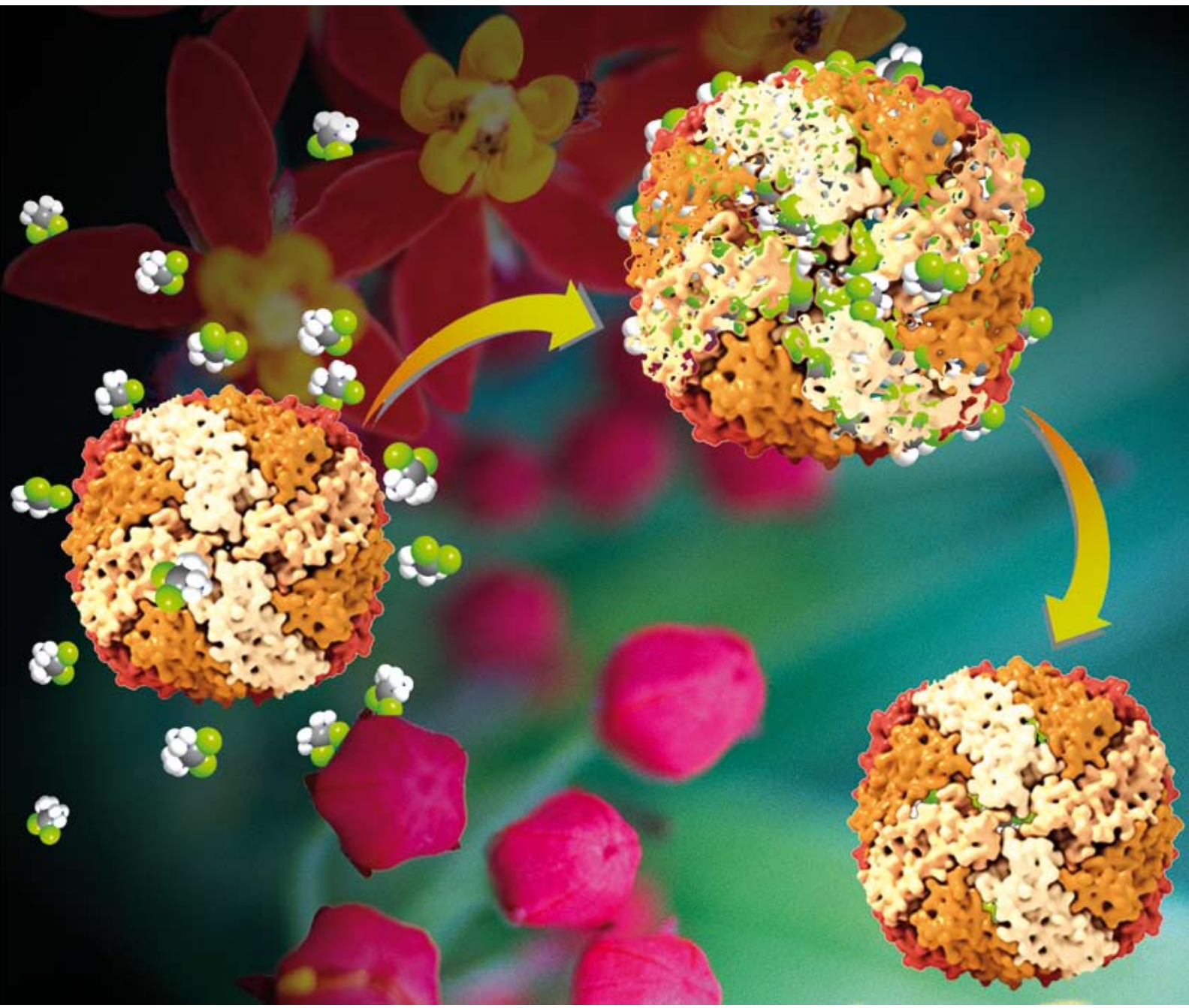
ChemComm

Chemical Communications

www.rsc.org/chemcomm

Number 33 | 7 September 2007 | Pages 3409–3500



ISSN 1359-7345

RSC Publishing

1359-7345(2007)33;1-3

Encapsulation of platinum anticancer drugs by apoferritin†

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Received (in Cambridge, UK) 10th April 2007, Accepted 9th May 2007
First published as an Advance Article on the web 17th May 2007
DOI: 10.1039/b705326f

Apoferritin derived from the native protein ferritin was employed to encapsulate anticancer drugs cisplatin and carboplatin.

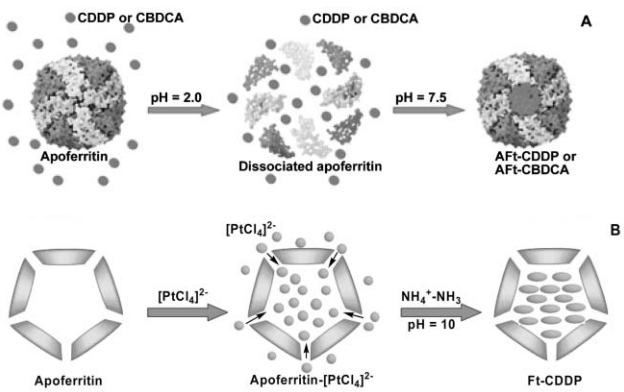
Cisplatin (CDDP) and carboplatin (CBDCA) are two major anticancer drugs used worldwide for the treatment of various malignancies.[1] However, their clinical applications are largely restricted by both high toxicity and severe tumor resistance.[2] Improving the tumor-selectivity of CDDP and CBDCA may mitigate the toxic effects and enhance the therapeutic index of these drugs. However, to develop a tumor-specific drug delivering system is quite challenging. The realization of this intention is largely dependent on the finding of suitable targeting carriers. In the past few years, ligand-receptor-mediated delivery systems have received major attention because of their non-immunogenic and site-specific targeting potential to the ligand-specific bio-sites.[3]

The native iron-storage protein ferritin (Ft) could be a promising vehicle for targeted drug delivery. It was discovered that ferritin could be internalized by some tumor tissues, and the internalization was associated with the membrane-specific receptors.[4] Ferritin-binding sites[5] and the endocytosis of ferritin have been identified in neoplastic cells.[6] In fact, ferritin receptors have shown potential values in the delivery of anticancer drugs into the brain.[7] The 24 subunits of ferritin assemble automatically to form a hollow protein cage with internal and external diameters of 8 and 12 nm, respectively. Eight hydrophilic channels of about 0.4 nm, formed at the intersections of subunits, penetrate the protein shell and lead to the protein cavity.[8] A variety of foreign species such as gadolinium (Gd^{3+}) contrast agents,[9] desferrioxamine B,[10] metal ions,[11] and nanoparticles of iron salts[12] can be accommodated in the cage of apoferritin (AFt).

This paper describes two different processes of generating CDDP and CBDCA loaded AFt (AFt-CDDP or AFt-CBDCA, Scheme 1). The protein coated drugs are expected to improve the toxicity profiles of the naked ones and finally lead to a novel strategy to overcome the detrimental effects of platinum-based drugs.

AFt-CDDP and AFt-CBDCA were first prepared by a procedure used for trapping neutral molecules.[13] It consists of the dissociation of AFt into the subunits at pH 2.0 and the subsequent reformation at pH 7.5, thereby trapping CDDP or CBDCA within the cavity of AFt (Scheme 1A).‡ The initial concentrations of AFt, CDDP and CBDCA were 1×10^{-5} mol L^{-1}, 2×10^{-3} mol L^{-1} and 3×10^{-2} mol L^{-1}, respectively. After the pH-induced unfolding–refolding process, AFt-CDDP and AFt-CBDCA were separated from the bulk CDDP and CBDCA solutions by exhaustive dialysis. The concentration of the drug loaded AFt was 5×10^{-6} mol L^{-1} after this process.

The encapsulation of CDDP and CBDCA was confirmed firstly by ICP-AES. It was determined that *ca* 2 CDDP or 5 CBDCA molecules could be entrapped in each AFt molecule. The encapsulation was further confirmed by NMR spectroscopy using ^{15}N-labelled drugs (AFt-^{15}N-CDDP and AFt-^{15}N-CBDCA) instead of CDDP and CBDCA. A cryo-probe, with significantly enhanced sensitivity compared to traditional probes, enabled us to detect AFt-^{15}N-CBDCA at a concentration of *ca* 25 μM by 2D [^{1}H, ^{15}N] HSQC technique. As shown in Fig. 1A, three cross-peaks were observed in the spectrum. The major cross-peak (peak **a**) appeared at 4.20/−80.1 ppm was almost identical to that of ^{15}N-CBDCA in aqueous solution under similar conditions,[14] indicating that ^{15}N-CBDCA was entrapped in AFt and exists primarily in its intact form. Interestingly, two minor cross-peaks at 4.08/−86.7 ppm (peak **b**, 5%) and 3.95/−83.0 (peak **c**, 9%) were also observed in the spectrum. These peaks are shifted to higher field in both ^{1}H and ^{15}N dimensions compared to that of free ^{15}N-CBDCA. It is known that CBDCA can undergo a chelate ring-opening process in acidic solution,[15] which may result in **b** and **c**. The significant high-field shifting of the cross-peaks may be due to the interaction of hydrolysed ^{15}N-CBDCA molecules with the interior surface of Aft, which could exert strong shielding effects on



Scheme 1 Schematic representation of different ways to generate CDDP or CBDCA loaded apoferritin: A, unfolding-refolding method; B, *in situ* generation method.

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† Electronic supplementary information (ESI) available: experimental details, TEM images of Ft-Pt, 2D [^{1}H, ^{15}N] HSQC NMR spectrum, cytotoxicity data. See DOI: 10.1039/b705326f

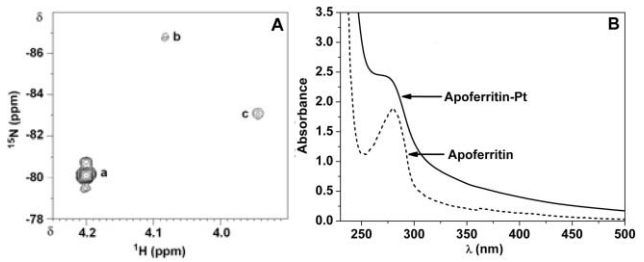

Fig. 1 The 2D [^{1}H, ^{15}N] HSQC NMR spectrum of AFt-^{15}N-CBDCA (5×10^{-6} M) at pH 5.2 and 298 K (A) and UV-vis spectra of apoferritin and apoferritin-Pt (B).

the $^{15}NH_3$ groups. The Ft-^{15}N-CDDP sample was also prepared using the same procedure. However, no signals can be detected by the [^{1}H, ^{15}N] NMR probably due to less ^{15}N-CDDP trapped in AFt (2.5 times less than CBDCA) and much faster hydrolysis of ^{15}N-CDDP than ^{15}N-CBDCA.

The AFt-CDDP was also prepared by an *in situ* procedure shown in Scheme 1B. Specifically, excessive K_2PtCl_4 (500 : 1, mol/mol) was added slowly to the solution of AFt (pH 8.5) to afford a homogeneous pale red aqueous solution containing AFt-$[PtCl_4]^{2-}$. AFt-CDDP was obtained after addition of NH_4^+–NH_3 buffer solution (0.3 mol L^{-1}, pH 10) to the Ft-$[PtCl_4]^{2-}$ solution (1 : 1, vol/vol). The entrapment of $[PtCl_4]^{2-}$ in AFt was confirmed by transmission electron microscopy (TEM),§ where reduced discrete spherical Pt particles with relatively homogeneous size of 2–3 nm were observed (see ESI†). The UV-vis spectrum of the AFt-Pt solution showed a shoulder-band at 280 nm due to the encapsulation of Pt particles which were formed by reduction of $[PtCl_4]^{2-}$. This is significantly different from that of AFt (Fig. 1B) as observed similarly for AFt loaded with other noble metal clusters.[16] The formation of CDDP was confirmed by a control experiment with the aid of 2D [^{1}H, ^{15}N] HSQC NMR technique (see ESI†).¶ On the basis of the ICP-AES and BCA protein assays (BCA™ Protein Assay Kit, Pierce), it is estimated that each AFt contains about 30 equiv. of Pt–NH_3 complex units, amongst which 15 equiv. are CDDP molecules (Table 1). Although this method generated a mixture of AFt-CDDP and Ft-TDDP (Ft-transplatin), it is much more efficient than the unfolding–refolding method in Scheme 1A. It is known that TDDP possesses little antitumor activity and has no toxicity at the same concentration of CDDP.[17]

To confirm the stability and the structural integrity of AFt-CDDP and AFt-CBDCA as described in Scheme 1, a native polyacrylamide gel electrophoresis (PAGE) experiment was carried out and the result was compared with that obtained by the normal sodium dodecyl sulfate (SDS) method.‖ Both AFt-CDDP and AFt-CBDCA showed a band at *ca* 440 kDa, indicating the samples contain AFt (*ca* 440 kDa) and the protein cage remains unchanged or well-folded after drug being loaded (Fig. 2).

Table 1 The Pt concentrations in the AFt-[Pt–NH_3] solutions [a] and the amount of Pt or CDDP in apoferritin[b,c]

Sample	Pt (μg mL^{-1})[a]	Pt/AFt (mol/mol)[b]	CDDP/AFt (mol/mol)[c]
1	60.5	30	15
2	60.0	30	15
Average	60.3	30	15

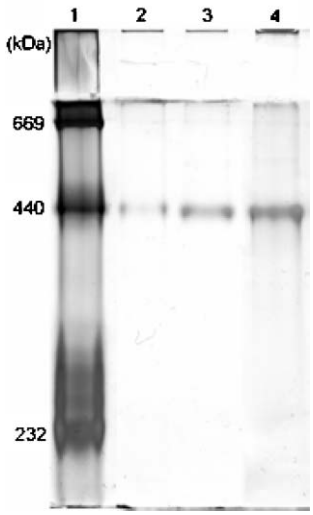

Fig. 2 The PAGE image of marker (lane 1), AFt-CBDCA (lane 2), Ft-CDDP (lane 3), and AFt-Pt-NH_3 complexes (lane 4) under native conditions (~8 h, silver-stained).

The cytotoxic activity of AFt-CDDP and AFt-CBDCA was preliminarily tested against the rat pheochromocytoma cell line (PC12) by MTT assay (see ESI†),[18] using AFt as a control. AFt-CDDP prepared *via* Scheme 1B was found to decrease the viability of PC12 cells at a concentration significantly lower than that needed for AFt. The inhibition effect of AFt-CDDP and AFt-CBDCA prepared by the Scheme 1A method was less pronounced, which may be due to the amount of drugs capsulated by this method being much less than that by the Scheme 1B method. Because the amounts of drugs released from Ft-CDDP and AFt-CBDCA are unknown, a direct comparison with free CDDP and CBDCA is not possible in this case.

Various macromolecular prodrugs have shown superior efficacy in preclinical models relative to their low-molecular-weight parent compounds.[19] In this paper, we have demonstrated that cisplatin (CDDP) and carboplatin (CBDCA) can be encapsulated in the cavity of apoferritin (AFt) and the drug-loaded protein has the potential to exert cytotoxic effect on tumor cells. Since the binding sites and endocytosis of ferritin have been identified in tumor cells, the involvement of AFt in CDDP and CBDCA delivery is likely to enhance their selectivity for those cell surfaces that express ferritin receptors. Chemical modification of native proteins is usually needed for efficient drug loading,[20] which undermines their affinity for cellular targets.[21] In this study, the protein shell of Ft-CDDP and Ft-CBDCA remains intact as it is in AFt, thus its potential recognition nature should not be affected. This approach may be further developed into a promising strategy for targeted delivery of CDDP and CBDCA.

We thank the National Natural Science Foundation of China (no. 20631020) and the Natural Science Foundation of Jiangsu Province (no. BK 2005209) for financial support.

Notes and references

‡ AFt was obtained from the horse spleen ferritin (85 mg mL^{-1}, 0.15 mol L^{-1} NaCl/H_2O, Sigma) by demineralization as reported in the literature (S. Stefanini, ref. 22).
§ The AFt-Pt sample used for the TEM (400 kV) was prepared by reducing the AFt-$[PtCl_4]^{2-}$ solution with a $NaBH_4$ solution (50 μmol, H_2O, 100 μL)

for 8 h at 8 °C. The Pt precipitate outside of ferritin was removed by centrifuging and dialysis (0.15 mol L^{-1} NaCl/H_2O, 24 h).

¶ A K_2PtCl_4 solution was treated with equal volume of $^{15}NH_4^+$–$^{15}NH_3$ buffer (pH 10) under the same conditions as in the presence of AFt. The products were examined by 2D [^1H, ^{15}N] HSQC NMR spectroscopy. The yielding of Pt–NH_3 complexes was thus confirmed and the major products were proved to be CDDP and TDDP, with a ratio of *ca* 1 : 1 by integration of the corresponding peaks (P. J. Sadler *et al.*[23]).

‖ Non-denaturing PAGE was performed according to a literature method, calibrated with high-molecular-mass native markers (Amersham Biosciences), and silver-stained (J. E. Coligan *et al.*, ref. 24).

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