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Effects of pH on Oxaliplatin-Induced Condensation of Single DNA Molecules *

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By using magnetic tweezers, atomic force microscope and mass spectrometry, we study the effects of pH on oxaliplatin-induced DNA condensation, the DNA persistence length, the amounts of micro-loops and of oxaliplatin bound to DNA. It is found that the DNA condensation degree, the amounts of micro-loops and of oxaliplatin bound to DNA increase with the decrease in the pH value while the DNA persistence length has an opposite behavior. The observed effects may be related to the drug resistance of cancer cells.

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Oxaliplatin is one of the third-generation platinum anticancer agents and has been approved for the clinical treatment of advanced colorectal cancer.[1] Its anticancer effect mainly results from the various types of covalent adducts formed between oxaliplatin and DNA molecules, which affect the processes of DNA replication and transcription and thus lead to cell death.[2,3] Like many other chemotherapy drugs, oxaliplatin can also cause drug resistance.[4,5] Nevertheless, the mechanism for the drug resistance is still not very clear.

Intracellular pH (pHi) plays an important role in biological functions such as cell metastasis, proliferation, and apoptosis. Although there are over 200 different known cancers, many of them have a common feature: the pHi of the drug-resistant cancer cells is more alkaline than that of the parental (drug-sensitive) cancer cells.[6–8] This feature prompts us to consider whether there is a relationship between the pHi and the drug resistance, and the latter is the major obstacle for effective treatments of cancers. Indeed, lots of studies showed that the change of the pHi is strongly associated with the change of the intracellular drug accumulation.[8–12] For platinum anticancer drugs, an interesting issue is whether the pHi has an effect on the interaction between platinum anticancer drugs and DNA. However, to the best of our knowledge, no related issue in vitro experiment, which avoids the effect of pHi on the intracellular drug concentration, is available. In this Letter, we study the effects of pH on the interactions between oxaliplatin and DNA mainly using single-molecule techniques.

[Pt(R, R-DACH)]$(NO_3)_2$ was obtained from the institute of precious metal in Kunming. The stock solution of [Pt(R, R-DACH)]$(NO_3)_2$ (5 mM) was stored in the dark at 4℃ for one month. The working solution was diluted by HEPES buffer (10 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, Sigma-Aldrich), 30 mM CH_3COONa (Sigma-Aldrich)) of different pH values (6.3, 6.8, 7.3 or 7.8). NaOH was used to adjust the pH of HEPES buffer. All of the pH values were measured at 29℃. The linear bacteriophage λ-DNA molecules (48502 bp, a contour length of 16.5 μm) with two 12-nt cohesive termini (New England Biolabs), which were annealed respectively with two 12-nt labeled single-stranded oligomers (Sangon Biotech), 3'-biotin-CCC GCC GCT GGA-5' and 3'-digoxigenin-TCC AGC GGC GGG-5', was used for magnetic tweezers experiments. The streptavidin-coated super-paramagnetic beads (with a diameter of 1 μm, MyOne, Dynabeads) were from Invitrogen. The 2-kb DNA fragment (2186 bp, with a contour length of 0.74 μm) generated by PCR with the forward primer 5'-CTA ACG GAT TCA CCA CTC CAA GA-3'and the reverse primer 5'-CGG CTG GCT GGT TTA TTG C-3' using pBR322 plasmid DNA (New England Biolabs) as a template was used for atomic force microscopy (AFM) experiments and inductively coupled plasma mass spectrometry (ICP-MS) experiments.[13] The PCR products were purified by using a QIAquick Gel Extraction Kit.

In AFM experiments, 2-kb DNA at a concentration of 2 ng/μL was incubated with 60 μM oxaliplatin in HEPES buffer of different pH values at 29℃ in the dark. After incubation for 1 h, 2.5 μL of the sample was taken out and added by 0.5 μL 100-mM $MgCl_2$ and 7 μL 10-mM Tris-HCl (pH 7.4). Then 10 μL of the solution was deposited onto a freshly cleaved mica. After 10 min, the mica surface was washed by 100 μL Milli-Q filtered ultrapure water for four times and then

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blown dry by a gentle stream of nitrogen gas. The scanning was performed in air by using the AFM from Veeco company (USA) in the tapping-mode. The silicon probe RTESP14 was employed. The E scanner was used. The scan frequency was 1 Hz per line, and the scan size was $4\times4\,\mu m^2$. The image analysis was performed by using the Nanoscope Ⅲ 5.31r1 software package.

In the ICP-MS experiments, 2-kb DNA at a concentration of 2 ng/μL was incubated with 60 μM oxaliplatin in HEPES buffer of different pH values at 29℃ in the dark. The amount of DNA was 200 ng. After incubation for 6 h, the samples were purified by using a QIAquick PCR Purification Kit to remove free oxaliplatin molecules, where the washing step was repeated three times. After wet-digestion, the amount of Pt of the sample was measured by ICP-MS (ELAN DRC Ⅱ, PerkinElmer, USA).[14] The detection limit of Pt was 0.01 ng/mL.

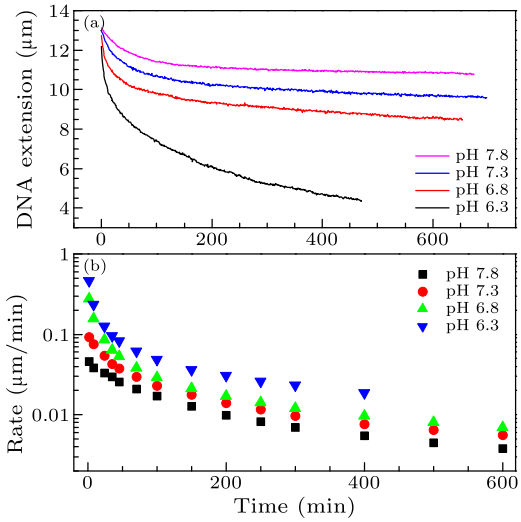

Fig. 1. Effects of pH on DNA condensation induced by oxaliplatin. (a) DNA extension versus incubation time of λ-DNA with 60 μM oxaliplatin in HEPES buffer of different pH values. The data in each curve are obtained by an average of at least four independent measurements. (b) The calculated average DNA condensation rate.

The extensions of DNA molecules incubated with 60 μM oxaliplatin in HEPES buffer of different pH values were measured with time by using magnetic tweezers (Pico Twist company, France) (Fig. 1(a)), where the stretching force was fixed at about 0.6 pN. As shown in Fig. 1(a), the DNA extensions decrease with time and the DNA condensation degree increases with the decrease of pH value. Particularly, the condensation degree at pH 6.3 is significantly larger than those at other pH values. By comparison, we also measured the extensions of DNA molecules in HEPES buffer of different pH values in the absence of oxaliplatin (data not shown). The results show that the DNA extensions remain nearly invariant, indicating that the DNA molecules are stable in HEPES buffer of different pH values. Thus, it is expected that the DNA condensation is solely induced by the interaction of oxaliplatin potentially via the formation of Pt-DNA adducts, which include mainly the 1,2-GG/AG diadducts and micro-loops.[15,16] The average DNA condensation rate was calculated (Fig. 1(b)). As shown in Fig. 1(b), the average rate decreases with the pH value. Particularly, the average rate at pH 6.3 is significantly larger than those at other pH values.

To further study the above phenomenon, the effects of pH on DNA persistence length, the amount of micro-loops and the amount of oxaliplatin bound to DNA are studied.

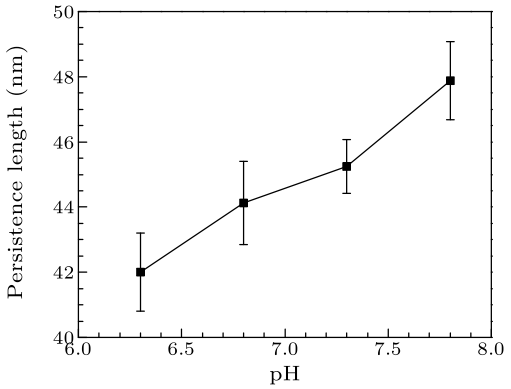

Fig. 2. Effects of pH on DNA persistence length in the absence of oxaliplatin. The force-extension curves of single λ-DNA molecules in HEPES buffer of different pH values were measured and fitted by the WLC model to obtain the persistence lengths. Each data point is obtained by an average of thirty independent measurements. The errors correspond to 95% confidence intervals.

The force-extension curve of single DNA molecules in HEPES buffer of different pH values in the absence of oxaliplatin was measured and fitted by the WLC model[17,18] to obtain the persistence length. The fitting formula is[19]

$$F=\left(\frac{k_{\rm B}T}{P}\right)\left[\frac{1}{4(1-L/L_0)^2}-\frac{1}{4}+\frac{L}{L_0}+\sum_{i=2}^{i\leq 7}\alpha_i\left(\frac{L}{L_0}\right)^i\right], \quad (1)$$

with $\alpha_2=-0.5164228$, $\alpha_3=-2.737418$, $\alpha_4=-16.07497$, $\alpha_5=-38.87607$, α_6=39.49944, and $\alpha_7=-14.17718$, where F is the stretching force, L is the DNA extension, $k_{\rm B}$ is Boltzmann's constant, T is the absolute temperature, P is the persistence length and L_0 is the contour length of DNA molecule. The persistence length as a function of pH value is shown in Fig. 2. The persistence length increases with the pH value, which is consistent with the previous experimental data.[20] The result can be simply explained as follows. The backbone of DNA carries negative charges in neutral pH buffer. When the pH value of

the buffer decreases (pH 6.3), some of the charges on the backbone are neutralized by the increasing H^+, resulting in the decrease in the electrostatic repulsion between the phosphates on DNA and thus the decrease of the persistence length. Similarly, when the pH value of the buffer increases (pH 7.8), the electrostatic repulsion increases, thus increasing the persistence length.

Here it is necessary to discuss the effect of the change in the concentration of Na^+ and/or the ionic strength of the buffer due to the pH adjustment by the addition of NaOH on the persistence length. Our calculations show that the concentration of Na^+ at pH 7.8 is about 36 mM and that at pH 6.3 is about 30 mM. Previous studies[21–24] showed that in the moderate-to-high ionic strength region (above 10 to 20 mM Na^+, the ionic strength here actually means the concentration of Na^+), the persistence length changes slightly with the increase of the ionic strength. Thus, if we consider only the effect of Na^+, the difference in persistence length between pH 6.3 and pH 7.8 should be slight. In addition, we speculate that the change of the ionic strength with the pH value is very small. Thus, the effect of the change in the concentration of Na^+ and/or the ionic strength is ignored.

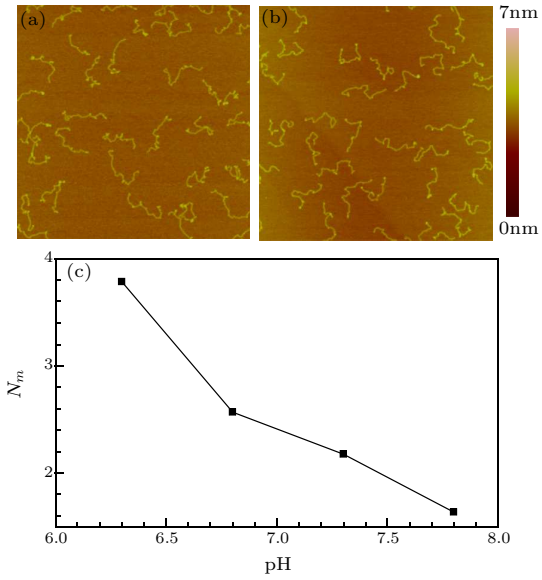

Fig. 3. Effects of pH on the amount of micro-loops. Here 2-kb DNA at a concentration of 2 ng/μL was incubated with 60 μM oxaliplatin in HEPES buffer of different pH values at 29℃ for 1 h. The AFM images of DNA molecules in HEPES buffer of pH 6.3 (a) and pH 6.8 (b). The size of each image is $2 \times 2\,\mu m^2$. (c) The average number of micro-loops formed in one DNA molecule (N_m) as a function of pH value. At least 400 DNA molecules are analyzed for each data point.

DNA molecules incubated with 60 μM oxaliplatin in HEPES buffer of different pH values were scanned by using AFM to study the effects of pH on the amount of micro-loops. Two typical AFM images are shown in Figs. 3(a) (pH 6.3) and 3(b) (pH 6.8). The amount of micro-loops, which are characterized as high and large protuberances in the images,[16] is statistically analyzed and the average number of micro-loops formed on one DNA molecule as a function of pH value is shown in Fig. 3(c). The average number of micro-loops formed on one DNA molecule decreases with the increase of the pH value. Particularly, the number at pH 6.3 is significantly larger than that at pH 6.8. By comparison, DNA molecules in the absence of oxaliplatin in HEPES buffer of different pH values are also scanned and no micro-loops are formed (data not shown).

To investigate the effects of pH on the amount of oxaliplatin bound to DNA, the amount of oxaliplatin bound to DNA in HEPES buffers of different pH values was detected by ICP-MS, and the average number of oxaliplatin molecules bound to one DNA molecule as a function of pH value is given in Fig. 4. The number decreases with the pH value in the range of 6.8 to 7.8. However, the difference between the number at pH 6.3 and that at pH 6.8 is not statistically significant.

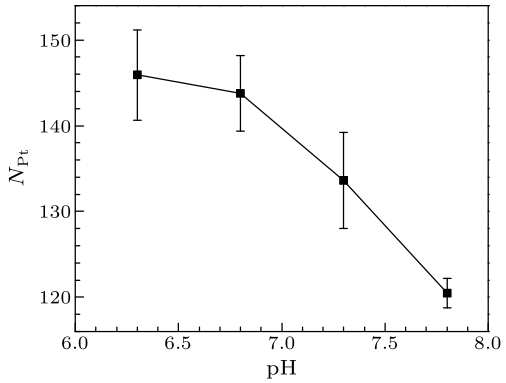

Fig. 4. Effects of pH on the amount of oxaliplatin bound to DNA. N_{Pt} was the average number of oxaliplatin molecules bound to one DNA molecule. 2-kb DNA at a concentration of 2 ng/μL was incubated with 60 μM oxaliplatin in HEPES buffer of different pH values at 29℃ for 6 h. Each data point is obtained by an average of eight independent measurements. The errors correspond to 95% confidence intervals.

Based on the above results, now we may explain the observation in Fig. 1. The decrease of DNA persistence length implies the reduction of the stability and rigidity of DNA. On the other hand, the major 1, 2-GG Pt-DNA adduct bends the helix axis by about 30° toward the major groove.[25,26] Since DNA molecules of low persistence lengths are more flexible and easier to bend than those of high persistence lengths, oxaliplatin molecules show a higher tendency to bind DNA molecules of low persistence lengths (Fig. 4) and more micro-loops are formed on such DNA molecules (Fig. 3(c)). Therefore, the DNA condensation degree increases with the decrease of the pH value (Fig. 1). As for the results at pH 6.3, we conclude that the

extremely low persistence length (42±1.2 nm, mean ± 95% confidence interval) at pH 6.3, which is very close to the intrinsic (bare) persistence length (40 nm) due to the vertical base-stacking interactions[27,28] and reduced significantly the repulsive interaction between the different segments along the DNA molecule, is responsible. Many more micro-loops are formed due to the fact that DNA molecules at pH 6.3 are much more flexible and easier to make two distant bases being close to each other to form loops. The contribution of micro-loops to DNA condensation is larger than that of diadducts.[16] Therefore, although the number of oxaliplatin molecules on one DNA molecule at pH 6.3 is nearly the same as that at pH 6.8, the DNA condensation degree at pH 6.3 is larger than that at pH 6.8 due to the fact that more micro-loops are formed at pH 6.3.

Based on the above results and analyses, we speculate that the effects of pH on the interaction between platinum anticancer drugs and DNA may constitute one of the origins for the drug resistance of cancer cells. To be more specific, for most of the known cancers, the pHi of the drug-resistant cancer cells is more alkaline than that of the parental (drug-sensitive) cancer cells.[6–8] Our experiments indicate that the increase of the pH value reduces the amount of the platinum anticancer drug oxaliplatin bound to DNA. This phenomenon may imply that the increasing pHi could be one possible mechanism for the resistance of cancer cells to platinum drugs.

In summary, we have studied the effects of pH on the interaction between oxaliplatin and DNA in vitro. The effects of pH on DNA condensation induced by oxaliplatin are studied by using magnetic tweezers. We find that the DNA condensation degree and the average DNA condensation rate increase with the decrease of the pH value. Particularly, the condensation degree and the average rate at pH 6.3 are significantly larger than those at other pH values. To further study this phenomenon, the effects of pH on DNA persistence length, the amounts of micro-loops and of oxaliplatin bound to DNA are studied. We find that the persistence length increases with the increase of the pH value. The amount of micro-loops decreases with the increase of the pH value. Particularly, the amount at pH 6.3 is significantly larger than that at pH 6.8. The amount of oxaliplatin bound to DNA decreases with the increase of the pH value in the range of 6.8 to 7.8. However, the difference between the amounts at pH 6.3 and pH 6.8 is not statistically significant. The present studies shed new light on the mechanism for drug resistance of cancer cells.

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