against the CSPG4 melanoma antigen was conjugated to ferritin NPs containing cisplatin and used to provide the proof of principle that mAb–NP conjugates specifically deliver anticancer drugs to $CSPG4^{+}$ melanoma, but not to $CSPG4^{-}$ cells, both in vitro and in vivo [35] settings. This kind of mAb–NP conjugates may favorably impact the management of tumors that, like melanoma, once metastatic are highly resistant to conventional chemotherapy, and only transiently responsive to newly developed targeted therapies, including immunostimulatory mAbs to CTLA4.

3.3. Co-delivery platforms of cisplatin and other molecules

Another possibility often considered when using nanocarriers is the co-delivery of different molecules simultaneously. Among them, the most common approach is the co-delivery of two different therapeutic drugs in multicomponent chemotherapy [36]. However, the possibility of incorporating biomolecules that serve as sensitizers for the response to cisplatin like RNA/DNA sequences, enzymes or different ROS inducers has been also reported (some examples are included in Fig. 2). Combinations of cisplatin with doxorubicin [37], paclitaxel [38], metformin [39] and gemcitabine [40] have been described as a co-delivery platform combined with different nanocarriers (mostly biopolymers-based structures). Also, natural products like vitamin E or curcumin have been co-delivered using this type of platform. In particular, curcumin is a natural compound that has been shown to induce cytotoxicity in cervical cancer cells through multiple pathways including inhibition of telomerase or inhibition of cyclin D1 and CDK4 via acetylation and upregulation of p53, for instance. Moreover, curcumin can reverse the multi-drug resistance of cancer cells. Thus, the co-incorporation of cisplatin and curcumin has been attained using lipid-polymer hybrid NPs which combine the mechanical advantages of biodegradable polymeric NPs and biomimetic advantages of liposomes, both described previously. The authors reported higher cytotoxicity to cervical cancer cell models concerning the single formulation [41].

There is a pressing need to engineer nanocarriers that are capable of delivering combination therapeutics involving siRNA since its systemic delivery remains challenging. A recent paper describes an integrated nanodelivery system with NPs prepared through self-assembly of PLGA-PEG capable of simultaneously delivering cisplatin prodrug and siRNAs against *REV1* and *REV3L* to enhance the chemosensitivity of tumors. REV1 is a translesion DNA polymerase, while REV3 is the catalytic subunit of the translesion DNA polymerase Polζ (REV3L/REV7). Recent studies using mouse lymphoma and lung cancer models have shown that knocking down *Rev1* or *Rev3L* can inhibit drug-induced mutagenesis so that relapsed tumors remain sensitive to subsequent treatment. The authors demonstrated that siRNA-containing NPs can successfully lower expression levels of target genes in vitro and in vivo without any evidence of associated toxicity [42].

An interesting application shows the use of IONPs as carriers of cisplatin but also as agents to deliver iron and a glutathione peroxidase 4 (GPX4) small interfering RNA (si-GPX4) for the highly efficient synergistic induction of ferroptosis/apoptosis in glioblastoma (GBM) cells. Ferroptosis was recently identified as an innovative target for the treatment of malignant cancers. Ferroptosis is a type of iron-dependent programmed cell death that is distinct from other forms of cell death, such as necroptosis, apoptosis, autophagy, and paraptosis. Functional si-GPX4 was loaded into our nanodrug to maximize the antitumor effect of ferroptosis. Thus, the three main components enabling two pathways of attack, namely, Pt, si-GPX4, and the iron NPs themselves, were included in our nanodrug. The NPs entered the cells, were degraded, and induced increased intracellular levels of Fe^{2+} and Fe^{3+}. In this scenario, Pt did attach to both nuclear DNA and mitochondrial DNA, leading to apoptosis and simultaneously producing H_2O_2. These changes laid the foundation for the efficient production of ROS (notably hydroxyl radicals) through the Fenton reaction. Finally, GPX4, a key negative regulator of the ferroptotic process was synchronously knocked down by co-loading si-GPX4, increasing the extent of ferroptosis initiation [43].

3.4. Cisplatin prodrugs versus active cisplatin

Intravenous administration is the leading route to deliver antineoplastic agents in cancer therapy, owing to the immediate and complete bioavailability of drugs. However, most anticancer drugs with high toxicity display a narrow therapeutic window because of their nonspecific distribution in the body, resulting in undesirable side effects and reduced patient compliance. In response to these obstacles, the use of prodrugs, which are inactive conjugates metabolized in vivo to release the parent bioactive components, has been shown to improve the efficacy of existing anticancer agents [44]. In particular, the anticancer potential of platinum (IV) compounds has been recognized from the time of the original discovery of the biological properties of cisplatin, but their clinical value has only more recently been acknowledged. The physicochemical properties of platinum (IV) agents differ significantly from those of their platinum (II) counterparts. Unlike square-planar platinum (II) complexes, platinum (IV) complexes show relatively lower reactivity (less unwanted side reactions). Moreover, the two extra ligands present in platinum (IV) compounds provide a means to impart and fine-tune desired biological properties such as lipophilicity and cancer-cell targeting and also facilitate attachment to NPs and other

carrier systems [45]. The conjugation of cisplatin prodrugs into nanocarriers represents an ongoing area of research. Thus, several of the previously described nanodelivery systems make use of the cisplatin (IV) prodrug analogues that release functional cisplatin under specific stimuli (e.g. the presence of reducing molecules like GSH, see Fig. 2). In some cases, the prodrug is covalently conjugated in either ultrasmall IONPs [46] or polymeric NPs [34]. Further research focused on the incorporation of the Pt(IV) prodrugs into the building blocks of polymeric NPs through a stimuli-sensitive bond (e.g., an acid-responsive bond). In this case, a polymer of cisplatin prodrug conjugate is formed and precipitated afterwards in the form of < 100 nm NPs. Such polymer-cisplatin prodrug conjugate NPs exhibited well-controlled drug loading yield, excellent acid-responsive drug release characteristics, and potent cytotoxicity against ovarian cancer [47].

4. Analytical methods to evaluate cisplatin nanodelivery systems

The evaluation of drug nanodelivery systems must be thoughtfully executed to ensure their optimal performance. For this aim, there is a growing need to develop analytical strategies that permit to control the different parts of the process, from the characterization of the nanodelivery system to its behavior in biological systems like cells or tissues. In the following parts, we will summarize the most interesting approaches to address each one of them as seen in Fig. 3.

4.1. Assessment of the drug-nanostructure conjugation

As the use of these different types of nanodelivery systems continues to grow in the field of biomedicine due to the advantages previously discussed in this review, it is necessary to develop proper analytical strategies for the characterization of drug-carrying nanoconjugates [48]. Such methodologies can be classified according to the type of generated data. Thus, infrared (IR) spectroscopy, which allows the identification of functional groups in molecules, provides information about the chemistry behind the formation of the nanosystem [49]. This technique has been applied, for instance, in the characterization of complex systems, such as a chitosan-based self-assembly nanocarriers for cisplatin delivery. In particular, *N*-benzyl-*N,O*-succinyl chitosan (BSCT) nanocarrier was studied to evaluate the coordination formed after its incubation with cisplatin. Here, IR spectroscopy determined the characteristic absorption bands of each compound involved and the conjugation-derived new functional groups. Likewise, this IR approach in combination with DLS and z-potential techniques was employed to assess the complete characterization of the system for its test as drug delivery system in human carcinoma cell lines (HN22 and HT29) [50].

Surface-enhanced Raman spectroscopy (SERS) is another spectroscopic analytical technique that has been reported for its effectiveness in biochemical and medical applications [51,52]. The methodology makes use of rough metallic surfaces to enhance the Raman scattering, which occurs thanks to the chemical and electromagnetic interactions between the metal and the laser light (surface plasmon resonance phenomenon). Its characteristic wavelength depends on the roughness of the nanometric surface, the metallic nature, and the size and shape of the NPs of study [53].

On the other hand, microscopy techniques provide direct information about the size and morphology of nanoconjugates based on the interaction of the sample material with radiation, which depends on the type of microscopy [54]. In the nanocarrier field, the study of morphology is essential to evaluate the drug release process. In this context, the high resolving power offered by electromagnetic radiation enables reaching carrier sizes in the nm scale. Among the multiple types of microscopic techniques, transmission electron microscopy (TEM) is one of the most popular to study structures. This technology is based on the acceleration of a beam of electrons (80–200 KeV voltage) over a sample with enough electron density, in such a way that the transmission of some electrons generates an image of the material's interior in the nm scale. To obtain these images, very thin layers of the sample are needed, which must be subjected to a prior dehydration process and could be a handicap for certain sample types. Abdel-Bary et al. employed this technology for the characterization of different cisplatin-loaded NPs made up of different nanocomposites (e.g., chitosan, silicon oxide), confirming their near-spherical nature [55]. In other studies, different coatings of gold NPs [56] were evaluated by TEM: PEG functionalization covered with cisplatin drug [57] and L-aspartate for the non-covalent bonding for three different drugs [58]. This TEM-based

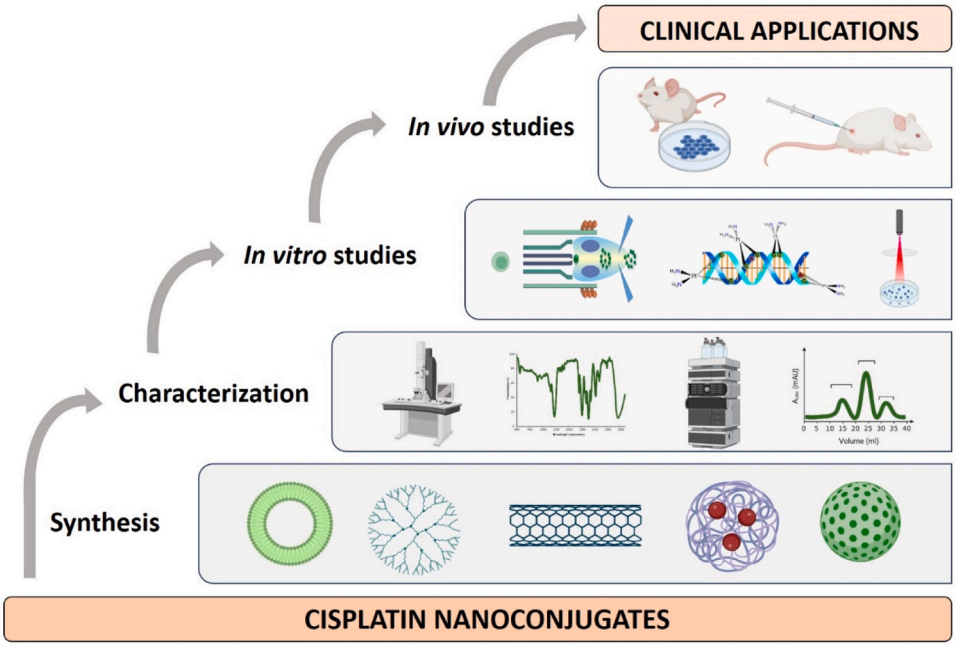

Fig. 3. Bioanalytical pyramid for nanodrug-delivery systems. Summarizes all the bioanalytical strategies that have to be developed/carried out, from synthesis to final clinical application, for the different nanostructures studied in cisplatin chemotherapy.

characterization is frequently complemented with elemental sample analysis by energy-dispersive X-ray spectroscopy (EDX), which informs about the percentage of the elements of interest in a sample in a punctual mode along the microscopic image. This technique also reports on the functionalization of drug nanocarriers and the variations in the element composition during the coating process, as to verify the presence of the drug [59].

Nowadays, the fastest method to study the particle size of any nanometric system is the dynamic light scattering (DLS) approach. It is based on the study of the dispersion of the light that occurs due to the difference between the refractive index of the sample and the solvent in which it is contained when it is stroked by a monochromatic laser. The scattered light by the NPs fluctuates with time due to the Brownian motion of the particles, which is related to the particle size [60]. Despite being a fast technique, as it does not require any prior sample preparation, DLS is highly dependent on the temperature and viscosity of the solvent. Since this might be an issue in certain cases, complementary techniques are needed for result validation (e.g., TEM, SEM, atomic force microscopy, 3D X-ray microscopy) [61]. For instance, DLS and TEM have been employed in the study of PEG monomethyl ether artesunate (mPEG-ART) nanocapsules, reporting differences up to 2 nm between the unloaded nanocapsule and its version with cisplatin as a dye [62]. In another study, the loading efficiency of cisplatin in magnetic nanocapsules made up of PGLA was optimized by investigating the effect of the solvent removal on the size of the capsules, ranging from 142 to 384 nm, allowing for a 62% efficiency increase [63].

As for the separation of nanostructures according to their size, charge or shape, diverse analytical techniques are available, such as centrifugation [64], dialysis [65] or centrifugal ultrafiltration [66]. However, these methodologies are laborious and show low recovery rates due to aggregation during sample processing. Alternatively, chromatographic techniques appear as improved separation approaches although they have not been widely used for the separation of nanoparticles. They are based on the interaction of the sample with a stationary phase while passing through a mobile phase. In this way, the sample components interact with the stationary phase so they get separated depending on the affinity differences with time [67]. Within this type of methodology, size exclusion (SEC) and reverse phase (RP) liquid chromatography (LC) techniques are included. SEC separates the sample components according to their differences in hydrodynamic size, which enables the distinction of different surface coatings [68,69]. Thus, SEC-inductively coupled plasma (ICP)-MS can be applied to understand the relation between the Pt (II) loading amount in NPs and its molecular geometry [70]. Also, SEC was employed to characterize a ferritin-based system for carrying cisplatin. Here, an SEC column with a fractionating range between 10 and 600 kDa was used to distinguish between the protein (UV-Vis detection) and the non-encapsulated drug (ICP-MS to discriminate between the encapsulated and the free drug by measuring Fe and Pt levels [71]). In addition to SEC, Helfrich et al. described a chromatographic method for nanoparticles separation based on a C_{18} reverse phase column, that separates according to hydrophobicity levels, modified with a surfactant agent (sodium dodecyl sulfate, SDS) to obtain an SEC-like mechanism [72] allowing the detection of ionic species as well as NPs within the 5–20 nm range [73–75]. SDS chemically modifies the silanol groups of the stationary phase to separate by the hydrodynamic range and also prevents the formation of irreversible interactions inside the column when working with metallic NPs [75,76]. Based on this principle, we have developed a multielemental strategy to conduct HPLC-ICP-MS the evaluation of the loading level of cisplatin (IV) prodrug into iron oxide nanoparticles as ultrasmall nanodelivery systems with successful results to isolate it from the excess of the free prodrug [46].

One non-chromatographic technique that is widely used for nanoconjugate characterization is the asymmetric flow field-flow fractionation (AF4), which allows the identification of NPs ranging from 1 nm to 100 μm. It applies a perpendicular force to the channel through which the sample is transported so that, by diffusion, the particles in that channel are separated depending on their size. Thus, particles with smaller sizes and greater diffusion rates are concentrated in the center of the channel, while the larger ones showing worse diffusion rates remain in the periphery [77]. Despite AF4 presenting low recovery rates, it is a fast and versatile technique for the characterization of cell-secreted nanoparticles extracellular vesicles for target drug delivery. For instance, Zhang et al., transfected HEK293T cells with si-RNA (small interfering RNA) and incubated the isolated exosomes with gastric cancer cell lines. They demonstrated that exosome-delivered si-RNA could reverse chemoresistance to cisplatin in gastric cancer. However, the application of this technique for the characterization of the NPs-drug conjugates in the case of cisplatin is relatively scarce.

Overall, all described methods in this section are usually employed in a complementary way to each other to obtain a comprehensive characterization of the nanocarrier.

4.2. Study of drug-nanocarrier intracellular incorporation

The incorporation of drug-carrying nanosystems in the cell can happen through diverse mechanisms and are directly related to the physicochemical properties of the NPs [78]. In this regard, several techniques can be used to investigate the cellular incorporation of cisplatin and its derivatives, although special attention requires ICP-MS. This elemental technique is based on the formation of ions by a plasma source for their subsequent detection based on their mass/charge (m/z) ratio. Depending on the type of mass analyzer, ions can be detected either sequentially (as in a quadrupole instrument) or quasi-simultaneously (as in a Time-of-Flight (TOF) or multi-collector instrument) [79]. ICP-MS can operate for bulk analysis where a large population of treated cells are lysed or digested to further quantify the total elemental content. Then, knowing the cell's dry weight, protein content or cell concentration, results can be relatively quantified at cell level [80].

Some of the advantages of ICP-MS include a high sensitivity in the low range, great versatility and multi-elemental detection performance [81]. In addition, in recent years, there has been a growing interest in determining molecular or elemental differences at individual-cell level [82] and for this aim, the concept of "single cell ICP-MS" has been widely evaluated. Particularly, in the case of cisplatin [83] and its nanocarriers, single-cell-ICP-MS (SC-ICP-MS) methodology has shown a good performance. The working principle is that when the sample contains NPs and/or cells in suspension, once they reach the plasma (individually), they give rise to a cloud of ions (event) and each event corresponds to a NP/cell. Therefore, the frequency of the events is related to the cell/particle number concentration in the sample, and the event intensity is proportional to the mass of the element in the NP or cell [84]. This methodology requires the complete and intact introduction of the cell to the mass analyzer. To successfully achieve this, cells can be introduced in a liquid suspension or detached from a support where they were previously fixed using a laser beam. In this context, the possibility of having simultaneous or quasi-simultaneous m/z detection (like in the previously mentioned ICP-TOF-MS analyzers) permits the more complete characterization of cells by addressing their multielemental composition (see Fig. 4).

For single cell analysis using liquid sample introduction, it is necessary that each cell is introduced into the plasma within a very fine drop through a pneumatic nebulizer or a microdroplet generator, being the cell transport to the plasma a crucial parameter whose efficiency is determined by the type of nebulizer used [85]. This liquid introduction method has been broadly applied for the study of the effect of cisplatin and other homologues at the single cell level, reporting quantifiable results even with very low drug concentrations [83,86,87]. Also, it has been compared the cytotoxic activity of $Gd@C_{82}(OH)_{22}$ NPs vs cisplatin by analysing ^{82}Gd and ^{195}Pt, respectively, at the pg/cell level [88]. Based on this principle, in our research group, we have demonstrated the

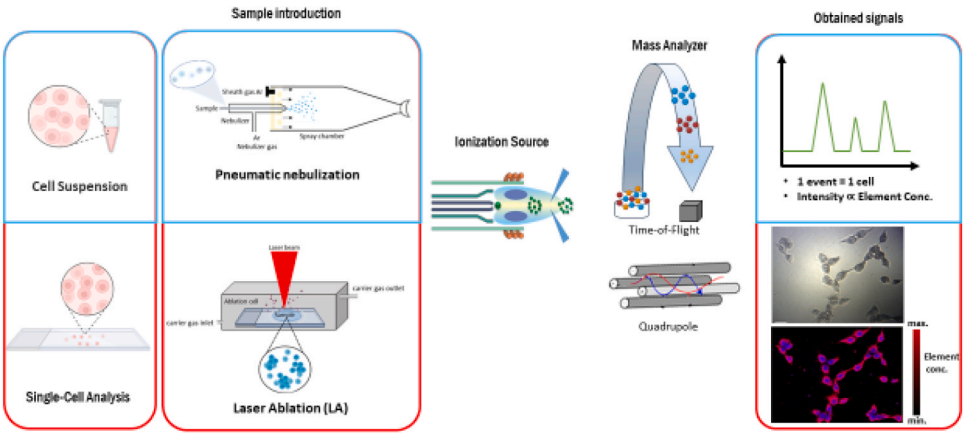

Fig. 4. Elemental mass spectrometry-based strategies to address cellular uptake of Pt-nanoformulations in single cells. Summarizes the two most commonly used strategies based on ICP-MS to obtain individual cell information on the uptake of the different Pt-trasporters. Blue line shows the use of cell suspensions and a red line reflects the use of laser ablation as mean to introduce cells previously immovilized into glass slides.

efficiency of a nanodelivery system made up of ultrasmall IONPs decorated with a Pt(IV) prodrug vs free cisplatin in both a drug-sensitive (A2780) and resistant (A2780cis) tumoral cell line by SC-ICP-MS, quantifying the intracellular Pt content. Data showed a significant difference (up to 4x in the resistant cell line) when using the nanotransporter [46].

The use of ICP-MS permits the introduction of cells, not only in a suspension but immobilized on a glass slide by carrying out the ablation of the spot using a laser beam and generating a plume of atoms that are further transported into the plasma. This technique, known as laser ablation mass spectrometry (LA-ICP-MS), allows direct elemental analysis of solid samples with high spatial resolution, sensitivity and reproducibility (see Fig. 4). Two working modes of detection are possible at the individual cell level: imaging at the subcellular level or complete individual cells, for which different laser beam sizes are needed [89]. LA-ICP-MS can be coupled to other non-destructive molecular techniques, such as SERS. Specifically, Leventi et al. combined SERS with LA-ICP-TOF-MS for the imaging of cells with cisplatin nanoconjugates demonstrating the internalization of the conjugate by the detection of the AuNPs-Cisplatin linker using SERS, and the simultaneous monitorization of ^{31}P, ^{197}Au and ^{195}Pt by LA-ICP-TOF-MS, with a spatial resolution of 10 μm [90].

ICP-MS technologies can also be used for the study of DNA platination, using mainly bulk analysis strategies. For instance, Cao et al. investigated the platination ability of a nanoplatform comprised of a biodegradable PEG-block-poly(lactide) (PLA) nanostructure, a hydrophobic polylactide-cisplatin prodrug, and a cationic lipid. The system was tested in a lung cancer cell line (A549) through the measurement of Pt in the DNA sample obtained from the treated cells. Also, a bulk analysis was performed to evaluate the total cellular drug incorporation. The study concluded that both DNA adduct formation and total cellular Pt uptake were significantly higher when using nanoconjugates compared to free cisplatin [91].

Likewise, a time-dependent comparative study of the use of ultrasmall iron NPs coated with a cisplatin (IV) prodrug vs conventional cisplatin was done using ICP-MS. In detail, the formation of DNA adducts was investigated in both treatment conditions after drug exposure at different time points (i.e., 24 h drug exposure followed by 3, 12 and 24 h resting). Results showed reduced adduct levels over time due to their repair by cellular mechanisms. Also, it was observed that the maximum drug release and activation occurs at 3 h after exposure and gradually lowers as adducts are repaired [92].

4.3. *Evaluation of drug release capabilities*

The rate of drug release from the nanocarrier is an extremely important parameter as it can influence the rate of clearance of the drug from circulation, the bioavailability and thus the activity of the drug at its site of action and the observed toxicities. So once the nanoconjugate has been fully characterized and its cellular penetration capabilities well established, it is necessary to evaluate its potential for drug delivery ("cargo potential").

In this regard, in vitro assays are the most convenient experiments since they do not require a living organism and multiple analyses can be afforded in this setting. For instance, the study of the nanocarrier pharmacokinetics is commonly performed by mimicking in vitro the tumor in vivo conditions. Usually, phosphate buffer saline (PBS), fetal bovine serum (FBS), cell culture medium or even blood plasma are selected to resemble the natural cellular environment [93]. Then, approaches such as dialysis are applied to study the release of loaded cisplatin in e.g. poly(D,L-lactide) coated Fe_3O_4 NPs at different time points by measuring the cisplatin absorbance by UV-Vis spectroscopy [94]. The same methodology enables the understanding of drug release levels under different pH conditions [95,96], which is critical in tumoral settings since they often show extracellular acidosis (pH values <6.0) [97]. Moreover, the presence of molecules in the tumoral environment can affect the drug release, as is the case for reducing substances like glutathione (GSH), which expression can be 1000x higher than in healthy cellular settings [98]. In this sense, Chen Q. et al. assessed the release performance of both cisplatin (IV) and oxaliplatin (IV) prodrugs encapsulated in biodegradable polymer methoxyl PEG-PLGA nanocapsules under physiological (pH 7.4) and reducing conditions. Data revealed that the latter produced 100% drug release, while the former resulted in 7x less efficiency, ensuring that only in a malignant cell environment the platin is discharged [99].

Finally, it is known that Pt(IV) prodrugs release Pt(II) species by removing their axial ligands in the presence of reducing agents, but since they are inert at normal conditions, understanding the cytotoxic functionality of the released species is critical. Pt (II), the active form of the drug, binds to the GG residues of DNA; therefore, a simple assay to define drug cytotoxicity would include the incubation of the released drug in a reducing environment with a GG-containing DNA strand. Then, the formation of the cytotoxic drug-DNA adduct could be also addressed by SEC-ICP-MS monitoring ^{31}P (DNA) and ^{195}Pt (drug) [46].

Molecular MS techniques (namely electrospray MS and/or MALDI-MS) have been seldom used for this kind of applications (probably due to the better capabilities of ICP-MS for these studies). However, some work has been focused on the characterization of the structure of the Pt-

containing molecules released from the nanoconjugate surface in the case of using pegylated gold-NPs after irradiation with a nanosecond-pulsed laser. The authors found that the oxidation of the Au–S bond to Au–S(=O)(=O) could be an important mechanism underlying Pt(II) release from NPs [100].

5. Tools to address the biological effects of the nano-conjugates

As earlier discussed, the inclusion of platinum compounds within nanostructures can notably reduce the side effects generated by the free drug in healthy cells and organs (e.g., high toxicity levels in the kidney and brain, or myelosuppression, see Table 1). Nevertheless, these nanoconjugates must maintain their anti-tumoral cytotoxic potential and remain stable in the tumoral microenvironment until targeted drug release. Thus, any physiological or immune reaction that could eliminate the nanostructured drug should be avoided. Bearing this in mind, the nano-conjugate effective toxicities and mechanisms of action might be consistently assessed when developing new nanoformulations (Fig. 5).

5.1. Cytotoxicity

Platinum-based nanoconjugates have been reported to be more effective than free platinum drugs, but their cytotoxic efficacy may depend on various factors such as the type of cancer, the NP formulation and the experimental conditions used. Cytotoxicity causes cell damage or death, consequently, the evaluation of cell membrane damage is a major indicator. Membrane disruptions can be determined by measuring enzyme leaks (e.g., lactate dehydrogenase) or using dyes that would only enter intact cells. Among these, fluorescent dyes staining the DNA (e.g., propidium iodide, DRAQ7™, 7-ADD) and non-fluorescent dyes (e.g., trypan blue) are widely used [101]. Also, metabolic activities can be measured to assess the cytotoxicity, such as the MTT or WST1 assays [102], where tetrazolium salts are converted into insoluble colored formazan products if cells are alive. Regarding the NP formulation, the conjugation of biodegradable NPs with cisplatin has reported the outperformance of this preparation over the free drug in a time-dependent manner. Thus, while at 24 h the free drug showed higher levels of cytotoxicity, at 72 h after treatment exposure, the cisPt-NP depicted better outcomes as the drug release rate increased. Moreover, studies on Pt uptake levels revealed enhanced incorporation of the drug when using the nanoconjugate [103]. Likewise, a graphene-oxide-based 2D nanoplatform functionalized with PEG and loaded with cisplatin (GO@PEG-Pt) allowed for cell proliferation inhibition in osteosarcoma reporting similar or improved levels compared to the free drug. Of note, the encapsulated formulation was less effective in a GBM model, probably due to its different metabolic profile [104]. This diversity in the effects of the formulations was also reported in other studies, such as those using noncovalent encapsulations [105] or poly-isobutylene-maleic acid (PIMA)-cisplatin NP formulations against lung cancer cells[106]. The latter nanoconjugate induced cell death but failed in generating tumoral cytotoxicity compared to free cisplatin, although cytotoxic levels were similar to those of carboplatin. To improve the efficacy of this nanodelivery system, the authors included glucosamine in the formulation (PIMA-GA-cisplatin) which helped in the release of Pt enhancing the efficacy of the nanoconjugate. Such surface modifications are widely applied in nanomaterials to improve drug delivery. Thus, Vaghasiya et al. [107] used collagen to functionalize the surface of mesoporous silica particles carrying Pt molecules. Only in the tumoral environment, where metalloproteinases are overexpressed, the collagen (the enzyme's substrate) would be detached releasing the anti-tumoral drug. This system reported higher cytotoxicity, cell cycle arrest and apoptosis levels in a lung cancer model vs the Pt-free alternative. In another study [108], iron-based NPs coupled to Pt and fluorophore molecules were evaluated for the treatment of colorectal and T-cell lymphoma tumors. Once again, results depicted a better nanoconjugate system performance than the free drug.

Moreover, coupling two or more drugs within the same nanodelivery system in combination with site targeting via specific receptors is also a promising approach to enhance the cytotoxic action of anti-cancer drugs. In these complex systems, it is also crucial to ensure the optimal performance of the combined nano-conjugate. Such is the case of conjugating silica nanocapsules with cis-diaquadiamino Pt (II) and pemetrexed drugs, further equipped with folic acid and rhodamine isothiocyanate moieties, which exhibited enhanced cytotoxic effect vs the unconjugated drug [109]. Likewise, the transmembrane glycoprotein CD44, overexpressed in diverse tumors (e.g., lung, stomach, breast, ovarian), is being utilized as a target molecule for nanodelivery systems carrying hyaluronic acid (HA; ligand of CD44) on their surfaces. In this line, Yu et al. [110] developed HA-functionalized micelles incorporating both doxorubicin and cisplatin drugs (HA-DOX-CDDP-NPs) showing higher uptake levels by tumoral cells and stronger inhibition of cellular growth than the free drug. Evaluation of $CD44^-$ tumoral cells confirmed the specificity of these nanodelivery systems. Other examples include the application of HA-cisplatin conjugates for the elimination of tumoral cells within draining lymph nodes [111], lung tumors [112], and head and neck squamous cell carcinoma [113] with successful outcomes. Another example combining cisplatin with paclitaxel in biocompatible polymer micelles of PEG coupled to polyaspartic acid (mPEG--PAsp-(PTX-Pt)) for the treatment of ovarian cancer unrevealed high cytotoxic activity via different mechanisms of action [114].

Table 1
Modifications on cisPt-loaded nanosystems to improve drug uptake, cytotoxicity and genotoxicity levels in target tumors.

Nanosystem modification	Target tumor	Reference
GO@PEG-Pt: Graphene-oxide-based 2D nanoplatform functionalized with polyethylene glycol and loaded with cisplatin	Osteosarcoma	104
PIMA-GA-cisPt NP: Poly-isobutylene-maleic acid, glucosamine-cisplatin nanoparticle	Lung cancer	106
Mesoporous silica-Pt particles functionalized with collagen	Lung cancer	107
IONPs-Pt-fluorophore: Iron-based nanoparticles coupled to Pt and fluorophore molecules	Colorectal and T-cell lymphoma tumors	108
Silica nanocapsules with cis-diaquadiamino Pt (II) and pemetrexed drugs equipped with folic acid and rhodamine isothiocyanate moieties	Lung cancer	109
HA-DOX-CDDP-NPs: Nanodelivery systems carrying hyaluronic acid-functionalized micelles incorporating both doxorubicin and cisplatin drugs	Lung tumors, head and neck squamous cell carcinoma	110–113
mPEG-PAsp-(PTX-Pt): Cisplatin with paclitaxel in biocompatible polymer micelles of polyethylene glycol coupled to polyaspartic acid	Ovarian cancer	114
cisPt-PLGA-NPs: Cisplatin-poly(lactic-co-glycolic acid) loaded nanoparticles	Glioblastoma	117
cisPt-MWNT: Cisplatin-loaded multi-walled carbon nanotubes	Lung tumor	123
cisPt-PBCA-NPs: Cisplatin-loaded polybutylcyanoacrylate biodegradable nanoparticles	Kidney tumor	124
PLcisPt: cisPt within pegylated liposomes	Bladder cancer	125
cisPt nanoconjugates combined with PD1/PD-L1 inhibitors	Leukemia	126
Gene therapy combined with cisPt-IONPs and si-GPX4	Glioblastoma	127

5.2. Genotoxicity

Cisplatin-based treatments cause cell genotoxicity via the formation of adducts between the DNA and the platinum drug. As earlier indicated,

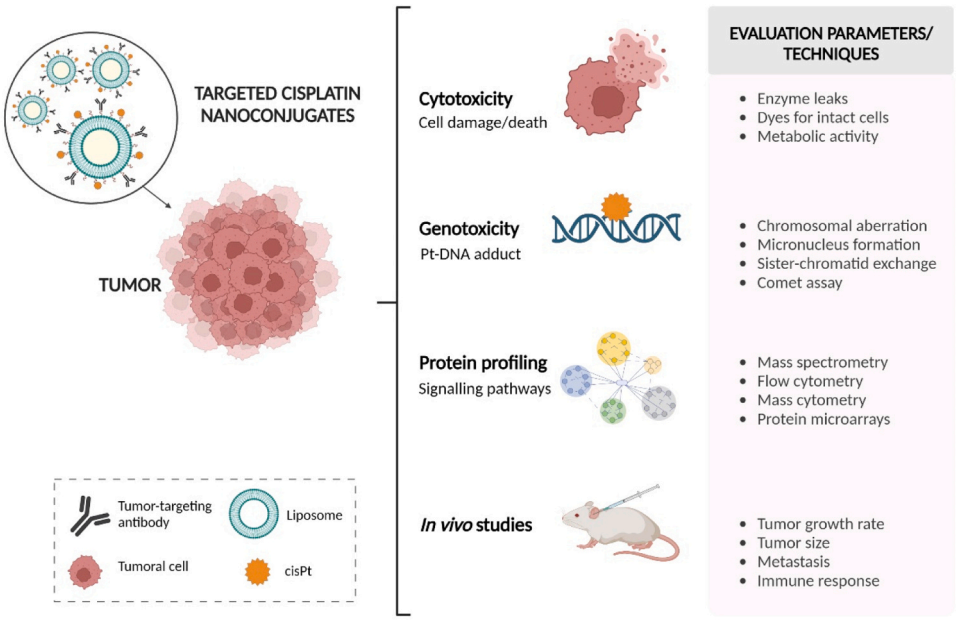

Fig. 5. Evaluation of biological effects of cisPt nanoconjugates. Created with BioRender.com.

these adducts inhibit replication and transcription processes and arrest the cell cycle. If cells cannot reverse this situation, the DNA cannot be repaired leading to cell death. Genotoxicity levels can be determined by evaluating diverse parameters [115], such as chromosomal aberration, an increase of sister-chromatid exchange or the formation of micronucleus. Also, using different techniques, like the comet assay, also called single-cell gel electrophoresis, where it is checked the formation of comet-like tails of relaxed supercoiled DNA, and the bacterial reverse test to identify mutations.

This adduct formation is dependent on the drug's presence, which is directly defined by the platinuḿs ability to enter and remain in the cell. Therefore, the usage of nanoconjugates would improve this toxic effect. Such is the case of Pt-NPs encapsulated within liposomes (Lipo-Pt-NPs) that showed greater genotoxicity (2.4x higher DNA damage) vs the free Pt, the Pt-NPs alone (without lipidic encapsulation) and the liposomes full of free drug [116]. Another example involves the usage of cisPt-PLGA-NPs for the treatment of GBM, which showed higher levels of DNA fragmentation compared to the free drug [117].

5.3. Nanoconjugate-triggered response at the protein level

Cisplatin-based systems not only induce tumoral cell death through cytotoxic and/or genotoxic effects but also by altering the normal functioning of the cellular pathways. Thus, it is also relevant to investigate the modifications of the protein profiles and identify the treatment response biomarkers to elucidate the cellular mechanisms affected. This characterization can be performed by applying high-throughput proteomics techniques such as mass spectrometry [118] that allow the simultaneous and unbiased identification of thousands of proteins. On a smaller scale, protein microarray platforms [119], mass cytometry [120], and flow cytometry approaches [121] also offer information at the protein level, although in a biased form. Despite the importance of proteomics profiling, limited studies have been performed in this line. Among them, a mass spectrometry-based analysis revealed the proteomic response evoked by bile-acid platinum derivatives conjugated with ferrofluids in osteosarcoma and T-cell lymphoma cells [122]. Thus, pathways related to cell repair mechanisms, apoptosis, redox processes, cellular structure rearrangements and vesicular transport were upregulated when treating the cells with the nanoconjugate. Also, specific proteins were found to be overexpressed under this condition. These nanoconjugates were also evaluated for the treatment of colorectal cancer cells by determining the newly synthesized proteins generated due to the treatment [108]. More than 1200 proteins were identified and related, among other functions, to DNA replication (to fix the Pt-DNA adducts), metabolic processes involving DNA repairing and autophagy response (as a response to Pt-mediated cellular stress). Overall, these proteomics studies further depict the altered cellular functioning triggered by drug nanoconjugates, which might help in the understanding of the action mechanisms of these new therapies.

5.4. In vivo studies

Thus far, the present review has acknowledged the advantages of combining platinum-based drugs with nanostructures to improve drug efficacy and performance. However, these nanoconjugates must optimally work in in vivo settings to be ultimately used in biomedical applications.

To address this topic,and considering the legal and ethical limitations of testing in humans, murine models are usually employed in the first stages of research. For instance, Qi et al.[123] reported that cisplatin-loaded MWNTS more effectively hindered lung tumor growth compared to the free drug in male BALB/c nude mice. In another study [124], kidney tumors were induced in male Wistar rats which were subsequently treated with cisplatin-loaded polybutylcyanoacrylate (PBCA) biodegradable NPs. This in vivo study showed the effectiveness of the nanoconjugate over the free drug, depicting a stable performance and time-dependent release of the platinum. Likewise, the inclusion of cisPt within pegylated liposomes (PLCispt) revealed a higher drug efficacy and decreased toxicity effects (4.8x and 3.3x, respectively) vs the free cisplatin in a rat model undergoing bladder cancer [125].

Remarkably, cisPt nanoconjugates have also been evaluated in combination with PD1/PD-L1 inhibitors in tumor-bearing animal models [126]. This powerful anti-cancer therapy reported slower tumoral growth and decreased tumor size compared to other treatments in mice suffering from leukemia. Thus, cisPt NPs upregulated the expression of PD-L1 in tumoral cells making them more accessible for PD1/PD-L1 inhibitors, which induced the infiltration of $CD8^{+}$ T cells that finally eliminated the tumor.

An even more complex system combined gene therapy with nanoconjugates to treat GBM malignancy [127]. In this therapeutic setting,

IONPs were linked to cisPt and a small interfering RNA sequence against glutathione peroxidase (si-GPX4). These Pt-si-GPX4 @IONPs depicted high therapeutic effects with low toxic side effects by mediating apoptosis and ferroptosis in tumoral cells but not in the normal counterparts. In detail, IONPs increased Fe^{2+}, Fe^{3+} and H_2O_2 levels within the cells generating ROS to initiate ferroptosis, which was synergistically improved by the inhibition of GPX4 by si-GPX4. Moreover, the formation of Pt-DNA adducts induced cell apoptosis, altogether resulting in a potent and safe anti-tumoral strategy with promising applications in clinical practice.

Thanks to these investigations, nanomedicine has been boosted during the last years resulting in the approval of up to 31 drug-based NPs for clinical usage [128]. Among them, two cisplatin nanoconjugates, previously detailed in this review, (LiPlaCis, for advanced or refractory tumors; NC-6004 Nanoplatin, for advanced solid tumors, lung, biliary, bladder and pancreatic tumors) are currently being used in 9 different clinical trials with promising results.

6. Conclusions and future perspective

CisPt drugs have been widely studied and applied in clinical practice for the treatment of tumors over the decades. However, their efficacies and toxicities have always concerned the clinical community and advances have been made in the field, including the design and synthesis of analogues of cisPt (e.g., oxaliplatin, carboplatin). Despite these developments, drug delivery levels remained low making necessary new approaches in this regard. Thus, the use of nanoconjugates coupled with platinum drugs, present in a diverse variety of formulations, appears as a promising strategy to re-launch the usage of this antitumoral compound with effective results. Multiple studies have been performed in this field offering outstanding technologies for specific conditions, which might also lead to personalized medicine soon. Therefore, further investigations are needed especially regarding the biological and functional assessment of such drug nanoformulations aiming at expediting their clinical application.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Maria Montes Bayon reports financial support was provided by University of Oviedo.

Data availability

No data was used for the research described in the article.

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Data statement

Since this is a review article, the data provided here can be revised in the corresponding reference and it is, in the current situation, unsuitable to post.

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