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Novel molecular drug carrier: encapsulation of oxaliplatin in cucurbit[7]uril and its effects on stability and reactivity of the drug†

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Oxaliplatin forms a stable 1 : 1 inclusion complex with cucurbit[7]uril as indicated by NMR, mass spectrometry, isothermal titration calorimetry and X-ray crystallography. The encapsulation of the drug results in a large enhancement in stability, a moderate decrease in reactivity toward guanosine but a much larger decrease in reactivity toward L-methionine, which suggests the encapsulation not only increases the stability of the drug but also may reduce unwanted side effects caused by protein binding of the platinum drug. A preliminary *in vitro* assay using various tumor cell lines reveals that the encapsulation results in a decrease in the antitumor activity of oxaliplatin.

Introduction

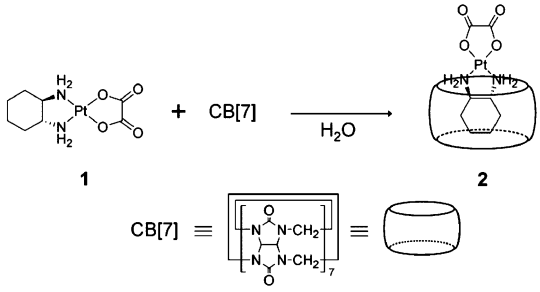
Molecular containers, rigid molecules with large cavities in which small molecules can be encapsulated, are of much interest because of their potential applications in separations, catalysis, sensors and drug delivery.[1] Among many synthetic receptors, cyclodextrins (CDs) and crown ethers have been extensively explored as drug carriers with the aim of enhancement of the solubility, stability, and bioavailability of drug molecules.[2] However, the quest for more efficient and safer drug carriers continues.

Cucurbit[6]uril (CB[6]), a macrocyclic cavitand comprising six glycoluril units, has a hydrophobic cavity accessible through two identical carbonyl-fringed portals. It has been widely used as a synthetic receptor[3,4] and as a building block for supramolecular assemblies.[5] Recent syntheses[6] of cucurbituril homologues, cucurbit[*n*]uril (CB[*n*], $n = 5$, 7, and 8) containing five, seven and eight glycoluril units, respectively, have widened the scope of cucurbituril chemistry.[7,8] Among them, CB[7] has a cavity large enough to form stable 1 : 1 inclusion complexes with 2,6-bis(4,5-dihydro-1*H*-imidazol-2-yl)naphthalene,[6] methyl-viologen,[7e] ferrocene,[8f,9] aminoadamantane,[7a] and carborane.[8b] Unlike CB[6] or CB[8], which are sparingly soluble in water, CB[7] has a moderate solubility in water ($2–3 \times 10^{-2}$ M), which is comparable to that of β-CD (1.6×10^{-2} M).[7a] The moderate solubility in water as well as cavity size of CB[7] prompted us to study its inclusion of drugs and explore its potential as a drug carrier. We first decided to study inclusion of platinum anticancer drugs[10] including cisplatin[10a] and oxaliplatin[10c,11] which has recently been approved by the US FDA for the treatment of advanced colorectal cancer.[12] Preliminary results have been disclosed in patent literatures.[13] When this work was near completion, a report on a dinuclear platinum complex encapsulated in CB[7] and its cytotoxicity was published.[14] Herein we report a detailed study of the inclusion complex of oxaliplatin and CB[7], its reactivities toward guanosine and L-methionine, and its antitumor activity toward various tumor cell lines. The potential of CB[7] as a drug carrier is also discussed.

† Electronic supplementary information (ESI) available: experimental procedure of ITC, NMR experiment, and characterization data of **2**. See http://www.rsc.org/suppdata/ob/b5/b504487a/

Results and discussion

Addition of 1 equiv of **1** into CB[7] in water results in the exclusive formation of inclusion complex **2** (Scheme 1) as indicated by ^{1}H NMR spectroscopy. All the peaks for the cyclohexyl ring protons of the guest are shifted upfield relative to those of the free guest, **1**, indicating that the cyclohexyl ring is now located inside the cavity of CB[7] (Fig. 1). The splitting of the CB[7] peaks into two sets indicates that the environment around the two portals is no longer equivalent upon



Scheme 1 Formation of inclusion complex **2** by mixing an equimolar amount of oxaliplatin **1** and CB[7] in water.

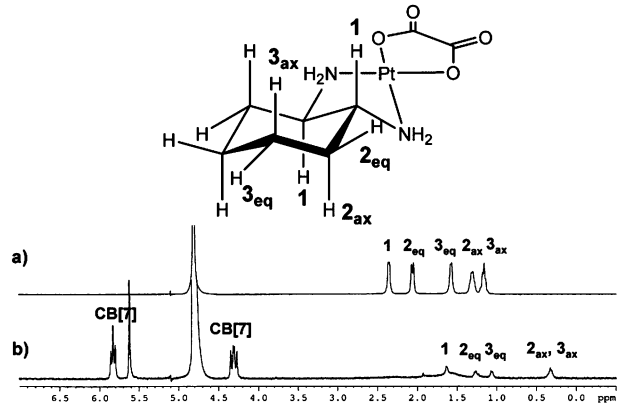


Fig. 1 Comparison of the ^{1}H NMR spectra in D_2O of (a) guest **1** and (b) inclusion complex **2**.

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encapsulation of oxaliplatin. The signal integration confirms a 1 : 1 stoichiometry of the host and guest. The formation of the 1 : 1 complex **2** has further been confirmed by ESI mass spectrometry.

Thermodynamic parameters associated with the inclusion phenomenon have been determined by isothermal titration calorimetry (ITC) (Fig. S1, ESI†). The association constant measured in TRIS buffer (pH = 7.2) is 2.3×10^5 M^{-1} which is similar to those for other guest molecules[7a] known to bind to CB[7]. The enthalpy and entropy changes ($\Delta H^\circ = -6.3$ (± 0.1) kcal mole^{-1}, and $\Delta S^\circ = 3.3$ eu) indicate that the inclusion process is driven not only by enthalpy but also by entropy. Such a positive entropy change associated with inclusion is in general rare,[15] but has often been observed in guest inclusion in CB[*n*].[7e,7f]

To investigate the kinetic stability of the inclusion complex in water, a 2D exchange NMR (2D EXSY) study was carried out (Fig. S2, ESI†). When 1.5 equiv of **1** were added to CB[7], two sets of the cyclohexyl ring peaks, one for free **1** and the other for **2** were observed at 273 K although the peaks are broad owing to exchange of the guests inside and outside the CB[7] cavity. The exchange rate constant k was estimated to be 130 s^{-1} at 273 K from 2D EXSY NMR data.[16] This result indicates that despite the high binding constant, the guest Pt complex can be slowly released from the CB[7] host.

For a better understanding of the inclusion phenomenon, the X-ray crystal structure of the inclusion complex **2** was determined (Fig. 2). Crystals of **2** suitable for X-ray work were obtained by heating a mixture of **1** and CB[7] in water at 100 °C in a sealed tube for 1 day followed by slow cooling to room temperature for 1 day. The crystal structure of **2** reveals three independent **2** supermolecules in the asymmetric unit, the structures of which are essentially the same. Most notably, the diaminocyclohexyl ligand of the oxaliplatin guest is embedded in the CB[7] host cavity, which is consistent with the solution structure suggested by NMR spectroscopy. The amine nitrogen atoms of the guest essentially lie on the plane made by seven oxygen atoms of the CB[7] portal while forming hydrogen bonds with the oxygen atoms, and the platinum atom is 1.53 (12) Å (on average) above the oxygen plane. Note that the oxalate group is pointing away from the CB[7] portal. The synergistic effect of the hydrogen bonds and hydrophobic interactions appears to be responsible for the remarkable stability of **2** in aqueous solution.

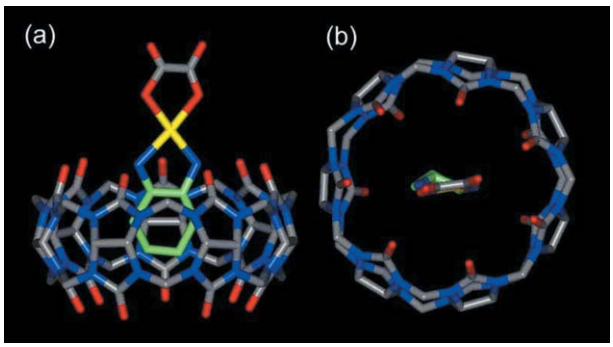

Fig. 2 X-Ray crystal structure of **2**: (a) side view and (b) top view. Only one of the three independent supermolecules in the asymmetric units is shown. The structures of the other two are essentially the same. Hydrogen atoms and solvent (H_2O) were omitted for clarity.

The stability of **1** is dramatically enhanced by forming the inclusion complex with CB[7]. It is known that once dissolved in water, **1** is stable for only 6 h at room temperature, and for 24 h under refrigeration.[11] In contrast, the inclusion complex **2** is stable even at a high temperature and pressure under which the crystals of **2** were grown for X-ray work. On a shelf, a white crystalline powder of **2** retains its original color for more than one year, while **1** changes its color in a much shorter period of time even in the dark.

To understand the effect of encapsulation of oxaliplatin in CB[7] on its binding to DNA, its reactivity toward guanosine (G) was investigated by ^{1}H NMR spectroscopy. An initial study on the reaction between **1** and G at 0.9 mM revealed that the peak at 8.2 ppm corresponding to the C8 proton of unreacted guanine base decreases while a new peak at 8.5 ppm grows (Fig. S3a, ESI†), which is consistent with binding of oxaliplatin to N7 of G. The reactivity of **1** toward G is almost the same as the one reported by a HPLC-MS study.[17b] However, the limited solubility of **2** in water (~0.1 mM) forced us to study the reactivities of **1** and **2** at a much lower concentration. The reaction was thus monitored at 310 K for 6 h after mixing **1** or **2** (0.1 mM) with G in a 1 : 1 ratio (Figs. 3a and 3b). Despite low signal-to-noise ratio, the NMR spectra (Fig. 3b) clearly showed upfield-shifted proton signals of the cyclohexyl ring of oxaliplatin (0–2 ppm), indicating that **2** retains CB[7] during the reaction. The change in concentration of unreacted G was calculated from the decrease of the peak at 8.2 ppm as the reaction proceeded. After 6 h, 50% of unreacted G was observed with **1**, whereas 72% of unreacted G remained with **2**, which indicates that the reactivity of **2** toward G is lower than that of **1** by a factor of 2–3 as judged by their second order rate constants (1.7 ± 0.3 mM^{-1} h^{-1} for **1** and 0.6 ± 0.1 mM^{-1} h^{-1} for **2**). Hence it is expected that the cytotoxicity of **1** may decrease slightly upon encapsulation of CB[7].

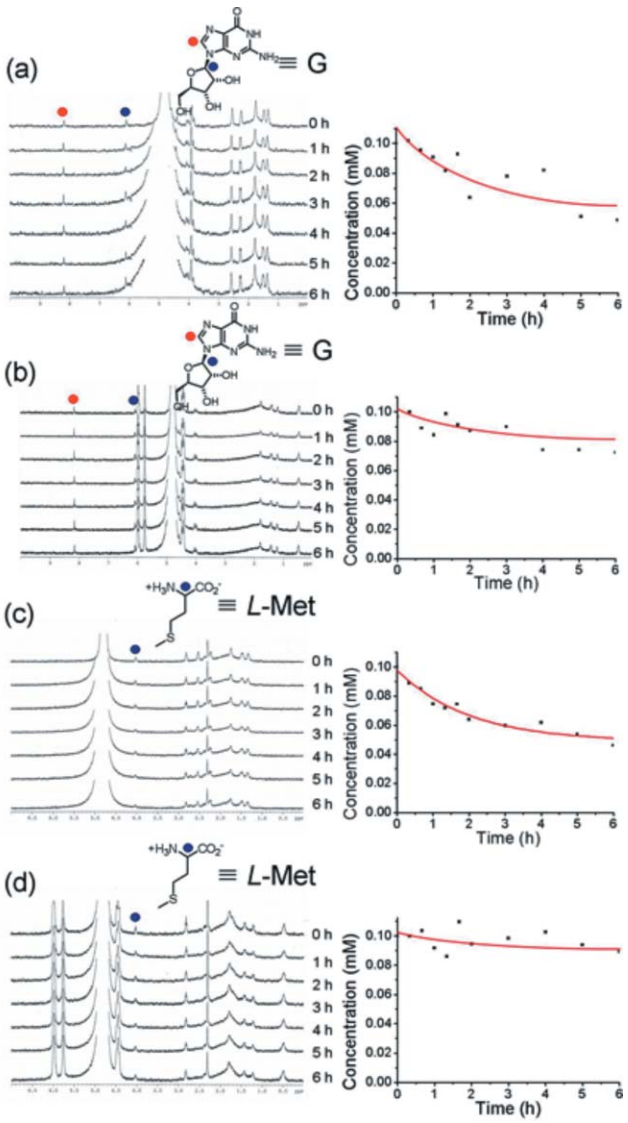

Fig. 3 Progression of ^{1}H NMR spectra (left) and concentration of unreacted G or L-Met (right) with time: (a) **1** and G and (b) **2** and G (c) **1** and L-Met and (d) **2** and L-Met in D_2O.

We then decided to investigate the reactivities of **1** and **2** toward L-methionine (L-Met), a model compound for sulfur

Table 1 Comparison of the growth-inhibitory activities (ED_{50}/M) of cisplatin, **1, 2** and CB[7][a]

Cell line[b]	Cisplatin	**1**	**2**	CB[7]
A549	1.2	0.4	2.4	>100
SKOV-3	2.9	0.7	4.9	>100
SKMEL-2	3.1	1.0	18.7	>100
XF-498	0.5	0.5	4.9	>100
HCT-15	1.3	0.9	14.3	>100

[a] Growth-inhibitory activity assessed by the SRB (sulforhodamine B) assay. [b] A549 human non-small cell lung; SKOV-3 human ovarian; SKMEL-2 human melanoma; XF-498 human CNS; HCT-15 human colon.

containing proteins which are thought to be responsible for appreciable side effects, mainly nephrotoxicity and ototoxicity, caused by Pt–S bond formation.[17] The reactivities of **1** and **2** toward L-Met were tested under the same conditions as the above experiments involving G (Figs. 3c and 3d). After 6 h, 55% of L-Met was reacted with **1**, whereas only a small decrease (~10%) in L-Met was observed in the case of **2**; the reactivity of **2** toward L-Met is thus lower than that of **1** by a factor of ~15 as estimated by their second order rate constants (1.7 ± 0.2 mM^{-1} h^{-1} for **1** and 0.1 ± 0.1 mM^{-1} h^{-1} for **2**). The lower reactivity of **2** toward G and L-Met compared with **1** apparently arises from the bulkiness of CB[7] encapsulating oxaliplatin, but why the encapsulation affects the binding of the drug to L-Met more than to G is not clear at the moment. Nevertheless, this result suggests that encapsulation of oxaliplatin with CB[7] may reduce unwanted side effects caused by protein binding of the platinum compound.

Finally, to investigate the effect of the encapsulation of the drug in CB[7] on cytotoxicity, a preliminary *in vitro* assay of **1, 2** and cisplatin was carried out against various cell lines including A549 human non-small cell lung, and SKOV-3 human ovarian. The result shows that **2** is less cytotoxic than **1** or cisplatin against all the tumor cell lines (Table 1). Upon encapsulation, in general, **1** loses its antitumor activity by about five-fold in some cases, and more than ten-fold in other cases. No decrease or moderate decrease in activity by encapsulation of CB[7] has been reported for a dinuclear platinum complex.[14] At the moment, the origin of the much lower activity of **2** is not clear, but we cannot rule out the possibility of significantly reduced cellular uptake as a result of encapsulation. Note that CB[7] itself has no cytotoxic effect on the cell lines. Further investigations on the *in vitro* and *in vivo* antitumor activities of **2** are in progress.

In conclusion, we have examined CB[7] as a potential drug carrier. CB[7] forms a stable 1 : 1 inclusion complex with oxaliplatin in aqueous solution. The encapsulation of the drug results in a large enhancement in stability, a moderate decrease in reactivity toward G but a much larger decrease in reactivity toward L-Met, which suggests that the encapsulation not only increases the stability of the drug but also may reduce unwanted side effects caused by protein binding of the platinum drug. We are currently investigating encapsulation of other drugs in CB[7] and their pharmacological activities.

Experimental

Materials

All the chemicals were purchased from commercial sources and used without further purification. Cucurbit[7]uril was produced according to the method that we reported previously.[6] Oxaliplatin was prepared according to the literature.[12] ^{1}H NMR spectra were recorded on a Bruker DRX 500 spectrometer operating at 500.23 MHz for ^{1}H. ESI-mass spectrometry was performed on a JEOL JMS-700T mass spectrometer.

Synthesis of inclusion complex 2

A mixture of oxaliplatin (3.0 mg, 7.64 μmol) and cucurbit[7]uril (9.0 mg, 4.3 μmol) in 10 mL of water was heated at 100 °C in a teflon-lined hydrothermal reactor. After slowly cooling to room temperature, the crystalline product **2** was collected by filtration and dried in air (4.5 mg, 67%). ^{1}H NMR (500 MHz, D_2O, 25 °C) $\delta = 5.82$ (14H, dd, CB[7]), 5.54 (14H, s, CB[7]), 4.30 (14H, dd, CB[7]), 1.56 (4H, br, cyclohexane), 1.24 (2H, m, cyclohexane), 1.07 (2H, m, cyclohexane), 0.34 (2H, m, cyclohexane). ESI-MS: m/z 786.7 $[M + 2\ Li]^{2+}$, 823.3 $[M + 2\ Li + DMF]^{2+}$, 1566.5 $[M + Li]^+$, 1700.5 $[M + Li + LiI]^+$, 1834.4 $[M + Li + 2\ LiI]^+$, 1968.4 $[M + Li + 3\ LiI]^+$. Elemental analysis calcd for $[(C_8H_{14}N_2O_4Pt)\cdot(C_{42}H_{42}N_{28}O_{14})]\cdot 8H_2O$: C 34.87; H 4.33; N 24.40; found: C 34.47; H 4.43; N 24.65%.

ITC experiment

Isothermal titration calorimetric experiments were performed on a Microcal® microcalorimeter. Freshly prepared oxaliplatin (20 mM) was dissolved in TRIS buffer (pH = 7.2) and titrated with CB[7] (1.0 mM) in TRIS buffer (pH = 7.2) at 25 °C (Fig. S1, ESI†).

X-Ray crystallographic study

Crystals of **2** suitable for X-ray work were obtained by heating a mixture of **1** and CB[7] in water at 100 °C in a sealed tube for 1 day followed by slow cooling to room temperature for 1 day. The data collection was performed on a Siemens SMART CCD diffractometer with MoKα radiation ($\lambda = 0.71073$ Å). After the data integration (SAINT) and semi-empirical absorption correction based on equivalent reflections (SADABS), the structure was solved by direct methods and subsequent difference Fourier techniques (SHEXLTL). The asymmetric unit contains three independent **2** supermolecules. Two of them behave well, whereas the oxaliplatin guest molecule in the remaining supermolecule is disordered. A two-site disorder model was employed and the site occupancy was refined to be 0.545 and 0.455. All the non-hydrogen atoms were refined anisotropically except the disordered C, N, and O atoms which were refined isotropically. Positions of hydrogen atoms were calculated and their contributions were included except those of water molecules. Crystal data of **2**. $C_{50}H_{82}N_{30}O_{31}Pt$, $M = 1794.55$, orthorhombic, $a = 23.6543(5)$, $b = 30.23670(10)$, $c = 31.2827(6)$ Å, $U = 22374.3(6)$ Å^3, $T = 223(2)$ K, space group $P2_12_12_1$ (no. 19), $Z = 12$, μ(Mo–Kα) = 1.986 mm^{-1}, 89939 reflections measured, 35014 unique ($R_{int} = 0.0993$) which were used in all calculations. The final $R1$ ($I > 2\sigma(I)$) = 0.0827, $wR(F^2) = 0.2162$ (all data).

Reactivity study with guanosine and methionine

^{1}H NMR spectroscopy was used to monitor the reaction between **1** or **2**, and G or L-Met. In initial studies, L-Met or G (45 mM, 20 μL) and **1** (0.9 mM, 1000 μL) were mixed in an NMR tube and NMR spectra were recorded every 1 h over 6 h. The reaction between **1** and G was monitored by the decrease in the peak at 8.2 ppm corresponding to the C8 proton of unreacted guanine base (Fig. S3a, ESI†). Similarly, the reaction between **1** and L-Met (0.9 mM) was monitored by the decrease in the peak at 4.1 ppm (Fig S3b, ESI†). However, the limited solubility of **2** in water (~0.1 mM) forced us to study the reactivities of **1** and **2** at a much lower concentration. Thus G or L-Met (5 mM, 20 μL) and **1** or **2** (0.1 mM, 1000 μL) were mixed in an NMR tube and NMR spectra were recorded every 1 h over 6 h at 310 K (Fig. 3). The reactions were monitored by the decrease in the characteristic signals of G or L-Met as described above. However, due to a low signal-to-noise ratio, the quality of the data was not as good as the one measured with 0.9 mM solutions, but the trend was

the same. Despite some scattered points, the data were fitted to second order reaction kinetics, and second order rate constants were calculated using the following equation:

$$1/[C] = 1/[C]_0 + kt$$

where $[C]$ is the concentration, $[C]_0$ is the initial concentration of G and L-Met, and k is the second-order rate constant.

In vitro antitumor activity assay

An *in vitro* antitumor activity assay of cisplatin, **1, 2**, and CB[7] was performed by a SRB (sulforhodamine B) assay method at the Pharmaceutical Screening Laboratory of the Korea Research Institute of Chemical Technology (KRICT, Daejon, Korea).

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