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Coordination-Driven Self-Assembly of a Pt(IV) Prodrug-Conjugated Supramolecular Hexagon

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This article presents a new strategy to engage coordination-driven self-assembly for platinum drug delivery. The self-assembled supramolecular hexagon is conjugated with three equivalents of Pt(IV) prodrugs and displays superior therapeutic index as compared to cisplatin against a panel of human cancer cell lines.

Cisplatin, carboplatin, and oxaliplatin are the FDA-approved platinum-based chemotherapeutics commonly used for cancer therapy in clinics.[1] Their anticancer activity arises from the formation of Pt-DNA adducts through covalent bonds of the Pt atoms with the purine nucleobases. These cross-links block transcription, leading to apoptotic cell death.[2, 3] Patients treated with Pt-based drugs usually suffer from a variety of side effects including kidney damage, nerve damage, hearing loss, and suppression of bone marrow activity. To overcome theseundesired side effects, it is necessary to develop a suitable drug delivery system for carrying anticancer agents to the target location.[4, 5] Pt(IV) prodrugs in combination with nanodelivery is an approach that has received much attention, due to the versatility of Pt(IV) prodrugs to be chemically modified and the superior therapeutic index of nanodelivery. A large variety of delivery platforms have been investigated over the last decades,[6] including polymeric nanoparticles,[7] carbon-based nanomaterials,[8] inorganic nanoparticles,[9-11] etc. However, a delivery system with well-defined size and shape that demonstrates consistent drug loading is still limited.

Coordination-driven self-assembly is a well-established methodology for constructing macromolecular/supramolecular inorganic structures with well-defined size and geometry.[12] Unlike many other macromolecular/supramolecular species, e.g. polymers or liposomes, their structural features can be determined by NMR spectroscopy, mass spectrometry, and X-ray crystallography.[13, 14] By virtue of their unique structural features, these self-assembled structures have led to a variety of innovative research in chemistry, material science, and biology, such as the development of new catalysts, novel energy storage platforms, etc. In recent years, studies about applying coordination-driven self-assembly in the area of cancer research has attracted increasing attention.[15-19] Lippard, Therrien, Crowley, and Isaacs have respectively published their works on designing self-assembled metal-organic cages for anticancer drug delivery.[20-23] In all of these reports, the therapeutic components were introduced via non-covalent interactions.

In this report, we present the first example of using covalent conjugation and coordination-driven self-assembly to develop a delivery platform with well-defined size, geometry, and Pt drug loading. As shown in Figure 1A, the Pt(IV) prodrug is covalently conjugated to a 120° dipyridyl building block. Subsequently, the drug-conjugated building blocks (**1**) self-assemble with the 120° organoplatinum linkers (**2**) and generate a supramolecular hexagon (**3**), each of which carries a payload of three Pt(IV) prodrug molecules. As a negative control, the hexagon (**5**) without Pt(IV) prodrugs was prepared via the self-assembly of **2** and **4** (Scheme S1 in the Supplementary Information).

The 120° Pt(IV) prodrug conjugated dipyridyl ligand (**1**) was prepared via the amide-bond-formation reaction between [*c,c,t*-Pt(NH_3)Cl_2(ethyl carbamate)(succinate)] and the amino dipyridyl linker with 45% yield (Scheme S2). The amino linker was synthesized by the reaction between 3,5-di(2-(4-pyridyl ethynyl)) phenol and 2-(Boc-amino)ethyl bromide and subsequent deprotection in dichloromethane and trifluoroacetic acid (*v/v* 2:1). Details of the synthesis can be found in the Supplementary Information. Ligand **1** is anticipated to release cisplatin, ethylamine, CO_2, and a

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Electronic Supplementary Information (ESI) available: Experimental details regarding the synthesis and characterization of **1**, **3**, **5**, and **9**, cell culture, the MTT assay, cellular uptake, real-time PCR, flow cytometry, and HPLC analysis are presented in the ESI. See DOI: 10.1039/x0xx00000x

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succinate dipyridyl ligand (**9**) upon reduction (Figure S1A). As shown in Figure S1B, the HPLC peak (11.1 min) for **1** disappeared upon incubation with 20 eq. of ascorbate (10 mM) in aqueous solution for 15 h at 37 °C, and a new peak (9.2 min) corresponding to the reduction product (**9**) was observed.

For the self-assembly, the Pt drug-conjugated 120° dipydriyl linker (**1**) was reacted with the 120° organoplatinum building block (**2**) in a 1:1 ratio in DMSO-d_6, and the mixture was stirred at R.T. for 2 h. As a result of coordination-driven self-assembly (Figure 1A), the reaction generated the supramolecular hexagon (**3**). As depicted in the 3D model (Figure 2B), the hexagonal structure of **3** bears three Pt(IV) prodrug components. The structure of **3** was characterized by multinuclear (^{1}H and ^{31}P) NMR spectroscopy and electrospray ionization mass spectrometry (ESI-MS). In the ^{1}H NMR spectra (Figure 2C), the signals of the α- (peak a') and β-pyridyl (peak b') protons of **3** have been downfield shifted by 0.22 ppm and 0.32 ppm as compared to those (peak a and b) of **1**, and is attributed to the coordination to the platinum centers of the acceptor (**2**). Likewise, in the ^{31}P{^{1}H} spectrum (Figure S2 in SI), a predominant singlet at δ = 13.61 ppm with two ^{195}Pt satellites ($^1J_{Pt\text{-}P}$ = 2632 Hz) was observed corresponding to the formation of the hexagon (**3**). The NMR evidence is consistent with that observed in other supramolecular hexagons reported previously.[14, 24] High-resolution ESI-MS was employed to further confirm the formation of the supramolecular hexagon. As shown in Figure 2B, the MS signals at m/z = 934.76, m/z = 1151.47, m/z = 1476.60, and m/z = 2018.44 are corresponding to [**3** – 6OTf]$^{6+}$, [**3** – 5OTf]$^{5+}$, [**3** – 4OTf]$^{4+}$, and [**3** – 3OTf]$^{3+}$ respectively, and all the isotopically resolved patterns match with the theoretical simulations (Figure S3). Combined evidence from NMR and MS support the coordination-driven self-assembly of the supramolecular hexagons conjugated with the three Pt(IV) prodrugs per ensemble.

The *in vitro* anticancer activity of the supramolecular hexagon was evaluated using the MTT assay (Figure 2A). Six human cancer cell lines were tested, including the non-small cell lung cancer cell line A549, ovarian cancer cell line A2780, and cisplatin-resistant ovarian cancer cell line A2780cis, ovarian cancer cell line SKOV-3, colon cancer cell line HT29, and triple-negative breast cancer cell line MDA-MB-231. Cancer cells were treated with cisplatin or the different test compounds for 72 h and cell viability was assessed. Additionally, various control groups have been used in order to clarify that the obtained cytotoxicity profiles represent the intrinsic properties of the Pt prodrug-loaded hexagons, and

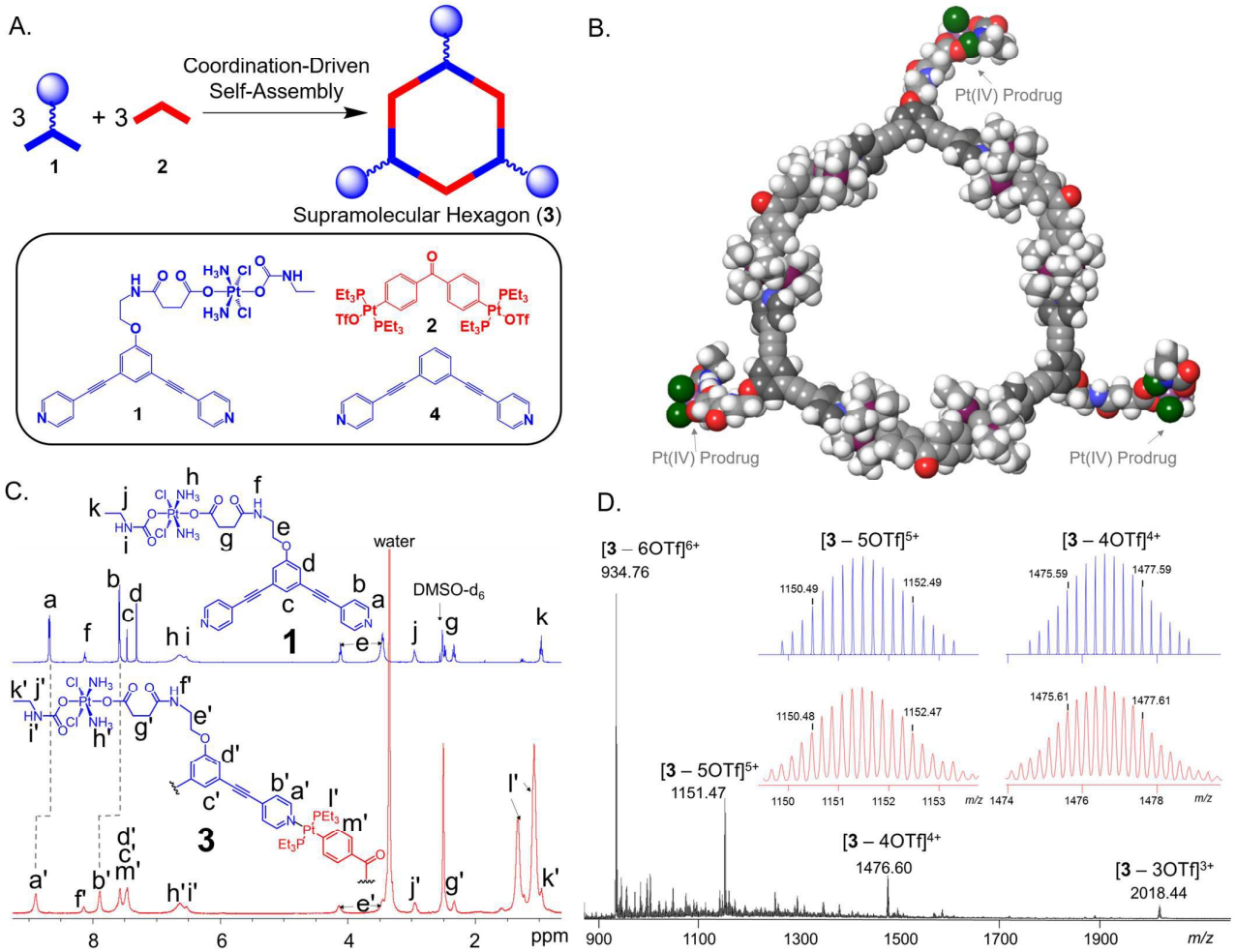

Figure 1. Coordination-driven self-assembly of the Pt(IV) prodrug-conjugated supramolecular hexagon: (A) illustration of the self-assembly of the hexagon (**3**) using a 120° Pt(IV) prodrug-conjugated dipyridyl ligand (**1**) and 120° organoplatinum acceptor (**2**); (B). 3D computational model of the self-assembled supramolecular hexagon (**3**), to which, three molecules of Pt(IV) prodrugs are conjugated (Carbon: grey; Oxygen: red; Nitrogen: blue; Phosphorous: purple; Chlorine: green; Platinum: pink); (C). ^{1}H NMR spectra of **1** (top) and **3** (bottom) in DMSO-d_6; (D) ESI-MS spectra of **3**. (Theoretical simulations in blue and experimental data in red).

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not from the individual components. These controls include the individual building blocks (**1** and **2**), the mixture of **1** and **2** in cell culture media without self-assembly, and Hexagon **5** that does not carry Pt(IV) prodrugs (Scheme S1). Details of the synthesis and characterization of **5** can be found in Supplementary Information (Figure S4 and S5). The IC_{50} values, which represent the Pt concentration required to inhibit growth by 50%, are given in a table shown in Figure 2A. It is clear that **3** has the lowest IC_{50} values among all tested cell lines and all test compounds. For example, in A549 lung cancer cell line (Figure 2B), IC_{50} (**3**) = 0.87±0.13 μM is 18 times lower than that of cisplatin (IC_{50} = 16.38±2.02 μM) and 100 times lower than that of the Pt(IV) prodrug (**1**) itself (IC_{50} = 75.5±20.5 μM). The *in vitro* anticancer activity of **3** was also confirmed by using fluorescent microscopy and the LIVE/DEAD cell assay, a combination of the ethidium homodimer-1 assay and staining with acetomethoxycalcein, or calcein AM (Figure 2C). Live cells stain with calcein AM and yield a green fluorescence signal, whereas dead cells exhibit no fluorescence or a red signal due to the ethidium homodimer-1. A549 lung cancer cells treated with **3** ([Pt] = 7 μM) for 48 h were mostly dead, but those treated with cisplatin (7 μM) under the same conditions had mostly survived. The observation in live/dead cell assay agrees with the above-mentioned cytotoxicity profiles. Notably, as shown in Figure 2D, **3** displays a lower resistance factor ($IC_{50(A2780cis)}/IC_{50(A2780)}$ = 1.7) in ovarian cancer cell lines than cisplatin ($IC_{50(A2780cis)}/IC_{50(A2780)}$ = 7.0). To sum up, all these results suggest that the supramolecular hexagon **3** shows superior therapeutic index as compared to cisplatin *in vitro*.

A.

IC_{50} (μM)*	A2780 Ovarian Cancer Sensitive to cisplatin	A2780cis Ovarian Cancer Resistant to cisplatin	SKOV-3 Ovarian Cancer	A549 Lung Cancer	HT-29 Colon Cancer	MDA-MB-231 Breast Cancer
cisplatin	1.19 ± 0.02	8.35 ± 1.65	18.6 ± 2.17	16.38 ± 2.02	24.85 ± 0.17	30.40 ± 1.78
1	2.68 ± 0.35	>100	171.1 ± 11.7	75.5 ± 20.5	163.1 ± 68.1	>100
2	4.92 ± 0.87	6.81 ± 0.55	8.95 ± 3.37	20.19 ± 0.11	14.38 ± 5.87	17.91 ± 4.43
2 and 1	1.34 ± 0.06	8.42 ± 0.10	21.22 ± 2.82	12.18 ± 3.38	22.19 ± 2.70	11.26 ± 0.81
3	0.48 ± 0.02	0.82 ± 0.11	2.08 ± 0.18	0.87 ± 0.13	4.09 ± 0.58	1.03 ± 0.12
5	2.35 ± 0.06	6.01 ± 0.34	12.56 ± 0.21	8.16 ± 3.52	8.55 ± 3.21	7.68 ± 3.57

*IC_{50} values are reported based on the Pt concentration

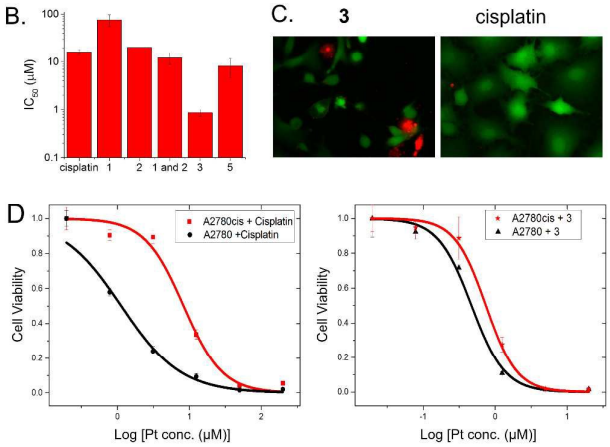

Figure 2. Cytotoxicity profiles of the supramolecular hexagon (**3**): (A) A table of the IC_{50} values of cisplatin, **1**, **2**, and the mixture of **1** and **2** in media without self-assembly, the hexagon conjugated with Pt(IV) prodrugs (**3**), and the hexagon without Pt(IV) prodrugs (**5**) determined by MTT assays; (B) bar graph of IC_{50} values obtained using A549 cell line; (C) fluorescence microscopic images obtained for the live/dead cell assay using A549 cells treated with **3** (7 μM [Pt], 48 h) or cisplatin (7 μM [Pt], 48 h). Dead cells were labelled with red and live cells were labelled with green; (D) representative killing curves of cisplatin and **3** against A2780 and A2780cis ovarian cancer cell lines.

Further mechanistic studies were carried out to gain a better insight into the anticancer activity of the supramolecular hexagon **3**. As shown in Figure 3A, the cytotoxic effect of **3** is hypothetically attributed to three key steps, including cellular uptake, DNA damage triggered by cisplatin released from reduction of the Pt(IV) prodrug, and subsequent apoptosis. Cellular uptake of **3** and other compounds has been determined using A549 cell line and graphite furnace atomic absorption spectroscopy (GFAAS). As a result (Figure 3B), **3** shows up to 20 times higher uptake as compared to cisplatin and ligand **1**. The high uptake of **3** is believed to contribute to its high potency as discussed above. To confirm DNA damage caused by cisplatin release, we monitored changes in expression of biomarkers related to DNA damage pathways. A549 cells incubated with **3** ([Pt] = 7 μM) for 48 h showed a marked increase in phosphorylated H2AX (γH2AX) as determined by flow cytometry (Figure 3C), and increases in p21 and p53 mRNA expression determined by real time PCR (Figure S6). Consequently, the supramolecular hexagon **3** was able to kill cancer cells by triggering programmed cell death (apoptosis). As shown in Figure S6, the mRNA levels of BAX and Apaf-1 were upregulated in **3**-treated A549 cells. By using a dual staining Annexin V/PI flow cytometry assay, the occurrence of apoptosis was investigated in A549 cells treated with **3** or cisplatin. The results in Figure 3D clearly indicate that **3** can efficiently induce apoptosis in A549 cells. Hexagon **3** ([Pt] = 7 μM) prompts A549 cells to undergo early (9.49%) and late (6.89%) stage apoptosis after 48 h of incubation, the populations of which were much higher than those of cisplatin (Figure 3D). The evidence compiled from the cell-based experiments well supports the conclusion that **3** can readily enter cancer cells and release cisplatin to damage DNA and trigger apoptosis to kill cancer cells.

In conclusion, we demonstrate that coordination-driven self-assembly can serve as a novel, precisely engineered drug delivery platform and provide superior therapeutic index. For the first time, through covalent conjugation of Pt(IV) prodrugs, coordination-driven self-assembly allows for precise control of drug loading, by which, three equivalents of the Pt payload are incorporated onto each supramolecular hexagon. The drug-loaded supramolecular complex has been well characterized by multinuclear NMR spectroscopy and high-resolution mass spectrometry. In cell-based experiments through MTT assay and live/dead cell assay, we found that such supramolecular

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complexes have superior therapeutic properties, including sub-μM potency against various human cancer cell lines and low cross-resistance with cisplatin. According to our mechanistic studies, such therapeutic properties mainly attribute to the high cellular uptake of the hexagon **3**, which promotes DNA damage-induced apoptosis. The success of these novel structures not only demonstrates the potential for applications of coordination-driven self-assembly in cancer research, but also provides new strategies to develop innovative Pt-based drug delivery platforms.

Conflicts of interest

There are no conflicts to declare.

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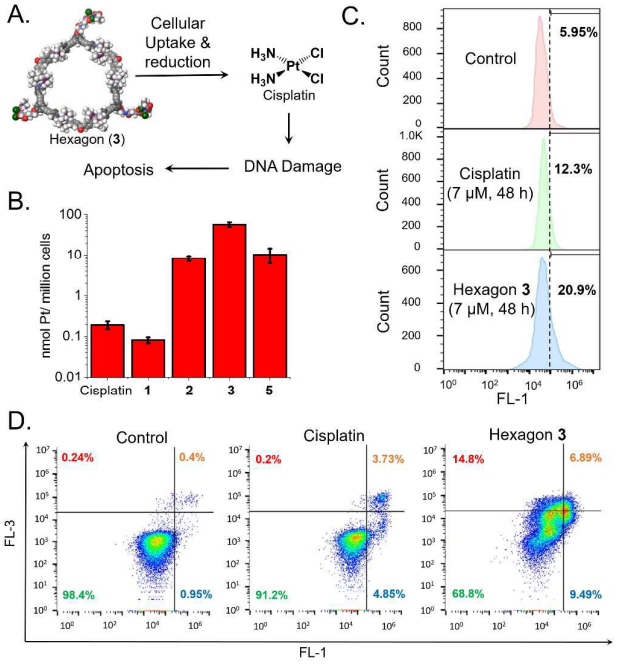

Figure 3. Mechanistic studies of the anticancer activity of the Pt(IV) prodrug-conjugated hexagon (**3**): (A) Schematic representation of the key steps associated with the cytotoxic effect of **3**; (B) Cellular uptake determined by GFAAS. A549 cells were incubated with different samples ([Pt] = 50 μM) for 4h; (C) Flow cytometric analysis of phosphorylation of H2AX (γH2AX) level. A549 cells were incubated with **3** ([Pt] = 7 μM) for 48 h; (D) Annexin V/PI flow cytometric analysis of the apopotic events of A549 cells treated with **3** or cisplatin ([Pt] = 7 μM, 48 h).

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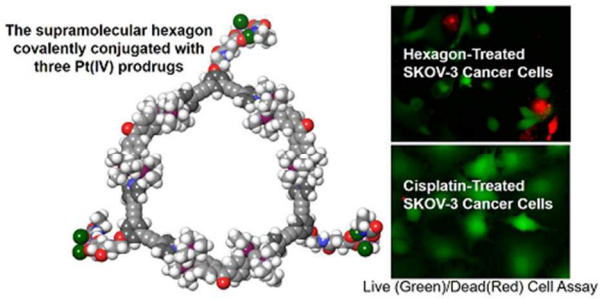

A supramolecular hexagon obtained from coordination-driven self-assembly can be used to deliver platinum-based anticancer agents and promote their therapeutic index