Assessment of the relative toxicity of Cu^{2+} by measuring structural changes of supercoiled DNA

Gang Pan^{a,*}, Guohua Chang^a, Hao Chen^a, Lorenzo Giusti^b

^a State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-environmental Sciences, Chinese Academy of Sciences, 18 Shuangqing Road, Beijing 100085, China

^b Centre for Research in Environmental Sciences, Faculty of Applied Sciences, UWE, Bristol BS16 1QY, United Kingdom

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RC₅₀ values (concentration at which 50% of the supercoiled DNA relaxed) can be used to reflect the relative toxicity of Cu in aquatic environment.

Abstract

A method for the measurement of the relative toxicity of Cu^{2+} in aquatic environments is proposed. It is based on the quantitative measurement on the shape change of the supercoiled DNA after it is contacted with different levels of Cu^{2+} for various time intervals. In the absence of any redox reagents, all supercoiled DNA degraded into other forms of DNA after 24 h incubation in the presence of 5.13×10^{-3} , 5.08×10^{-4} and 5.35×10^{-5} mol/L Cu^{2+} . At a lower Cu^{2+} concentration (10^{-6} mol/L), 44% of supercoiled DNA retained its original supercoiled form after 24 h, and 29% after 48 h. The concentration of RC_{50} , i.e. concentration of pollutants at which 50% of the supercoiled DNA was relaxed compared to control samples, can be obtained from the dose–response curves at different exposure time, which may provide a rapid and convenient approach to assess the relative toxicity of environmental pollutants.

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1. Introduction

One of the goals of ecotoxicology is to understand the concentration of chemicals at which organisms in the environment are affected. This concentration is often determined by in vivo tests on individual organisms. Other toxic endpoints (survival, growth, or reproduction) can be obtained using a tiered effects process. Since such toxicity data for a given pollutant vary with the type and characteristics of the organisms tested, it is difficult to establish a unique relationship between the concentration of chemicals and the toxicity data based on conventional methodology. Therefore, the commonly used concept of concentration of pollutants has an ambiguous meaning since a given concentration can be harmful to one organism but non-harmful to another. This limits

the application of chemical methodologies (e.g. concentration) to ecotoxicology quantitatively. For example, when additive or synergistic effects strongly depend on the concentration of chemicals in a certain environment, it may be very difficult to make an accurate modeling of environmental risk assessment without knowing the qualitative and quantitative dependence of such concentration effects. This problem may be further complicated in practice since it is often difficult to identify the chronic hazards associated with contaminants and to choose the representative species that inhabit a particular geographic area. These difficulties could be minimized if toxicity data were based on a property that applies to all the organisms in a given environmental system. When a dose–response relationship for different chemicals is assessed against a standard material that is representative for such a property, then the harmful effect of different chemicals on this particular property can be quantitatively compared among different organisms using the concentration of

* Corresponding author. Tel.: +86 010 62849686; fax: +86 010 62923563.
E-mail address: gpan@rcees.ac.cn (G. Pan).

potentially toxic chemical substances. Although such defined “relative toxicity” provides a tool for studying toxicity on a certain comparable property (structural change of supercoiled DNA in this case) among different organisms in a system, it does not replace the conventional toxicity data where the real, integrated toxicity for individual organisms in the system was reflected. The potential DNA structural change may be measured independently from the fact that these changes may be reduced by protection mechanisms (e.g. bio-availability, DNA repair mechanisms) at the cellular level. In another word, conventional toxicity assessment based on the whole cells or organisms represents the real, integrated toxic effect, while the relative toxicity based on a single but common property of different organism provides insights into the mechanisms of the real toxicity. Such a methodology (idea) has never been seen before in the studies in aquatic environment. The purpose of the paper is to study whether this idea is achievable from technical point of view.

Single-property or mechanism-based studies, such as DNA damage, are well documented in genetic toxicology (Lee and Steinert, 2003). Many routine assays are based on the change of DNA structure, such as studies of gene mutation and chromosome aberration. Over the last two decades, single cell gel electrophoresis and comet assay have been used as rapid, sensitive methods to assess the genetic toxicity of pollutants on individual cells (Fairbairn et al., 1995). Recently, plasmid DNA assay has been used to evaluate environmental toxicities of atmospheric aerosols and respirable fibers (Gilmour et al., 1995; Donaldson et al., 1996; Greenwell et al., 2002). The latter method is to assess the toxicity by measuring changes of supercoiled DNA structures caused by free radicals present on particle surfaces. These developments prompted us to use the change of DNA supercoiled structure as a specific criterion to assess and compare the relative toxicity of pollutants (Cu in this case) in aquatic environmental systems.

Among the studies of DNA damage caused by copper, most results indicate that Cu can only cause DNA damage either in the cells or outside the cells when redox reagents are added or when free radicals are presented (Sagripanti and Kenneth, 1989; Aruoma et al., 1991; Goel et al., 2003). However, natural aquatic environment is significantly different from that inside the cell and that of redox solutions. No results were found as to whether plasmid DNA structure can be changed by the existence of Cu in pure water solutions.

In this work, we will use plasmid DNA assay as a tool to detect whether the structure or the shape of plasmid DNA can be changed by the existence of Cu in pure water solutions, and if so, whether there is any dose–response relationship that can be used to quantify the idea of relative toxicity in the field of aquatic environment.

2. Materials and methods

2.1. Materials

ϕ X174 RF DNA (Form I, 5386 bp, 0.25 μ g/ μ L), which is purified from *Escherichia coli* C infected with bacteriophage ϕ X174 am3cs70, was obtained

Fig. 1. Distribution diagrams of aquatic copper (II) species.

from Invitrogen Co. (USA), and stored in buffer solution containing 10 mM Tris–HCl (pH 7.4), 5 mM NaCl and 0.1 mM EDTA. Agarose (ultrapure, Promega) is DNase-free and RNase-free. Ultrapure ethidium bromide and Tris were obtained from USB Co. (USA). Analytic grade $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (s) and autoclaved ultrapure water were used throughout the experiments.

A Hoefer HE 33 mini submarine electrophoresis unit (Amersham Biosciences Co.) and GelDoc 2000 gel documentation system were used for electrophoresis analysis. An ORION 868 pH meter was used for pH measurements. Concentrations of copper were measured using a Hitachi Z-6100 atomic absorption spectrophotometer. All plasticware and glassware used in the experiments were autoclaved for 30 min at 0.12 MPa.

2.2. Methods

A 5×10^{-3} mol/L CuCl_2 stock solution (pH 5.23) was prepared by dissolving 0.2131 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (s) into a 250 mL flask. Aliquots of stock solution were diluted to make 5×10^{-4} , 5×10^{-5} , 5×10^{-6} , 5×10^{-7} and 5×10^{-8} mol/L CuCl_2 solutions. These solutions were autoclaved and the pH adjusted to 5.20 with 0.1 mol/L HCl sterilized solutions. The Cu^{2+} concentrations of autoclaved solutions were 5.13×10^{-3} , 5.08×10^{-4} , 5.35×10^{-5} and 8.07×10^{-6} mol/L by AAS. Solutions of 5×10^{-7} and 5×10^{-8} mol/L levels were not re-determined accurately since preliminary tests indicated that Cu^{2+} at these levels showed no detectable effect on supercoiled DNA. Reaction mixtures (20 μ L) were made up with 1 μ L DNA solution (0.25 μ g/ μ L, pH 7.4) and 19 μ L of Cu^{2+} solutions. Reaction mixtures were incubated for 1, 4, 8, 12, 24 and 48 h at 37 °C. Control experiments were carried out at the same conditions, with sterilized, ultrapure water or sterilized hydrochloric solutions instead of Cu^{2+} solutions. Loading buffer (0.25% bromophenol blue in 40% sucrose) was added to each sample and mixed gently. Samples were immediately loaded onto a 7 cm $\times$ 10 cm slab agarose gel (1.0%). Electrophoresis was performed in

Fig. 2. The effect of incubation time on supercoiled DNA in the control sample. Lanes 1–7 are results of 0, 1, 4, 8, 12, 24, and 48 h, respectively.

Fig. 3. A plot of reaction period versus the percentage of different DNA forms in the control sample. Where error bars are not seen, they are within the range obscured by the data point. (■), Band 3; (○), band 2; (□), band 1; (▽), total bands.

a $1 \times$ TAE buffer containing 40 mmol/L Tris–acetate and 1 mmol/L EDTA. Finally, gels were run at 30 V for 6 h, subsequently stained with ethidium bromide for 30 min, and then photographed under UV illumination using a Bio-Rad Gel-Doc 2000 documentation system. Quantitative measurement of different forms of DNA was obtained using Quantity One Software (Bio-Rad).

2.3. Selection of pH and copper concentration

Copper (II) species change with the pH of a solution. Based on the solubility equilibrium conditions among various copper (II) species, Cu^{2+} is the main species at pH 5.0–5.5 when concentration of soluble Cu (II) is less than 10^{-3} mol/L (Fig. 1).

3. Results and discussion

3.1. Effect of incubation period on DNA structure

Untreated DNA showed a major band (band 3) and a minor band (band 2) corresponding to the supercoiled and a relaxed form of DNA, respectively (Fig. 2). There is a little change in

Fig. 4. The pH effect of Cu^{2+} -free solutions on supercoiled DNA after incubation periods of 24 h (A) and 48 h (B). Lane 1, pH = 4.8; lane 2, pH = 5.0; lane 3, pH = 5.2; lane 4, pH = 5.5.

the percentage of supercoiled DNA in the control sample with the increase of incubation period (Fig. 3). The mean percentage of supercoiled DNA in the control sample was 76.1%.

3.2. Influence of hydrochloric acid on DNA structure

The influence of the added HCl on supercoiled DNA was measured at all time intervals after treatment with sterilized Cu^{2+} -free hydrochloric solutions at pH 4.8, 5.0, 5.2 and 5.5 (Figs. 4 and 5). Over the pH range of 4.8–5.5, the added HCl did not cause any significant difference in the mean percentage of DNA forms (One-way ANOVA, $\alpha = 0.01$).

3.3. Effect of Cu^{2+} on DNA structure

When ϕX174 RF DNA was incubated with Cu^{2+} ions, the relative amount of band 3, band 2 and band 1 clearly responded to the change of Cu^{2+} concentration and incubation time (Fig. 6).

At $\text{Cu}^{2+} \geq 5.35 \times 10^{-5}$ mol/L, the supercoiled form of DNA (band 3) degraded rapidly into other forms and

Fig. 5. Plots of reaction period versus the percentage of different forms of DNA in Cu^{2+} -free solutions of different pH. (■), Band 3; (○), band 2; (□), band 1; (▽), total bands.

Fig. 6. Relaxation of supercoiled DNA after incubation for 1 h (A), 4 h (B), 24 h (C), and 48 h (D) in the presence of different Cu^{2+} concentrations. Lane 1, 5.13×10^{-3} mol/L; lane 2, 5.08×10^{-4} mol/L; lane 3, 5.35×10^{-5} mol/L; lane 4, 8.07×10^{-6} mol/L; lane 5, 5×10^{-7} mol/L; lane 6, 5×10^{-8} mol/L CuCl_2 .

completely disappeared within 24 h (Fig. 7A, B and C), suggesting that the DNA relaxed completely and converted to other forms. The background of gel images was subtracted using Quantity One (Bio-Rad) Software until the total percentage of bands in the lane was $\geq 90\%$. The plot of incubation time versus the percentage of different forms of DNA (Fig. 7) was obtained from electrophoresis data. At a lower level of Cu^{2+} (8.07×10^{-6} mol/L), this trend was less pronounced (Fig. 7D). At Cu^{2+} levels $< 5 \times 10^{-7}$ mol/L, no significant effect was observed on supercoiled DNA within 48 h (data not shown here).

With respect to band 2, the trends were different than those observed for band 3. The amount of band 2 increased gradually with incubation time when Cu^{2+} was more than 8×10^{-6} mol/L (Fig. 7A–D). The percentage of band 1 increased slowly with time when Cu^{2+} levels were high (Fig. 7A and B). No significant increase was found when the Cu^{2+} level was $< 5 \times 10^{-5}$ mol/L.

If the percentage of supercoiled form of DNA in the control sample is defined as one, then data normalization for band 3 can be performed and the relationships between DNA structure change, Cu^{2+} concentration and incubation time can be

established (Fig. 8). In the presence of $< 5.35 \times 10^{-5}$ mol/L Cu^{2+} , only 7.6% of supercoiled form of ϕX174 RF DNA relaxed within 4 h, while this percentage increased to 45% in the presence of 5.13×10^{-3} mol/L Cu^{2+} . After 24-h incubation, nearly 100% of supercoiled DNA relaxed when the Cu^{2+} concentration was above 5.35×10^{-5} mol/L (Fig. 8). In the presence of 8.07×10^{-6} mol/L Cu^{2+} , 56% the supercoiled form of DNA relaxed after 24 h and 71% relaxed after 48 h. No DNA relaxation was observed in the presence of 5×10^{-7} and 5×10^{-8} mol/L Cu^{2+} within 48 h.

3.4. Calculation of RC_{50}

Dose–response equations were obtained from linear regression based on the relationship between the probability unit (PU) and log (Cu^{2+} concentration) (Fig. 9). PU was obtained by adding five to the normal deviates of a standardized normal distribution of results from a dose–response study (Yan and Pan, 2002).

Fig. 7. Change of DNA structure as a function of reaction period and Cu^{2+} concentration. Initial concentrations for A, B, C, D are 5.13×10^{-3} , 5.08×10^{-4} , 5.35×10^{-5} , 8.07×10^{-6} mol/L, respectively. (■), Band 3; (○), band 2; (□), band 1; (▽), total bands.

Fig. 8. The percentage of relaxed supercoiled form of ϕ X174 RF DNA as a function of incubation time in the presence different concentrations of Cu^{2+} : (■), 5.13×10^{-3} ; (○), 5.08×10^{-4} ; (◇), 5.35×10^{-5} ; (—), 8.07×10^{-6} ; (△), 5×10^{-7} and (□) 5×10^{-8} mol/L CuCl_2 .

When supercoiled DNA was exposed to different concentrations of Cu^{2+} solution for 4 h, the following dose–response equation was obtained:

$$\text{PU} = 6.167 + 0.511 \times \log[\text{Cu}] \quad (R = 0.952) \quad (1)$$

Based on Eq. (1), when PU is 5.0, the RC_{50} (the concentration at which 50% of the supercoiled DNA relaxed compared to the control) of supercoiled DNA can be calculated as follows:

$$\log(4 \text{ h} - \text{RC}_{50}) = -2.283 \quad (2)$$

$$4 \text{ h} - \text{RC}_{50} = 5.20 \times 10^{-3} \text{ (mol/L)} \quad (3)$$

Similar calculations gave 8 h- RC_{50} and the 12 h- RC_{50} of 5.02×10^{-4} ($R = 0.978$) and 1.01×10^{-4} mol/L ($R = 0.930$), respectively.

With regard to the mechanism of copper induced DNA damage, many reports suggest that copper (II) is reduced to copper (I) by intracellular reducers such as ascorbate, and copper (I) is subsequently oxidized by H_2O_2 to copper (II), which generates reactive oxygen species (ROS). ROS can induce DNA damage and lead to single- and double-strand breaks, modified bases, and DNA–protein crosslinks (Chiou, 1983; Sagripanti and Kenneth, 1989; Rodriguez et al., 1995; Drouin

Fig. 9. Plots of probability unit versus the logarithm of Cu^{2+} concentration (mol/L). Lines are linear regression curves (95% confidence). Each data point represents the mean value from three separated experiments.

et al., 1996). Therefore, the catalytic role played by copper in initiating ROS was found to be the main mechanism for DNA damage by copper in cells. No direct effect of copper alone was detected in the past (Table 1). This may be due to the facts that the incubation time used in Table 1 (mostly <1 h) is much shorter than in this study or the Cu concentration used (mostly $<10^{-6}$ mol/L) is much less. In our work, no oxidants and reducers were present except sterilized Cu^{2+} solutions. The mechanism of supercoiled DNA degradation in the absence of oxidants and reducers needs to be investigated.

4. Conclusions

In a water solution prepared using Cu^{2+} , ϕ X174 RF DNA and distilled water only, supercoiled form of DNA decreased with the increase of incubation time and the concentration of Cu^{2+} . From the dose–response relationship between Cu^{2+} concentration and DNA structure change, RC_{50} values (concentration at which 50% of the supercoiled DNA relaxed compared to the control) can be obtained, which may provide a fast and simple method to assess the relative toxicity of pollutants in aquatic environment.

Table 1
No detectable damage of DNA was observed in listed reports

Cu conc.	DNA	Incubation time	Electrophoresis conditions	Reference
0.5 mmol/L	λ -DNA(1 μg), ϕ X174DNA(1 μg)	Minutes	25 V/35 mA, 14 h	Chiou (1983)
10^{-2} mol/L,	pUC8c2 DNA(1.6 μg)	20 °C, 30 min	—	Sagripanti and Kenneth (1989)
10^{-4} mol/L				
20 $\mu\text{mol/L}$	^{32}P -labeled DNA fragments	37 °C, 20 min	—	Yamamoto and Kawanishi (1989)
160 $\mu\text{mol/L}$	pUC18 plasmid DNA(0.4 μg)	37 °C, 4 h	—	Baudoin et al. (1998)
25 $\mu\text{mol/L}$	DNA from calf thymus (0.5 mg/mL)	37 °C, 1 h	—	Aruoma et al. (1991)
0.2 mmol/L	pBR332 (0.5 $\mu\text{g}/10 \mu\text{L}$)	37 °C, 1 h	—	Yoshino et al. (2002)
50 mmol/L	pBR332 (15 μm)	37 °C, 1 h	100 V (4 V cm^{-1}), 20 min	Jeżowska-Bojczuk et al. (2002)
50 $\mu\text{mol/L}$	pBR332 (1 $\mu\text{g}/20 \mu\text{L}$)	37 °C, 20 min	5 V cm^{-1} , 3.5 h	Goel et al. (2003)

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