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Direct intercalation of cisplatin into zirconium phosphate nanoplatelets for potential cancer nanotherapy

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Abstract

We report the use of zirconium phosphate nanoplatelets (ZrP) for the encapsulation of the anticancer drug cisplatin and its delivery to tumor cells. Cisplatin was intercalated into ZrP by direct-ion exchange and was tested *in-vitro* for cytotoxicity in the human breast cancer (MCF-7) cell line. The structural characterization of the intercalated cisplatin in ZrP suggests that during the intercalation process, the chloride ligands of the cisplatin complex were substituted by phosphate groups within the layers. Consequently, a new phosphate phase with the platinum complex directly bound to ZrP (cisPt@ZrP) is produced with an interlayer distance of 9.3 Å. The *in-vitro* release profile of the intercalated drug by pH stimulus shows that at low pH under lysosomal conditions the platinum complex is released with simultaneous hydrolysis of the zirconium phosphate material, while at higher pH the complex is not released. Experiments with the MCF-7 cell line show that cisPt@ZrP reduced the cell viability up to 40%. The cisPt@ZrP intercalation product is envisioned as a future nanotherapy agent for cancer. Taking advantage of the shape and sizes of the ZrP particles and controlled release of the drug at low pH, it is intended to exploit the enhanced permeability and retention effect of tumors, as well as their intrinsic acidity, for the destruction of malignant cells.

Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II), *cis*-Pt(NH₃)₂Cl₂), a square planar molecule, is a very potent anticancer agent widely used in advanced cancers of the ovary, head and neck, bladder, and non-small cell carcinoma.¹ The mechanism of action of cisplatin involves its binding to N7-sites of guanine bases in DNA, forming DNA–protein and DNA–DNA interstrand and intrastrand crosslinks.² This alkylating-like agent causes DNA damage

which activates transduction pathways that lead to induction of apoptosis.^{2, 3} Cisplatin-related toxicities are dose-dependent, and include myelosuppression, ototoxicity and nephrotoxicity.⁴ Another major limitation is the emergence of resistance to cisplatin in tumor cells.² Multiple mechanisms acting simultaneously account for cisplatin resistance including reduction in intracellular drug accumulation, increased drug efflux, inactivation by increased intracellular levels of glutathione (GSH), DNA repair and inhibition of cell death pathways.²

Delivery of anticancer drugs such as cisplatin to the tumor cells without damaging healthy organs or tissues is highly difficult, if not impossible. In recent years nanoparticle-mediated drug delivery is being studied as a valuable novel approach for overcoming this problem. The use of inorganic based nanoparticles as carriers for drug delivery is a newly emerging field and it is envisioned as a new alternative in cancer nanotherapy. One of the greatest advantages of these inorganic based nanoparticles is the robustness of the particle and the wide gamut of structural characterization techniques that can be employed to elucidate its structure before and after the drug is incorporated, in contrast with its organic based counterparts.

Recently, Sailor and coworkers reported the incorporation of an anticancer drug (doxorubicin) into luminescent porous silicon nanoparticles of spherical shape for therapeutic applications, and there are several other approaches using silicon-based nanoparticles for cancer treatment and detection.^{5, 6} Other than the extensively used silicon oxide nanoparticles, gold (or silver) nanoparticles have emerged as among the most successful inorganic based nanoparticles.^{7, 8} However, most of these nanoparticles have shown serious cellular toxicity and hemolytic activity.^{9–11} In addition to their intrinsic toxicity, these nanoparticles are usually spherical shaped and several studies suggest that spherical shaped nanoparticles suffer from poor penetration ability through vascular fenestrations, failure to adhere to the endothelial walls, and lack of margination properties.^{12–19}

The potential of using non-spherical inorganic layered structured nanomaterials (LSN) as non-viral vectors and drug carriers also is being explored.^{20–22} The nanoparticles of LSN can be prepared with the appropriate size and, thus, exploit the enhanced permeability and retention (EPR) effect of tumors by which macromolecules accumulate passively and preferentially in tumor tissues and are then retained.^{23, 24} The LSN-entrapped drug is excluded from the external medium and will remain biologically inactive, but once the drug-loaded LSN is inside the cancer cells the drug will be released selectively in the tumor cell. The drug release can ensue through dissolution of the LSN by the acidic microenvironment characteristic of cancer cells or the cell lysosomes; by delamination; and/or by direct ion-exchange of the intercalated drug with ions in the cells' interior.²¹

Zirconium phosphates (ZrP) are one of the best-characterized LSN. They are biocompatible inorganic ion-exchange materials and can intercalate a wide variety of species.^{25–28} ZrP is harmless to the body and is not associated with any metabolic function.^{25, 29, 30} The size of ZrP particles can be easily tuned synthetically, in a wide variety of ranges that go from 30 nm to up to 2 μ m, to avoid renal filtration and healthy tissue damage.³¹ Instead of having a

spherical shape, ZrP nanoparticles have a platelet-like shape, and therefore should show better adhesion, margination, and binding properties than spherical nanoparticles.^{14, 17, 32} Under standard biological conditions ZrP is stable and it is expected that once it reaches the low pH environment of the cancer cell, it will begin to release its loaded drug.^{25, 29, 33} In addition, as the drug-loaded ZrP nanoparticles reach the extreme conditions of the lysosome and the peroxisomes, they will dissociate the ZrP to form phosphate ion and harmless zirconium salt. Above all, ZrP seems to have the necessary characteristics to become a very stable, robust, inexpensive, and reliable drug carrier. Here we report our studies on the effectiveness of ZrP nanoparticles in the delivery of cisplatin. We successfully intercalated cisplatin into ZrP (cisPt@ZrP) and conducted cytotoxicity experiments in cancer cells. We studied the drug release profile of the platinum complex from the nanoparticles and the particle degradation under simulated body fluid (SBF) and artificial lysosomal fluid (ALF).

Experimental Section

Intercalation procedure

In a typical procedure the intercalation process was performed by the batch method, adding 50 mL of a solution with the desired quantity of cisplatin to a water suspension of θ -ZrP (1 mg/mL) at different molar ratios (loading levels). The typical loading levels used were 5:1, 1:1, 1:5, 1:10, and 1:20 cisplatin:ZrP. The mixture was stirred for five days at room temperature, monitoring each day the intercalation process by measuring the change in pH and by measuring the UV-vis absorption spectrum of the supernatant of a centrifuged aliquot of the suspension. When the measurements of pH and UV-vis absorbance were constant, indicative of the end of the ion-exchange process, the suspension was filtered using 0.22 μ m filters (Millipore), washed three times with abundant water and dried in a vacuum dryer for one day; then the solid sample was pulverized for characterization.

Instrumentation

X-ray powder diffraction (XRPD) experiments were performed from 2 to 45° (2 θ) using a Siemens D5000 X-Ray diffractometer system with a copper anode source (K_{α} , $\lambda = 1.5406$ Å) with a filtered flat LiF secondary beam monochromator. The divergence, receiver, and detector slits widths were 2 mm; the scatter slit width was 0.6 mm. The interlayer distance was determined using the Bragg's Law for the (002) diffraction plane of the diffraction pattern for α -ZrP, and the (001) diffraction plane of the diffraction pattern for the intercalation products. Diffuse reflectance spectra were obtained using a Cary 1E UV-vis spectrophotometer. The transmission electron micrographs (TEM) of the samples were acquired using a JEOL 2010 transmission electron microscope at an acceleration voltage of 200 kV. Samples were prepared by dispersing the solids in ethanol with an ultrasonic bath followed by deposition on a formvar/carbon coated copper grid (Ted Pella, Inc., Redding, CA).

The microprobe quantitative compositional analyses were carried out on a four spectrometer Cameca SX50 electron microprobe at an accelerating voltage of 15 kV at a beam current of 10 nA. All quantitative work employed wavelength-dispersive spectrometers (WDS). Analyses were carried out after standardization using very well characterized compounds or

pure elements. Qualitative analyses (spectra) were obtained with an Imix Princeton Gamma Tech (PGT) energy dispersive system (EDS) using a thin-window detector. Typical accuracy for major elements (> 10 wt %) is about ± 1 to 2% of the amount present; the uncertainty at low concentrations would increase as the concentration decreases, with the uncertainty reaching 100% at the lower limit of detection (LLD). The lower limit of detection for most elements under typical conditions would usually be about 0.05 to 0.10 wt %. Pressed powder samples will have some additional uncertainty based on surface roughness. The main effect of surface roughness will be to reduce analytical totals because of X-ray scatter. However, X-ray scatter is also wavelength dependent, which could result in a few percent changes in apparent elemental ratios if the surface is very rough and there are significant differences in wavelength. X-ray elemental distribution “maps” were obtained at 15 kV and 20 nA beam current in beam scanning mode. For the 1500x (62 μ m) maps, the beam was sweep in a 256 by 256 point grid, with a grid spacing of 0.24 microns and a total acquisition time of 600 seconds. Composite X-ray maps were generated by combining three 8-bit X-ray images into a false color 24-bit RGB image (ImageJ program). The ICP-MS analyses were performed on a Perkin Elmer NexIon 300 D instrument running in the standard mode with a quadrupole mass spectrometer (1% nitric acid matrix was used to digest the samples). Solid-state proton-decoupled CP/MAS ^{31}P NMR spectra were recorded using an AVANCE Bruker 400 spectrometer. Samples were packed into a sapphire tube and spun at 5000 Hz. ^{31}P spectra were acquired with broadband decoupling. The chemical shifts were measured relative to 85% H_3PO_4 with analytical grade $\text{NH}_4\text{H}_2\text{PO}_4$ ($\delta = 0.9$) used as the reference. Curve fitting was performed using the spectrometer software (LB = -150.00 Hz and GB = 0.25).

Inductively coupled plasma-mass spectrometry (ICP-MS)

The in vitro drug release profile experiment was performed by the dialysis method. In a typical experiment a suspension of about 0.00067% w/v (g/mL) of the intercalated material (cisPt@ZrP) was exposed with SBF (pH = 7.4) or ALF (pH = 4.5), simulating the same ionic concentration of human environment.^{34, 35} The suspension of the intercalated material was agitated and aliquots of 5 μL were taken at specified periods of time from the external volume. The aliquots were centrifuged for a period of 1 minute to avoid any possible light scatter. The release of the platinum complex from the layers was monitored by ICP-MS, by determining the Pt concentration in each aliquot. At the same time the concentration of Zr was also monitored by ICP-MS to determine the hydrolysis of the nanoparticles under biological conditions. The release profile was obtained by plotting the cumulative release (%) against time: Cumulative Release (%) = $[\text{Pt}]_t/[\text{Pt}]_o \times 100$ or $[\text{Zr}]_t/[\text{Zr}]_o \times 100$; where $[\text{Pt}]_t$ and $[\text{Zr}]_t$ are the concentrations of Pt and Zr, respectively, at time t and $[\text{Pt}]_o$ and $[\text{Zr}]_o$ are the total amount of Pt and Zr used in the experiment, respectively.

Cell viability assays

Cell viability was measured using the MTT assay (Sigma-Aldrich Co.). Human breast cancer MCF-7 cells were maintained in RPMI-1640 medium with HEPES (Lonza, Walkersville, MD, USA), 10% fetal bovine serum (Global Cell Solutions, VA, USA) and 1% penicillin-streptomycin-Amphotericin-B (Cell Gro, Mediatech, Manassas, VA, USA) at 37 °C. MCF-7 cells were cultured at a density of 1×10^4 cells/well in 96-well plates for 24

hours. Then, various suspensions of ZrP and cisPt@ZrP at varying loading levels (1:1, 1:5, 1:10) with equivalent concentrations of cisplatin in the solution (0.01– 100 μ M), were added and cells were grown for 24 h, and 48 h. Cisplatin was used as control and added in similar concentrations. At the end of the stated times 20 μ L of MTT solution (5 mg/mL in phosphate-buffered saline) was added to each well and cells were incubated for another 4 h at 37 °C. Formazan crystals that formed were solubilized in 150 μ L of 10% Triton X-100 in acidic isopropyl alcohol with 0.1 N HCl, and after 10 min the absorbance was read at 590 nm on a microplate reader (Bio Rad Model 680). Cells grown in medium without drug or ZrP were used as control. Cell viability was expressed as the percentage of viable cells as compared to the corresponding viable cell number in drug free controls. Assays were performed in triplicate and the median inhibitory concentration (IC₅₀) was determined from the dose-response curves.

Cell cycle analysis

Cell cycle alterations induced by ZrP as compared to untreated controls were analyzed using flow cytometry. Briefly, 2×10^4 MCF-7 cells were treated with void ZrP, at 10 μ M concentration. After 24 and 48 hours, the cell pellets were resuspended in PBS 1X at 4 °C. Then, cells were fixed in ice-cold 70% ethanol. The DNA content of the fixed cells stained with 1 mL of propidium iodide (PI) staining solution was analyzed using the Beckman Coulter Epics XL Flow Cytometer (Beckman Coulter Inc., Fullerton, CA). A total of 25,000 events were analyzed per sample. Cell cycle distribution of treated cells was analyzed using the Multi-Cycle DNA Content and Cell Cycle Analysis Software (Phoenix Flow Systems, Inc., San Diego, CA). Experiments were performed in duplicate and each result was confirmed by two independent experiments.

Apoptosis detection

Annexin-V/PI assay was used to determine the induction of apoptosis by the ZrP nanoparticles and cisPt@ZrP (1:1) at 10 μ M equivalent concentration in MCF-7 cells. Briefly, 100 μ L of cell suspension of 100,000 cells/mL was resuspended in 1X binding buffer, 1 μ L of Annexin V and 5 μ L of PI and incubated on ice in the dark for 30 minutes. After incubation, 400 μ L of 1X binding buffer was added and cells were analyzed with a Beckman Coulter EPICS XL Flow Cytometer (Beckman Coulter, Fullerton, CA); for each sample, 25,000 events were recorded. Experiments were performed in duplicate and each result was confirmed by two independent experiments.

Results and Discussion

In the past, biomolecules have been intercalated into the α phase of ZrP by using preintercalators to expand the layered material beforehand.^{26, 36–39} However, many of the preintercalators used to overcome the intercalation energy barrier are toxic and hamper the biological viability of the system. Alternatively, we used a hydrated phase of α -ZrP, θ -ZrP, which can easily intercalate large molecules without any preintercalators.^{29, 33, 40, 41} Upon drying, θ -ZrP dehydrates and converts to α -ZrP. Therefore, if intercalation is not successful, the material obtained after the intercalation reaction, upon being filtered, rinsed and dried,

will have a 7.6 Å interlayer distance, that of α -ZrP. A resultant interlayer distance greater than 7.6 Å indicates successful intercalation.

θ -ZrP was synthesized and cisplatin intercalated at cisPt:ZrP molar ratios varying from 5:1, 1:1, 1:5, 1:10 and 1:20, using the procedure reported by Santiago and coworkers.⁴¹ The resulting fine powders, referred to by the solution cisPt:ZrP molar ratio used in their preparation (loading levels), were analyzed by X-ray powder diffraction (XRPD), diffuse reflectance spectroscopy, microprobe analysis, and TEM. The XRPD patterns show the formation of a new phase with an interlayer distance of ca. 9.3 Å at all loading levels (Fig. 1). Since α -ZrP has a layer thickness of 6.6 Å,^{42, 43} in this new phase the interlayer distance is increased by 2.7 Å, confirming that intercalation has taken place.

A simple dimensional analysis of cisplatin intercalated into ZrP, based on the cisplatin and the interlayer galleries dimensions, predicts an interlayer distance of ca. 8.3 Å if the square planar cisplatin molecule is laying parallel between the ZrP layers along the gallery plane, and 10.4 Å if the square planar molecular plane is perpendicular to the layers. A 9.3 Å interlayer distance would be expected for molecules oriented with their square planar molecular plane inclined 45° with respect to the ZrP plane. However, at low loading level of cisplatin (1:20 or 5%) the expected interlayer distance is 8.3 Å, because the molecule has enough room to lie parallel to the ZrP layers. On the other hand, if the concentration of cisplatin increases (such as in the 1:5, or 20% material) the interlayer distance is expected to increase as more molecules orient themselves with a progressive increase in the inclination angle, with respect to the layers, to accommodate more molecules. The maximum level of intercalation would be reached when all molecules are oriented with their square planar molecular plane perpendicular to the ZrP layers. In contrast, in our case every intercalation product produced at all loading levels has an interlayer distance of 9.3 Å, instead of showing at low loading levels an interlayer distance of 8.3 Å for molecules parallel to the ZrP layers planes that increases in distance up to 10.4 Å at maximum loading if the cisplatin molecules then orient with their square plane perpendicular to the layers to maximize the loading. The observation of the 9.3 Å interlayer distance at all loading levels suggest that this distance is not from materials with cisplatin molecules oriented at 45° with respect to the ZrP planes. Instead, we believe that the intercalation process does not follow a simple ion exchange mechanism. Diffuse reflectance spectra were consistent with phosphate coordination to Pt (*vide infra*)⁴⁴ and microprobe analyses up to the 1:1 cisPt:ZrP sample indicated much less chloride present than expected if all cisplatin molecules retained their chloride ligands inside ZrP (see Fig. 1 and Fig. 2). The labile nature of the chloride ligands in the cisplatin complex makes it likely that in the new phase many of the chloride ligands are substituted by the phosphate groups from the ZrP layers.⁴⁵

A preliminary structure analysis was performed to elucidate how the Pt complex might accommodate in the interlayer galleries with coordination to the phosphate groups. Taking into account the interlayer distance (obtained by XRPD) two different kinds of structures are predicted: one with the Pt coordinated to two phosphates of the same layer and another with the Pt coordinated to phosphates in adjacent layers in a cross-linking fashion. The distance between the two closest hydroxy groups within the same ZrP layer is ca. 4.6 Å. If the Pt complex coordinated to two phosphates of the same layer, assuming that the O-Pt-O angle

was still 90° as is the Cl-Pt-Cl angle in free cisplatin, the Pt-O bond length would be ca. 3.25 Å, too long for this kind of complex (experimental Pt-O bond lengths in Pt phosphate complexes are 1.96 – 2.09 Å).^{46, 47} In contrast, the alternative crosslinked structure can be produced where the Pt complex is coordinated with a phosphate group from one layer and another phosphate from the adjacent layer. In addition, monoadducts between the platinum complex and the layers can also be formed, where one of the chlorine ligands of cisplatin is substituted by a water molecule and the other by a phosphate group of the ZrP layer. This case will be more probable within nanoparticles with low loading levels; wider peaks will then be expected in the XRPD patterns and they are observed experimentally as shown in Figure 1.

The diffuse reflectance spectra of the cisplatin intercalation products (Fig. S1, [†]) show bands at 278 and 360 nm corresponding to new phosphate coordination complex within the layers, plus the characteristic 417 nm band of free cisplatin for the 5:1 cisPt:ZrP intercalation product, suggesting that at high loading level some of the cisplatin molecules retain their chloro ligands. This result could be attributed to an excess of cisplatin at those loading levels and the possible agglomeration of a platinum excess in the surface of the nanoparticles. Therefore, the diffuse reflectance spectroscopy results are consistent with chloride ligand substitution in the Pt complexes by phosphate groups of the ZrP layers. The chloride ligand substitution by phosphate groups of the ZrP layers was further confirmed by microprobe and ICP-MS quantitative compositional analyses.

Table 1 shows the results of microprobe and ICP-MS quantitative compositional analyses of the intercalation products at all loading levels. The chemical composition of the intercalation products indicates that the amount of chloride in the intercalation products is clearly far lower than what would be expected for free cisplatin, which has a Pt:Cl ratio of 1:2 (i.e., 0.5:1). For instance, the sample prepared with a 1:1 cisPt:ZrP molar ratio has a Pt:Cl ratio of 31:1. In addition, the presence at the surface of ZrP of cisplatin molecules that retain their chloride ligands for the 5:1 cisPt:ZrP intercalation product was confirmed by the Pt:Cl molar ratio obtained at that loading level. Therefore, the quantitative compositional analyses results are consistent with the hypothesis that cisplatin molecules that retain their chloride ligands are present in the 5:1 intercalation product resulting from the excess amount of cisplatin used in the synthesis procedure for that loading level, causing agglomerations on the surface of the ZrP nanoparticles. Finally, the chemical formula obtained indicates that a maximum loading of cisplatin where the chloride ligands were substituted by the phosphate groups of the ZrP layers was achieved when the intercalation reaction was performed with a cisPt:ZrP solution molar ratio of 1:1 (Table 1). In contrast, when an excess amount of cisplatin was used in the intercalation reaction (i.e., cisPt:ZrP = 5:1), no further uptake of the platinum complex inside the layers occurred.

Further evidence was obtained from ³¹P MAS NMR experiments. The alpha phase of zirconium phosphate shows a single ³¹P signal at –18.7 ppm corresponding to the phosphate in the interlayer space.⁴⁸ In the case of the cisplatin-intercalated ZrP we expect at least two

[†]Electronic Supplementary Information (ESI) available: Diffuse reflectance and ³¹P-MAS NMR spectra of the nanoparticles, cell cycle analysis, and table with apoptosis data. See DOI: 10.1039/b000000x/

signals in the ^{31}P MAS NMR spectrum of the intercalation product. One shifted upfield with respect to the original $\alpha\text{-ZrP}$ signal, attributed to the change in the H-bond strength resulting from the substitution of the water by the amino groups in the platinum metal complex, and the other downfield due the deprotonation of the phosphate and the new bond interaction between the platinum(II) and the phosphate. The ^{31}P MAS NMR spectra of cisplatin:ZrP at various loading levels shows two distinctive chemical shift signals, as predicted, one upfield and the other downfield (Fig. S2, [†]). The characteristic chemical shift of $\alpha\text{-ZrP}$ at -18.7 ppm is absent in all the ^{31}P NMR spectra for the cisplatin:ZrP intercalation product. This is typical in a successful intercalation reaction, which is in agreement with the XRPD. On the other hand there are at least three signals that can be identified in the spectra and can be assigned based on the literature. The first signal at ca. -21.2 ppm is assigned to the interaction, *via* H-bond, between the amino groups in the platinum complex and the deprotonated phosphate of the layer. In all the intercalation products this peak shows minimal positional variation, but the full width at half maximum (FWHM) of this peak is considerably larger (ca. 2.6 ppm) especially at lower loading levels (1:20). The second chemical shift at ca. -13.5 ppm is attributed to the phosphate bonded to the platinum(II) of the cisplatin molecule, causing a significant deshielding of the phosphorus.

Figure 2 shows the X-ray elemental distribution “mappings” obtained for the 5:1, 1:1, and 1:5 intercalation products which show the atomic distribution of Pt, P, and Cl in a $62 \times 62 \mu\text{m}^2$ section of the surface of our intercalation products. A very uniform distribution of Pt and P is observed in the 1:1 intercalation product while agglomeration of $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$ is clearly seen on the surface of the 5:1 intercalation product. In addition, a lack of Pt atoms for the 1:5 intercalation product is identified, where 1/3 of the available intergallery spaces are unfilled in the layered material as indicated by the chemical formula obtained from the microprobe analysis.

The TEM images of the cisPt@ZrP intercalation product at loading levels of 5:1, 1:1, and 1:5 show that the hexagonal-like shape of the ZrP crystallites is partially retained in the intercalation product with particle size of ca. 180 nm (Fig. 3). That particle size can be easily modified by changing the synthesis procedure if needed.^{31, 49} In order to avoid renal filtration and keep the particles in the blood stream for prolonged time until they reach their targets, the particle size should be in the range of 20 to 200 nm.⁵ Moreover, particles sizes in the range of 100 to 200 nm are desirable to target cancerous tissue through the enhanced permeation and retention effect.⁵⁰

The controlled release experiments were carried out at pH 7.4 and 4.5 in simulated biological fluids to study the release of the Pt complex from the ZrP layers using pH and chemical stimulus. Simulated body fluid (SBF) was used to simulate the suspension of the nanoparticles within human plasma (pH = 7.4) and artificial lysosomal fluid (ALF) was used to simulate the nanoparticles in the lysosome of the cells (pH = 4.5). Figure 4 shows the release profile of cisPt@ZrP with different loading levels (1:1, 1:5, and 1:10 cisPt:ZrP) upon agitation in SBF (Fig. 4A) and ALF (Fig. 4B). The release was monitored via ICP-MS by observing the platinum (Pt^{195}) signal over time. At pH = 7.4 under the SBF condition the Pt release takes place within the first 12 h. It is only between 2% to 3% of the theoretical maximum, if all the intercalated Pt complexes were released. The release profile reaches a

plateau after the first 12 h that is maintained for several days. We assign the Pt released under these conditions to the accessible platinum on the surface and edges of the ZrP nanoparticles, not from the Pt intercalated complexes within the ZrP layers. The slow release of cisplatin at pH = 7.4 under blood simulated condition is highly desirable in order to avoid the general administration of the drug and the many side effects related to this phenomena. On the other hand, rapid release of the Pt complex from the ZrP galleries at low pH is desirable since it approaches the typical pH of the acidic environment of the tumor endosomes, and lysosomes.^{8, 51}

The release profile of the platinum complex from the ZrP nanoparticles under the pH = 4.5 ALF condition (Fig. 4B) shows a much faster release, compared to the release in SBF (pH = 7.4), with almost 50% of release after the first 12 hours for the 1:5 molar ratio. The release is faster for the 1:5 molar ratio sample than for the 1:10 and the 1:1 molar ratios samples. This difference can be explained by taking into account the structure of the cisPt@ZrP nanoparticles for each molar ratio. The 1:1 molar ratio sample is a fully loaded material, where most of the platinum complexes are covalently bonded to the layers in a crosslinked fashion, making the protonation of the phosphate to release the Pt complex and ligand exchange of the platinum complex more difficult by the steric hindrance of the system. In the case of the cisPt@ZrP 1:10 molar ratio sample, the platinum complex is less hindered, but is more diluted throughout the whole nanoparticle, making the release slower. In the case of the cisPt@ZrP 1:5 molar ratio sample there is a higher concentration of the Pt complex than the 1:10 molar ratio sample but less hindrance than in the 1:1 molar ratio sample because the system has not reached saturation, producing a faster release.

The hydrolysis of the Zr atoms from the ZrP layers was also monitored while the release of Pt takes place in the ALF. The release of Zr atoms into the medium is indicative of hydrolysis of the particles, which is important for the clearance of the inorganic nanoparticles from the body. The hydrolysis of the nanoparticles produced a soluble Zr salt and inorganic phosphate that can be reused in the biological system. Figure 4B shows the release of Zr atoms with time. The release of the Zr is slower in comparison to the release of the Pt from the particle, which implies that the hydrolysis of the platinum complex is faster than to the hydrolysis of the nanoparticles under lysosomal conditions.

Inhibition of MCF-7 cancer cells growth by ZrP nanoparticles was evaluated after 24-h and 48-h treatment using the MTT assay (Fig. 5). ZrP alone did not affect the viability of MCF-7 cells (Fig. 5 A and C). However, treatment with cisplatin alone for 48 hours reduced by 50% the viability of MCF-7 cells ($IC_{50} = 7 \mu M$; Fig. 5 D). A higher concentration of cisPt@ZrP (1:1 molar ratio) was needed to reduce MCF-7 cell growth by 40%. Results of cell viability inhibition were consistent with the cisplatin release profile results (Fig. 4). At 24 and 48 hours the effective cisplatin concentration released from cisPt@ZrP was much lower and, thus, its inhibitory concentration. Recent studies show that burst release profiles of drug-loaded polymeric nanoparticles and liposomes are undesirable due to increased local and systemic toxicity, low drug availability at the tumor site, and consequently a reduced therapeutic effect.⁵² Therefore, the slow release profile of cisPt@ZrP may be pharmacologically advantageous by increasing the time of exposure of the tumor cells to the drug. Due to the enhanced permeability and retention effect (EPR) when using ZrP

nanoplatelets as carriers, cisplatin will be released mainly into the tumor cells that might result in lower systemic toxicity to the patient.

DNA analysis showed that cells treated with ZrP display a normal cell cycle very similar to untreated control cells which is indicative that ZrP does not produce DNA damage or alterations in cell cycle (Fig. S3, [†]). Apoptosis induced by ZrP was limited because early apoptotic population of cells was 17.2% after treatment with ZrP with a net increase of 5% over control cells (Table S1, [†]). The apoptotic effect of cisPt@ZrP (1:1 molar ratio) is 1.8-times stronger than ZrP alone. Moderate cisplatin-induced apoptosis was evident in the cells treated with ZrP-intercalated cisplatin for 48 hours. The side scatter plot (not shown) showed MCF-7 cells carrying ZrP alone were displaced to the right suggesting an increased granularity that can be associated with the internalization of ZrP nanoparticles. The slow release profile of cisplatin@ZrP intercalation products may increase both the time that tumor cells are exposed to maximum drug levels and the drug penetration distance, compared with free drug allowing a reduction of cisplatin exposure to healthy tissue *in vivo*.

Conclusions

The *in vitro* studies suggest that application of ZrP nanoparticle-mediated drug transport as exemplified by cisPt@ZrP, is highly promising for cancer chemotherapy. Intercalation of cisplatin by direct ion exchange, using α -ZrP as a precursor, produced a new material that has a strong therapeutic potential. The intercalation reaction of cisplatin involves the substitution of the cisplatin chloride ligands by the layers phosphate groups producing a loaded nanoplatelet with a very strong anticancer drug crosslinked within the layers. We also demonstrated the *in vitro* release of the Pt(II) complex after the intercalation by a pH stimulus. The Pt-complex release profile shows a direct dependency on the pH of the medium for the release of the complex where at low pH the cisplatin is released from the layers and at higher pH the complex remains much longer in the interlayer region. This behavior is ideal for their use in cancer nanotherapy, where the tumor cell uptake of large nanoparticles is favorable and the pH is lower than in normal cells, avoiding the side effects of cisplatin to healthy tissue. In addition, the drug release experiment show particle degradation with time, producing soluble Zr ions, that can be potentially cleared from the system, and inorganic phosphate that can be used in important biological processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. X-ray powder diffractograms of α -ZrP and of the intercalation products of the reaction of ZrP and cisplatin at several cisPt:ZrP molar ratios. The molar ratios are (from top to bottom): 1:20, 1:10, 1:5, 1:1, and 5:1.

Fig. 2.

X-ray elemental distribution “maps” of cisPt@ZrP at 5:1, 1:1, 1:5 molar ratios. The microprobe image composites X-ray maps were generated using the program Image J® by combining three 8-bit X-ray images into a false color 24-bit RGB image, where red was selected for Pt, blue for P, and green for Cl.

Fig. 3.

TEM images of cisPt@ZrP intercalation products for the 1:5, 1:1, and 5:1 molar ratios.

Fig. 4.

In-vitro platinum and zirconium release from cisPt@ZrP nanoparticles (1:1, 1:5, and 1:10 molar ratio) in SBF (A) and ALF (B), at pH 7.4 and 4.5, respectively.

Fig. 5.

Effects of ZrP, cisplatin, or cisPt@ZrP on MCF-7 cell growth viability. Cells were treated with varying concentrations of ZrP, cisplatin, or cisPt@ZrP for 24 (A and B) and 48 h (C and D). The cisplatin concentration in μM was calculated from cisPt@ZrP based on the molecular formula at different loading levels as shown in Table 1. Results are presented as mean $\pm$ SEM of three assays.

Table 1

Molecular formula determination for cisPt@ZrP at different loading levels based on microprobe and ICP-MS.

Molar Ratio ^a	Chemical Formula	Pt:Cl ratios ^b
1:20	$\text{Zr}(\text{H}_{0.9935}\text{PO}_4)_2(\text{Pt}(\text{NH}_3)_2)_{0.008}\text{Cl}_{0.003} \cdot 0.8\text{H}_2\text{O}$	17:1
1:10	$\text{Zr}(\text{H}_{0.9920}\text{PO}_4)_2(\text{Pt}(\text{NH}_3)_2)_{0.012}\text{Cl}_{0.008} \cdot 0.3\text{H}_2\text{O}$	23:1
1:5	$\text{Zr}(\text{H}_{0.8635}\text{PO}_4)_2(\text{Pt}(\text{NH}_3)_2)_{0.140}\text{Cl}_{0.007} \cdot 0.3\text{H}_2\text{O}$	27:1
1:1	$\text{Zr}(\text{H}_{0.6870}\text{PO}_4)_2(\text{Pt}(\text{NH}_3)_2)_{0.318}\text{Cl}_{0.010} \cdot 0.2\text{H}_2\text{O}$	31:1
5:1	$\text{Zr}(\text{H}_{0.7525}\text{PO}_4)_2(\text{Pt}(\text{NH}_3)_2)_{0.286}\text{Cl}_{0.077} \cdot 0.5\text{H}_2\text{O}$	4:1

^a molar ratio based on the initial reaction ratio used between Pt and Zr.

^b based on microprobe analysis.