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PAPER

In situ analysis of cisplatin binding to DNA: the effects of physiological ionic conditions†

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Platinum-based anti-cancer drugs form a major family of cancer chemotherapeutic agents. Cisplatin, the first member of the family, remains a potent anti-cancer drug and exhibits its clinical effect by inducing local DNA kinks and subsequently interfering with DNA metabolism. Although its mechanism is reasonably well understood, effects of intracellular ions on cisplatin activity are left to be elucidated because cisplatin binding to DNA, thus its drug efficacy, is modified by various ions. One such issue is the effect of carbonate ions: cisplatin binding to DNA is suppressed under physiological carbonate conditions. Here, we examined the role of common cellular ions (carbonate and chloride) by measuring cisplatin binding in relevant physiological buffers *via* a DNA micromanipulation technique. Using two orthogonal single-molecule methods, we succeeded in detecting hidden monofunctional adducts (kink-free, presumably clinically inactive form) and clearly showed that the major effect of carbonates was to form such adducts and to prevent them from converting to bifunctional adducts (kinked, clinically active). The chloride-rich environment also led to the formation of monofunctional adducts. Our approach is widely applicable to the study of the transient behaviours of various drugs and proteins that bind to DNA in different modes depending on various physical and chemical factors such as tension, torsion, ligands, and ions.

1. Introduction

Understanding the mechanism of the action of anticancer drugs is the first step towards development of improved drug agents for the treatment of cancer. Cisplatin, one of the most successful anticancer drugs, is believed to exert its cytotoxic effects by binding to DNA.[1–6] The interaction of cisplatin with DNA is a multi-stage process involving aquation of cisplatin, the pre-association of the aquated product with DNA, formation of a monofunctional adduct on DNA, and closure of the monofunctional adduct to a bifunctional adduct, which is the predominant end product from the reaction (Fig. 1).[7–9] Since cisplatin can react with a wide range of biologically common substances, each stage of the process can be controlled and modified by various ions that are present in blood and cells.[10–15] In particular, a series of recent studies have shown that carbonate ions at physiological concentrations greatly affect the speciation of cisplatin, which is important for cellular uptake, DNA binding, and the anti-tumor effects of the agent.[16–18]

However, the effect of carbonate ions still remains controversial. Binter *et al.* suggested that in carbonate buffer, the monofunctional cisplatin adduct is dominant over the bifunctional adduct based on their gel results.[19] Unlike the bifunctional adduct, the monofunctional counterpart, where cisplatin forms only one bond with DNA (and here forms the other bond with carbonate anions), cannot induce local kinking and unwinding of DNA (Fig. 1), and thus the gel mobility of a DNA duplex with monofunctional adducts would be similar to that of unplatinated DNA, and considerably larger than that of a DNA duplex with bifunctional adducts. They attributed the nearly unchanged gel mobility of the DNA in carbonate buffer to the dominant presence of the monofunctional adducts. In contrast, Todd *et al.* argued that carbonates do not affect the preferred bifunctional mode of cisplatin binding to DNA, but rather only suppress the overall binding efficiency.[20] Defining the prevalence of monofunctional adducts is an important issue because the adducts directly affect the anti-cancer activity of cisplatin. This controversy originated from the intrinsic difficulty in verifying the nature of cisplatin–DNA adducts. Furthermore, since platinum–carbonato (when an anion is bound to a metal ion, its name ends with 'o') complexes which

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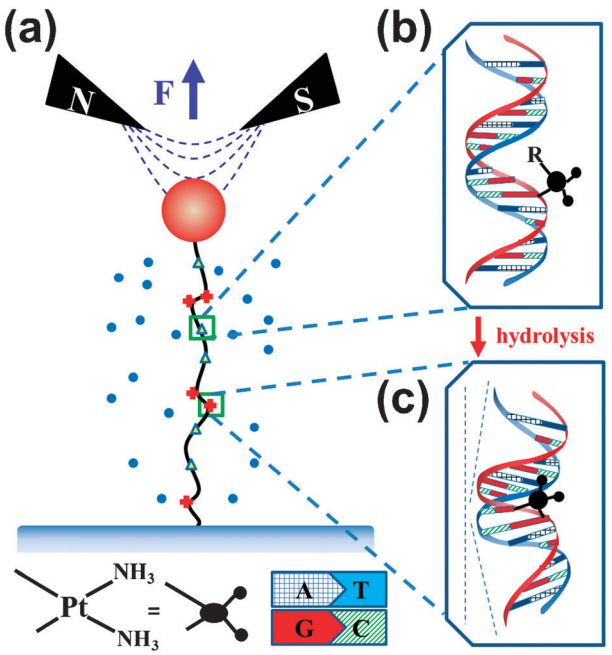

Fig. 1 (a) Schematic of monofunctional (blue triangles overlaid on DNA) and bifunctional (red crosses at the apex of kinked DNA) cisplatin adducts formed on a single dsDNA prepared in magnetic tweezers and blow-ups of (b) mono- and (c) bi-functional cisplatin–DNA adducts. Blue dots in (a) are free cisplatin. In (b), R is either CO_3 or Cl depending on whether monofunctional adducts are formed by a cisplatin–carbonato complex or mono-hydrolyzed cisplatin. As illustrated in the bottom of this figure, cisplatin is simplified as a ball with four arms in (b) and (c), and four bases are represented by four different shapes and colors: A (adenine: cross-hatching in blue), T (thymine: solid blue), G (guanine: solid red), and C (cytosine: oblique hatching in green).

form in carbonate buffer are in equilibrium with dissolved carbon dioxide gas, experimental approaches designed to identify the nature of cisplatin–DNA adducts in carbonate media should not disturb the carbonato–carbon dioxide equilibrium. Thus, without specific consideration of this subtle equilibrium issue, the past experimental validation or invalidation of the existence of mono-functional adducts would be disputable.[19,20]

Here, we demonstrated that the existence of the monofunctional mode can be revealed *via* a properly designed experimental approach under various salt conditions. We investigated the mono- and bi-functional modes of cisplatin–DNA adduct formation using the manipulation of single DNA molecules with magnetic tweezers and determined the degree of cisplatin binding to double-stranded DNA (dsDNA) in each mode. From this, we directly verified that monofunctional cisplatin–DNA adducts commonly exist under physiological carbonate buffer conditions. We also observed that high chloride conditions conferred a significant amount of mono-functional cisplatin adducts. In addition, our *in situ* method does not require lengthy purification of the reaction product, which could easily disturb the chemical conditions of the sample and consequently alter the population of species. Therefore the method can be used to measure the degree of cisplatin binding under constant, physiological buffer (carbonate or chloride) conditions *in situ*. This technical advantage over conventional methods renders our conclusion highly reliable.

2. Materials and methods

2.1 Sample preparation

Cisplatin and salts used in this study were all purchased from Sigma-Aldrich. After cisplatin was dissolved in purified water at 37 °C for 24 h (2 mg ml^{-1}) and subsequently diluted with either solutions containing various concentrations of NaCl or a carbonate solution, the final concentration of cisplatin was 1.65 mM or 500 μg ml^{-1} with the indicated concentrations of [NaCl] or with [$NaHCO_3$] = 24 mM. Our carbonate buffer contained 5 mM NaCl. The pH of the carbonate buffer was adjusted to 7.4 by adding a small volume (a few μl) of 60% nitric acid. Or we also adjusted the buffer pH with hydrochloric acid and obtained similar results as shown in Table S1 (ESI†).

The ~15 kb DNA molecule used in our experiments was prepared as described elsewhere.[21] A ~14.8 kb DNA fragment obtained by digesting a plasmid with BamHI and SalI restriction endonucleases (New England Biolabs, Inc.) was ligated to two DNA linkers (~500 bp), each of which was obtained by digesting PCR products modified by either biotin-16-dUMP or digoxigenin (Dig)-16-dUMP residue (Roche Molecular Biochemicals). The ends of the DNA molecule were then attached to a streptavidin-coated magnetic bead (1 μm diameter, MyOne, Dynal, Invitrogen) and an anti-digoxigenin-coated glass surface, respectively. The DNA tether molecule was then used for DNA manipulation experiments.[22]

2.2 DNA manipulation measurements with magnetic tweezers

The detailed description of our magnetic tweezers setup was given in ref. 21, so the essential information will be summarized here. Magnetic-bead-bound DNA tethers were pulled by a pair of magnets. In our setup, the magnets can exert a force up to ~6 pN to a 1 μm sized magnetic bead. To measure the force to the beads, we utilized Brownian fluctuations of the beads monitored at 20 Hz in real time by a CCD camera (Sensicam em, PCO). The force exerted on a DNA molecule was calculated by the usual equipartition theorem, $F = k_BTl/\langle x^2\rangle$, where k_BT is the thermal energy, l the extension of DNA tethers, and $\langle x^2\rangle$ the lateral fluctuations of the bead. The vertical position of the bead necessary to determine the extension (l) of the DNA molecule was obtained by analyzing the diffraction ring pattern of the bead. By measuring DNA extensions under various tensional forces, we obtained the force–extension data of DNA. In order to characterize the force–extension behavior and thus determine the persistence length of DNA, we used the Worm-Like Chain (WLC) model given by $F\xi/k_BT = l/L + 1/(4(1 - l/L)^2) - 1/4$, where ξ is the persistence length (for bare DNA, it is about 50 nm) and L is the contour length of DNA.[23]

In DNA-twisting measurements, we first characterized each DNA tether molecule by measuring the persistence length to make sure that the DNA tether is normal and usable for further analysis. In this analysis, we measured a set of three σ-*vs.*-extension curves (σ is the superhelical density of DNA). The σ-*vs.*-extension curve for bare DNA has a maximum peak at $\sigma = 0$ at the tensional forces used in our experiments. First, a σ-*vs.*-extension curve was obtained for the bare DNA by measuring the extension for various σ values under constant force.

Then, the sample was incubated with 1.65 mM cisplatin dissolved in 24 mM carbonate buffer. After 30 min incubation, the sample chamber was washed with the same buffer minus cisplatin in order to remove any residual free cisplatin. Then, the second σ-*vs*.-extension curve was obtained. After washing the sample chamber with 10 mM NaCl, the last σ-*vs*.-extension curve was obtained. The three σ-*vs*.-extension curves were compared to find changes in peak positions.

2.3 Data analysis

The detailed description of estimation of the degree of cisplatin binding was given in ref. 24 and 25. In those papers, the kink angle was set to be $\theta_k = 40^\circ$ according to ref. 26. Here, we used a more recent, accurate value (32°) reported in ref. 27. Based on the new calculation, we drew a new graph relating the effective persistence lengths and the degree of cisplatin binding (p) (see Fig. S1, ESI†). The p values obtained thereby appear to be approximately 50% larger than the values which would be obtained by previously reported graphs in ref. 24 and 25. These values were then compared to the values deduced from DNA-twisting measurements.

3. Results

3.1 Formation of monofunctional cisplatin–DNA adducts in a physiological $NaHCO_3$ buffer

To resolve the controversy regarding the role of carbonate ions, we examined the effect of carbonate ions on cisplatin binding to DNA. To fulfill the task, we should be able to characterize the amounts of mono- and bi-functional adducts in a straightforward manner, especially, in the presence of buffering carbonates. Here, we utilized magnetic tweezers to access the degree of (bifunctional) cisplatin binding to DNA *via* elasticity measurements.[24,25,28–30] Bifunctional cisplatin–DNA adducts produce DNA kinks, which change the elastic properties of DNA. According to the WLC model, the elastic response of DNA can be characterized by a single parameter, persistence length (ξ), which is related to the bending modulus (A) of DNA by $\xi = A/k_BT$. At physiological salt concentrations ([NaCl] = 10 – 180 mM), the persistence length of dsDNA is typically $\xi_0 \approx 50$ nm (black lines in Fig. 2 and 3). The formation of bifunctional cisplatin adducts causes a reduction in the persistence length, which is manifested as a shift in the elastic response curve with respect to that of bare DNA. On the other hand, monofunctional adducts bring no noticeable changes in the elastic response of DNA. The analysis of such shifts in the elasticity measurement allowed us to quantitatively estimate the degree of cisplatin binding (p), that is, the ratio of bifunctionally bound platinum adducts to the number of base pairs of a DNA molecule.[24,25] In the low-tension regime (<0.4 pN), the reduced ξ reflects less fluctuation in the end-to-end length of DNA due to the inserted kinks. In the high tension regime (>0.4 pN), the kinks are aligned by the external tension and still store some chain length. Thus the chain extension is reduced accordingly with the increasing number of kinks and this behavior can be expressed as the reduced value of ξ of a worm-like chain.

In detail, the ξ values in the high- and low-tension regimes were independently and consistently related to the degree of cisplatin binding according to ref. 24 and 25. In the low tension regime, the effective persistence length ξ' is related to p by $\frac{1}{\xi'} = \frac{1}{\xi_o} + \frac{p}{a}(1 - \cos\theta_k)$, where a is the base pair distance (= 3.4 Å) and θ_k the kink angle. In the high tension regime, the effective persistence length ξ' is given by $\frac{1}{\xi'} = \frac{1}{\xi_o}(1 + \theta_k^2\frac{p\xi_o}{8a})^2$. Using this method, we can directly measure the amounts of bifunctional adducts. If monofunctional adducts are present in carbonate buffer, they can be readily converted to bifunctional adducts in a buffer favorable to cisplatin hydrolysis. Thus, if there is a difference between bifunctional adducts before and after the buffer exchange, the difference should be attributed to the amount of monofunctional adducts, which existed in the presence of carbonate buffer.

The total concentration of all carbonate species such as carbonate ions, bicarbonate, carbonic acid, and dissolved carbon dioxide in blood and cytosol is about 24 mM and 12 mM,

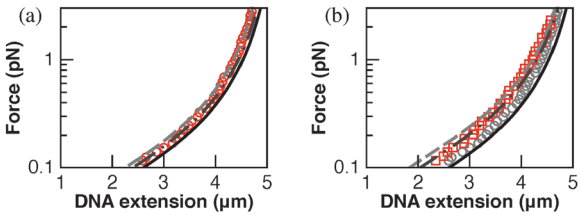

Fig. 2 Monofunctional cisplatin adducts in the presence of 24 mM $NaHCO_3$ and their conversion to bifunctional adducts. (a) The force–extension curves of a dsDNA molecule measured at $t = \sim 30$ min after the DNA was incubated with cisplatin in [$NaHCO_3$] = 24 mM (red circles). (b) After washing with [NaCl] = 10 mM, ξ dramatically decreased resulting in a large shift in the force–extension curve (red squares). Gray circles in the right panel indicate the same data shown in the left for easy comparison. Dot-dashed dark gray and dashed light gray lines are fits to the WLC model for the low and high tension regimes, respectively. Black lines represent bare DNA. The degrees of cisplatin binding were obtained according to ref. 24 and 25 and summarized in Table 1.

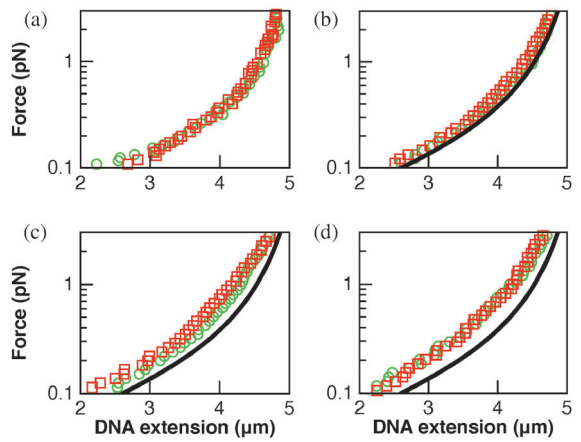

Fig. 3 The force–extension curves of platinated dsDNA molecules generated under various NaCl concentrations ([NaCl] = 180 mM (a), 120 mM (b), 90 mM (c), and 60 mM (d)). Green circles and red squares represent the curves before and after washing with 10 mM NaCl. Black lines represent bare DNA. The degrees of cisplatin binding under various experimental conditions were obtained according to ref. 24 and 25 and summarized in Table 1.

Table 1 The degree of cisplatin binding in percentage and the ratio of mono- and bi-functional cisplatin–DNA adducts at various ion concentrations. $p_{Bi,x}$ and $p'_{Bi,x}$ ($x = h$ or l) denote the degrees of cisplatin binding in the bifunctional mode before and after washing with [NaCl] = 10 mM, respectively, and the indices h and l indicate that the value was estimated from the high- and low-tension parts of the force–extension curve, respectively. Since $p_{Bi,h}$ and $p_{Bi,l}$ should be, in principle, the same, the nominal value (p_{Bi}) is given by the mean of $p_{Bi,h}$ and $p_{Bi,l}$. p'_{Bi} was obtained in a similar manner. p_{Mono} is the degree of monofunctional cisplatin binding prior to the washing step, which is $p'_{Bi} - p_{Bi}$. Errors in p are given in parentheses

Ion (mM)	$p_{Bi,l}$; $p_{Bi,h}$ (%)	p_{Bi}	$p'_{Bi,l}$; $p'_{Bi,h}$ (%)	p'_{Bi}	$p_{Mono} = p'_{Bi} - p_{Bi}$	p_{Mono}/p_{Bi}
$NaHCO_3$						
24	0.6; 0.8 (±0.1)	0.7	2.0; 2.5 (±0.1)	2.3	1.6	2.3
NaCl						
180	<0.15	<0.15	<0.3	<0.3	<0.3	NA
120	0.6; 0.6 (±0.1)	0.6	0.9; 1.2 (±0.1)	1.1	0.5	0.8
90	1.5; 1.7 (±0.1)	1.6	2.3; 2.9 (±0.1)	2.6	1.0	0.6
60	2.6; 3.4 (±0.2)	3.0	2.6; 3.4 (±0.2)	3.0	0	0

respectively.[31] Since the carbonate ion concentration used in the two conflicting reports[19,20] was about 24 mM, we used the same concentration. When [$NaHCO_3$] = 24 mM, the persistence length of unplatinated dsDNA was 50 nm. After 30 min incubation with cisplatin (1.65 mM) in the 24 mM carbonate buffer followed by rinsing with the same buffer, the ξ decreased to 44 (42) nm in the low (high) tension regime, corresponding to $p \approx 0.7\%$ (Fig. 2a). For simplicity, the nominal values of p are only presented in the text and a complete list of the determined values of p is given in Table 1. During the reaction, the DNA molecule was pulled at 6 pN to avoid the formation of accidental DNA loops, which can be covalently pinched by cisplatin. Thus, only local cross-links were considered. This level of force should not preclude bifunctional binding of cisplatin as can be seen from the bifunctional binding data under the ionic conditions favorable to cisplatin hydrolysis (Fig. 2 and 3). At low salt conditions ([NaCl] ≈ 10 mM), monofunctional cisplatin–DNA adducts, if any, should be converted to bifunctional adducts, leading to a shift in the curve. Surprisingly, after washing the sample with [NaCl] = 10 mM, the ξ was dramatically reduced to 35 (30) nm in the low (high) tension regime, corresponding to a $p \approx 2.3\%$ (Fig. 2b). This result indicates that there were (undetected) monofunctional cisplatin adducts present before washing with [NaCl] = 10 mM. We also tested any spontaneous time-dependent conversion from mono- to bi-functional adducts before the washing step, and found that the elastic response curve remained unchanged (data not shown) after 2 h and 30 min, implying that no spontaneous transition from the mono- to bi-functional form took place, or such a transition occurred very slowly. We concluded that the conversion of mono- to bi-functional adducts in [NaCl] = 10 mM was responsible for the additional reduction of ξ upon the buffer exchange. Hence, we estimated that the ratio of mono- (p_{Mono}) and bi-functional (p_{Bi}) cisplatin adducts was about 2.3 : 1 (p_{Mono} and p_{Bi} were 1.6% and 0.7%, respectively; see Table 1) before the conversion. The ratio of the two adducts varied somewhat (>2) as summarized in Table S1 (ESI†). In any case, we are certain that a significant amount of monofunctional adducts was present.

3.2 Independent verification of formation of monofunctional cisplatin adducts in carbonate buffer: DNA-twisting measurements

To independently confirm the existence of monofunctional cisplatin adducts in carbonate buffer, we utilized the σ-*vs.*-extension relation of DNA. For bare DNA (no cisplatin binding), the σ-*vs.*-extension relation is symmetric: its peak is located at $\sigma = 0$. For cisplatin-bound DNA, the peak of the σ-*vs.*-extension curve shifts towards negative σ by the amount of bifunctional cisplatin adducts[32] because each bifunctional crosslink unwinds DNA by approximately $\varphi_k = 13°$ where φ_k is the unwinding angle per cisplatin binding.[27] The degree of cisplatin binding (p) can be related to the shift in σ ($\delta\sigma$) by the following simple relation. $p = (\delta\sigma \times 360°)/(\lambda \times \varphi_k)$ where λ is the pitch of DNA, *i.e.*, 10.5 bp. If there are mono-functional adducts to be converted to bifunctional adducts under hydrolysis-favoring buffer conditions, there will be additional peak shift upon switching to low salt conditions. Using the DNA-unwinding-sensitive single-molecule measurements, we observed a considerable conversion from mono- to bi-functional cisplatin adducts as shown in Fig. 4.

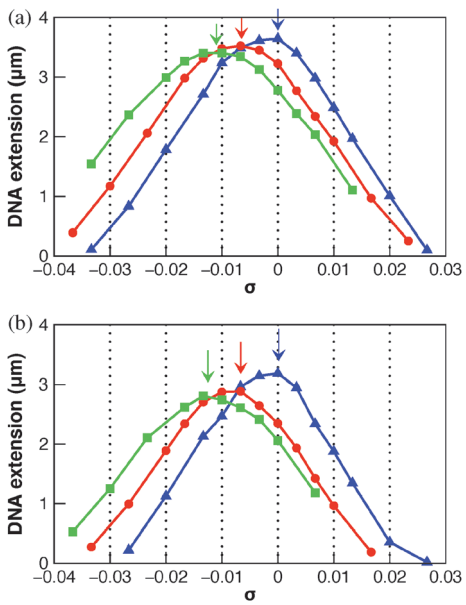

Fig. 4 DNA-twisting measurements show the existence of a considerable amount of monofunctional cisplatin adducts in carbonate buffer. A set of three σ-*vs.*-extension curves was obtained sequentially for each DNA molecule at (a) 0.28 pN and (b) 0.15 pN. Red circles and green squares show the σ-*vs.*-extension data of a DNA molecule incubated with cisplatin (1.65 mM) in 24 mM carbonate buffer before and after washing with 10 mM NaCl, respectively. The blue curve with triangles was obtained for the same DNA in 24 mM carbonate prior to any treatment with cisplatin. The arrows indicate the peak positions in the σ-*vs.*-extension curves.

After the DNA tether was incubated with cisplatin (1.65 mM) in the 24 mM carbonate buffer for 30 min in the presence of 6 pN's tension and subsequently washed by the same buffer, a set of three σ-*vs*.-extension curves was obtained sequentially for each DNA molecule at 0.28 pN (Fig. 4a) and 0.15 pN (Fig. 4b). Indeed, as bifunctional adducts were formed, the peak of the σ-*vs*.-extension curve shifted toward negative σ. After washing with 10 mM NaCl, an additional peak shift was observed, indicating the conversion of mono- to bi-functional adducts. Judged from the peak shifts, the amount of cisplatin bound bifunctionally is roughly doubled after the washing, which indicates that the amounts of mono- and bi-functional adducts were comparable under the reaction conditions. From the total peak shift, the degree of total cisplatin binding, initially either mono- or bi-functional, can be estimated by the relation introduced above. From Fig. 4, total $\delta\sigma$ is $\sim$1.1% and therefore p is $\sim$2.9%. From the elasticity measurements described in the previous section, p was $\sim$2.3%. The degrees of cisplatin binding evaluated from two separate methods are in reasonable agreement.

3.3 Formation of mono-functional adducts in the median range of physiological chloride concentrations

To generalize the presence of monofunctional cisplatin adducts, we examined the possibility of binding mode conversion in the absence of carbonate ions. We followed the same procedure in determining the degree of cisplatin binding as described for carbonate ions except that cisplatin (1.65 mM) was dissolved in various concentrations of NaCl. Under high salt conditions, cisplatin is not hydrolyzed and thus cannot bind to DNA. Therefore, the elastic response curve of dsDNA after the reaction with cisplatin in [NaCl] = 180 mM remained unchanged (Fig. 3a, green) compared to unplatinated DNA. Although there was no noticeable change in the curve, there might still be some monofunctional cisplatin adducts. Thus, we measured the elastic response curve after washing with [NaCl] = 10 mM. However, we found that the curve remained nearly unchanged (Fig. 3a, red), which implies that little cisplatin binds to DNA in either mode at [NaCl] = 180 mM.

When dsDNA was incubated with cisplatin in the presence of [NaCl] = 120 mM, the elastic response curve of dsDNA was moderately shifted, which corresponded to a $p \sim 0.6\%$ (Fig. 3b, green). After washing with [NaCl] = 10 mM, the response curve of the same cisplatin-bound DNA was further shifted to yield a $p \sim 1.1\%$ (Fig. 3b, red). This implies that mono- and bi-functional cisplatin–DNA adducts coexisted in [NaCl] = 120 mM. The ratio of cisplatin adducts in the mono- and bi-functional modes was estimated to be about 0.8 : 1. When the original buffer ([NaCl] = 120 mM) was restored, the curve remained reduced, indicating that the shift of the curve was not due to the buffer change *per se* and the reaction of cisplatin with DNA was irreversible (data not shown). When the reaction initially occurred at [NaCl] = 90 mM, p was $\sim$1.6% (Fig. 3c, green). After washing with [NaCl] = 10 mM, p increased to $\sim$2.6% (Fig. 3c, red). The ratio of cisplatin adducts in the mono- and bi-functional modes was determined to be about 0.6 : 1 in the original solution. When the initial concentration of NaCl was below 60 mM, the elastic response curve did not change after washing with [NaCl] = 10 mM (Fig. 3d), which indicates that the cisplatin in [NaCl] = 60 mM was almost fully hydrolyzed and could bind to DNA *via* the bifunctional mode. These data are summarized in Table 1 and other data under the same conditions are listed in Table S1 (ESI†).

4. Discussion and conclusions

Our experiments have revealed that the major effect of carbonate on cisplatin binding to DNA is the formation of the monofunctional cisplatin–DNA adducts although the presence of carbonate indeed reduces the amount of platinum that binds to DNA, as suggested in the study by Todd *et al.* Similarly, binding of cisplatin to DNA in the median range of physiological salt concentrations ([NaCl] $\sim$ 90 – 120 mM) occurred in both the mono- and bi-functional modes to a comparable extent, and monofunctional cisplatin adducts can be transformed to bifunctional adducts by switching to ionic conditions favorable to cisplatin hydrolysis ([NaCl] < 60 mM).

Interestingly, the fact that carbonate ions promote the formation of monofunctional cisplatin–DNA adducts is in contrast with the recent work by Todd *et al.*, where they suggested that the major role of carbonate ions was just to suppress cisplatin binding to DNA and the major product under physiological carbonate buffer conditions was still bifunctional.[20] This discrepancy could be attributed to the fact that their measurements were mainly conducted with 14-mer short single-stranded DNA (ssDNA) molecules containing a single GG site; thus, the activity of the cisplatin–carbonato complex might be different for ssDNA and dsDNA. This discrepancy may have also been due to different experimental conditions: in our experiment, cisplatin-bound DNA was characterized *in situ* in equilibrium with a physiological concentration of carbonate ions. In detail, our technique can measure the amount of bound cisplatin without additional sample handling or loss of carbon dioxide which may shift the chemical equilibrium in favor of the bifunctional adducts.[31] That is, our direct, *in situ* method minimizes the risk of losing cisplatin–carbonato complexes due to the formation of aqua- or hydroxo–cisplatin complexes which can be readily converted to bifunctional adducts. In this sense, our technique provides the most reliable evidence of the existence of monofunctional adducts under physiological conditions.

Besides this technical advantage, our single-molecule method is more sensitive to platinum–DNA adducts than electrophoresis-based methods (*e.g.* mobility shift assays), which have been primarily used to probe cisplatin binding to DNA.[19,20] Using our single-molecule method, we could measure the degree of cisplatin binding to DNA to be as low as p = 0.3%. This high sensitivity would enable quick detection of cisplatin binding in the early stage of the reaction. Taken together, the results of this study demonstrated that single-molecule measurements are a simple and powerful technique to estimate the degree of cisplatin binding to DNA and to directly observe the transient monofunctional state of cisplatin under various ionic environments.

In this study, we utilized the two independent experimental approaches in order to clearly show the existence of monofunctional adducts in the presence of carbonate buffer. The degrees of total cisplatin binding measured from the two methods

are in reasonable agreement, which further supports the validity of our experimental approach. In fact, the ratios of mono- to bi-functional adducts from the two methods differ quantitatively. One possibility is that supercoiled DNA may facilitate the conversion from mono- to bi-functional adducts. Local negative twist and its compact conformation may assist the conversion from mono- to bi-functional adducts. We hope to clarify this issue in future experiments. Nevertheless, it is obvious that a considerable amount of monofunctional adducts exist in carbonate media.

Our work will shed light on the role of ions in the activity of platinum-based anti-cancer drugs and pave the way for the development of more quantitative tools to characterize the pharmaceutical effects of a variety of drugs interacting with DNA from mechanistic points of view. Notably, the technique developed here will be widely applicable to the study of the transient behaviors of a broad range of drugs and proteins that bind to DNA in different modes depending on various physical and chemical factors such as tension, torsion, ligand, and ionic conditions.

Acknowledgements

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