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ORIGINAL RESEARCH

Improvement of DNA/Metal Particle Adsorption in Tungsten-Based Biolistic Bombardment; Alkaline pH is Necessary for DNA Adsorption and Suppression of DNA Degradation

Yuya Yoshimitsu · Kiwamu Tanaka · Takashi Tagawa · Yasushi Nakamura · Tomoaki Matsuo · Shigehisa Okamoto

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Abstract Tungsten particles have long been used as microcarriers in biolistic bombardment because of their cost-effectiveness compared to alternative gold particles—even if the former have several drawbacks, including their DNA-degrading activity. We characterized tungsten-induced DNA degradation to assess the value of this metal particle and to improve tungsten-based biolistic bombardment. Alkaline pH, low temperature, and high salt concentration were found to diminish tungsten-induced DNA breakdown. The pH was the most influential factor in this phenomenon, both in aqueous solutions and on the particles. Furthermore, alkaline pH greater than 9.4 of an adsorption mixture was found to be essential for DNA binding to metal particles. Based on these findings, we propose a new formula of DNA/tungsten adsorption by using TE buffers that keep alkaline pH (>9.4) of the mixture, in which tungsten-bound plasmid DNA cleavage was suppressed to half the level of that in the conventional DNA-binding condition.

Keywords Biolistic bombardment · Tungsten particle · Alkaline pH · DNA degradation · DNA adsorption

Abbreviations

cc	closed circular
GFP	green fluorescent protein
oc	open circular
Spd	spermidine-free base
TE	Tris–HCl/EDTA

Introduction

Small particles made from tungsten have long been used as microcarriers in a DNA delivery system referred to as "biolistic bombardment," which can be applied to most organisms (Sanford et al. 1993; Southgate et al. 1995; Veluthambi et al. 2003). Tungsten particles have the following merits in this system. First, they are much less expensive than alternatively used gold particles. In fact, the price of tungsten particles (per gram) is less than one-hundredth of that of gold particles purchased from Bio-Rad Laboratories Inc. Second, tungsten particles can penetrate target tissues for a desired depth with minimal mechanical injury because of their very high specific gravity and extreme hardness, in addition to their irregular shape (Buchowicz and Krysiak 2003). The tungsten particles, however, have several disadvantages for genetic transformation, including the cleavage of adhered DNA to the particle;

Y. Yoshimitsu · K. Tanaka · S. Okamoto (✉)
Department of Agricultural Sciences and Natural Resources,
Kagoshima University,
Kohrimoto 1-21-24,
Kagoshima 890-0065, Japan
e-mail: okamoto@agri.kagoshima-u.ac.jp

Y. Nakamura
Department of Food Sciences and Nutritional Health,
Kyoto Prefectural University,
Shimogamo, Sakyo-ku,
Kyoto 606-8522, Japan

T. Tagawa · T. Matsuo
Department of Biochemical Science and Technology,
Kagoshima University,
Kohrimoto 1-21-24,
Kagoshima 890-0065, Japan

oxidation of its own surface, which possibly affects DNA binding; cell toxicity against certain types of organisms; and medium acidification due to their chemical property as a Lewis acid (Russell et al. 1992; Sanford et al. 1993).

Among the drawbacks of tungsten particles, tungsten-triggered DNA degradation is the most serious dilemma; this is because the DNA itself is exactly what needs to be introduced into target cells for genetic transformation. However, this phenomenon has so far not been intensively studied. Only a few reports on tungsten-induced DNA cleavage have been published in the past decade (Krysiak et al. 1999a; Krysiak et al. 1999b; Mazuś et al. 2000); the nicking and linearization of plasmid DNA were reported to occur during incubation with tungsten particles, and DNA nicking with tungsten depends on pH of the incubation buffer. Therefore, we investigated minutely the effect of tungsten particles on plasmid DNA degradation to evaluate the value of this metal element and to diminish the disadvantage of tungsten particles in biolistic bombardment.

In this paper, we demonstrate that alkaline pH is crucial for the DNA/metal particle adsorption in addition to the suppression of tungsten-induced DNA degradation. We also show that additional Tris–HCl/EDTA (TE) buffers with alkaline pH in DNA adsorption mixture effectively suppress cleavage of tungsten-bound DNA.

Materials and Methods

Chemical Reagents, Tungsten, and Gold particles

The chemical reagents used in this study were purchased from Wako Pure Chemical Industries, Ltd., Japan, unless otherwise stated. Tungsten (M-17; average diameter of 1.1 μm) and gold (1.0 μm diameter) particles were purchased from Bio-Rad Laboratories, Inc.

Plasmids

Two plasmids were used in this study: pBI221 (5.7 kb; Jefferson 1987), which carries a *35S::GUS:NOS-T* (nopaline synthase terminator) chimeric gene encoding β-glucuronidase, and p35S-sGFP (4.1 kb; Chiu et al. 1996), which harbors a *35S::sGFP:NOS-T* gene coding for a modified green fluorescent protein. The plasmid DNA was amplified in *Escherichia coli* strain XL1 blue, extracted, and purified using a kit (Qiagen Plasmid Midi; Qiagen Inc.).

Evaluation of Tungsten-Induced DNA Degradation in Solution

The following assays were performed to characterize tungsten-induced DNA degradation in solutions. The ratio (*w*/*w*) of tungsten particles to plasmid DNA (600:1) were followed to the method of Sanford et al. (1993) but the reaction volume was reduced by half. Briefly, plasmid DNA was redissolved in autoclaved water (1 μg μl^{-1} Milli-Q water, Millipore Corp.) immediately before use, to replace 10 mM Tris–HCl (pH 8.0)/1 mM EDTA (10T1E) buffer. Next, the 2.5 μg of DNA was added to 60 μl of water, several kinds of buffers or the solutions of the chemicals used in DNA coating (Sanford et al. 1993). A set of buffers with a broad pH range (2.0–12.0) at intervals of 0.5 (Carmody 1961) was used to determine the pH dependency of tungsten-induced DNA degradation. In addition, diluted TE (0.4T0.04E) buffer was used because it can be brought to the adsorption mixture if the DNA is dissolved in 10T1E buffer. The mixture was then incubated at 25°C or 4°C for the specified period, with or without tungsten particles (1.5 mg) that had been pre-washed successively with 70% ethanol and water. After incubation, the supernatant containing DNA was separated from tungsten particles using two rounds of centrifugation, each for 3 min at 13,200×*g*; the pH of the supernatant was measured using a portable pH meter (twin pH; Horiba Instruments Ltd., Japan). After solvent-exchange from various solutions to 10T1E by the ethanol precipitation, the DNA samples were fractionated on a 0.7% agarose gel, and the gel was stained with ethidium bromide.

Preparation of Three Topological Isomers of Plasmid DNA

Freshly prepared DNA of pBI221 from *E. coli* was used as a closed circular (cc) molecule. In contrast, *Eco*RI-digested DNA of pBI221 was used as a linear molecule. An open circular (oc) molecule of pBI221 was prepared as follows. The pBI221 ccDNA was incubated at 25°C for 24 h in 10T1E buffer containing tungsten particles (tungsten:DNA ratio (*w*/*w*) is 600:1). The recovered pBI221 ocDNA from the supernatant was finally dissolved in water as described in the former section.

DNA Adsorption to Metal Particles and Recovery of the Bound DNA

The plasmid DNA were adhered to the surface of tungsten or gold particles, basically according to the method of Sanford et al. (1993) but on a half scale. Some further modifications were made, as described below. Metal particles were suspended in water, instead of 50% glycerol. Because it is reportedly not beneficial to DNA precipitation and transient GUS expression (Rasco-Gaunt and Barcelo 1999), we excluded glycerol from the adsorption mixture. When testing the effects of TE and borate buffers on DNA adsorption and DNA cleavage on tungsten particles, the particles were suspended in the 2.5 times concentrations of either TE or borate stock buffers adjusted to the specified

pH. While the suspension was mixed vigorously using a microtube mixer (MT-360; Tomy Seiko Co. Ltd.) to disperse metal particles uniformly, the other ingredients; plasmid DNA, $CaCl_2$, and spermidine-free base (Spd; Sigma-Aldrich, St. Louis, MO, USA) were added to the tube containing metal particles, in that order. The mixture was then incubated at 25°C or 4°C for 3 min with vigorous agitation and for one more min without agitation. When the structure of the DNA bound to the tungsten particles was examined, the DNA was eluted from tungsten particles by incubating in 50 mM Tris–HCl (pH 8.0)/1 mM EDTA (50T1E) buffer with agitation. After buffer-exchange from 50T1E to 10T1E, the eluted DNA was fractionated through a 0.7% agarose gel. The DNA levels were estimated, according to the method described by Tanaka et al. (2005). When preparing DNA-coated metal particles to be introduced to plant materials, two plasmids, pBI221 and p35S-sGFP were mixed with a ratio (*w/w*) of 9:1.

Plant Growth Conditions and Preparation of Target Tissues

Tobacco plants (*Nicotiana tabaccum* cv. bright yellow) were grown in black vinyl pots (18.0 cm diameter, 15.0 cm depth) filled with garden soil under natural day length. Leaves with blades of approximately 20 to 25 cm longitudinal length were detached from the plants. In a laminar flow hood, 3×3 cm sections were excised from the leaf blades by using a scalpel, surface-sterilized with 70% ethanol for 30 s, rinsed twice with sterilized water, and blotted onto sterilized paper towels to remove excess water.

Biolistic Bombardment and Histochemical Analysis

Biolistic bombardment was carried out using a particle delivery system (Biolistic PDS-1000/He; Bio-Rad Laboratories Inc.) in accordance with the supplier's specifications. Particles with plasmid DNA were shot to the abaxial sides of the tobacco leaf blades. The optimal bombardment conditions were 28 in Hg in the vacuumed chamber, a target distance of 9 cm, and 900 psi of He gas pressure.

After bombardment, the leaf sections were cultured at 26°C±3°C for 24 h, on two layers of paper towel soaked with sterile water in a 9 cm petri dish under 16 L:8D light conditions. The GFP spots on the leaf sections were counted under a fluorescence microscope (AX80; Olympus Optical Co. Ltd., Japan) using the 470/490 nm excitation and 515/550 nm emission filter set. The enzymatic GUS reaction was then performed in accordance with the protocol described by Jefferson (1987), except that 20% methanol was added in the substrate solution (Kosugi et al. 1990). The GUS spots on the leaf sections were counted under a stereomicroscope (StemiDV4, Carl Zeiss Inc.), after intensive washing with 100% ethanol.

Results

Tungsten-Induced DNA Degradation is Influenced by Temperature, pH, and Salt Concentration

To characterize the chemical properties of tungsten particles on plasmid DNA degradation, pBI221 ccDNA was incubated in the solution containing tungsten particles. The ccDNA in the tungsten-suspended water disappeared immediately after incubation at 25°C (Fig. 1a), although the same DNA remained in the tungsten-free water (Fig. 1a) and gold particle-suspended water (data not shown). At 4°C, however, DNA degradation in the tungsten-suspended water was delayed, and the ccDNA remained at 8 h after the initiation of incubation.

In the absence of tungsten particles, the pH of the DNA-containing water was maintained at approximately 6.0–6.3 throughout the incubation period; on the other hand, the pH value, as reported (Russell et al. 1992), rapidly decreased to around 3.5 in the presence of tungsten (Fig. 1b).

By using a series of borate–citrate–phosphate buffers with a broad range of pH (2.0–12.0), we examined whether tungsten-induced DNA degradation is affected by pH. In the absence of tungsten particles, pBI221 ccDNA did not topologically change in the buffers with pH between 3.5 and 12.0, when incubated at 25°C for 1 h or 24 h, but it was degraded at pH <3.0 within 24 h incubation (Fig. 2). In the presence of tungsten particles, pBI221 ccDNA was converted within 1 h to the corresponding ocDNA by half in the buffer with pH 3.5 to 11.5, and disappeared completely at pH <2.5. However, the plasmid DNA was mostly maintained at pH 12.0. After 24 h of incubation with tungsten, the pBI221 ccDNA changed to either its oc or linear form at pH 3.5 to 10.5, and the DNA disappeared

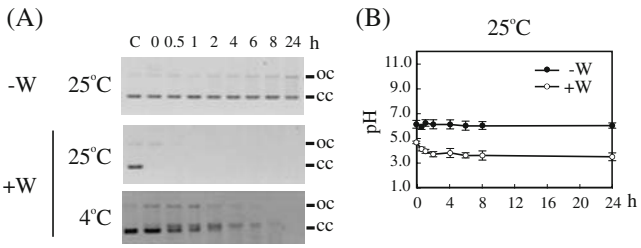

Fig. 1 Effect of tungsten particles on plasmid DNA integrity in water. **a** Plasmid pBI221 ccDNA was incubated with (+W) or without (−W) tungsten particles for the indicated periods, at 25°C or 4°C in pure water. The details of DNA recovery, electrophoresis, and detection are described in "Materials and Methods." **b** The pH in the solution at 25°C was measured at the times indicated and its value is shown as an average with SE of three independent experiments: *C*, non-treated pBI221 ccDNA loaded as a control. *Horizontal bars* along the right side of each photograph indicate the migration positions of the ocDNA (oc) and ccDNA (cc) from the top. *Open* and *closed circles* used in the graph indicate the presence and absence of tungsten particles, respectively

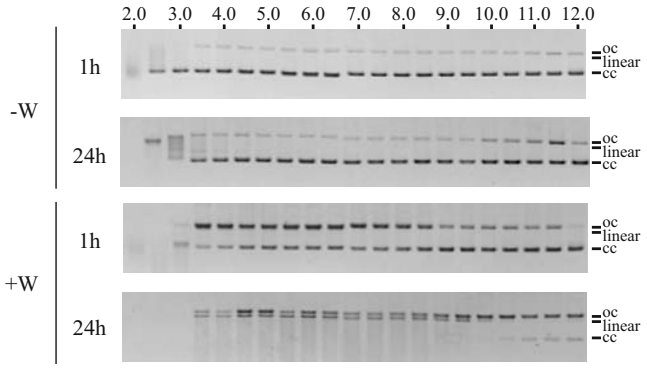

Fig. 2 Effect of pH on tungsten-induced DNA degradation. Plasmid pBI221 ccDNA was incubated with (+W) or without (–W) tungsten particles, at 25°C for 1 or 24 h in buffers with a broad pH range of 2.0–12.0, at 0.5 intervals. *Horizontal bars* along the right side of each photograph indicate the migration positions of the ocDNA, linear DNA, and ccDNA from the top

completely at pH <3.0. At alkaline pH >11.0, a small fraction of the ccDNA was retained.

The plasmid DNA was most resistant to tungsten-induced digestion in the borate–citrate–phosphate buffer (pH 12.0) containing 91.5 mM of tri-sodium phosphate (Fig. 2). In contrast, plasmid DNA was most susceptible to tungsten-induced digestion in the same buffer (pH 2.0) containing only 2.5 mM of tri-sodium phosphate. Therefore, we examined whether the concentration of salt—such as NaCl, which does not have a buffering action—affects this phenomenon in the absence or presence of TE buffer (Fig. 3). In water (no TE), pBI221 ccDNA was mostly digested in the presence of tungsten particles immediately upon the initiation of incubation; however, when >15 mM NaCl was added to the water, the ocDNA was retained, although its ccDNA diminished greatly at the same time point. One hour after incubation, in the solution with <63 mM NaCl, the plasmid DNA was degraded into small fragments, but at more than 125 mM NaCl its ocDNA slightly remained. However, plasmid DNA disappeared completely within 24 h, irrespective of the presence or absence of NaCl.

In contrast, any concentrations of NaCl did not affect the cleavage of plasmid pBI221 DNA by tungsten in 20T1E buffer. A fraction of the plasmid ccDNA changed to ocDNA after 1 h of incubation; most ccDNA converted to ocDNA after 24 h of incubation.

Chemicals Used for DNA/Metal Particle Adsorption Partially Inhibit Tungsten-Induced DNA Degradation

Next, we examined whether tungsten-induced DNA digestion is inhibited by the chemical ingredients used in the most common DNA-coating method (Sanford et al. 1993; Southgate et al. 1995)—namely, 16 mM Spd, 1 M $CaCl_2$, 20% glycerol, and 0.4T0.04E buffer. As shown in Fig. 4a, in solutions containing any of these four chemicals, tungsten-induced DNA degradation was retarded compared to that in water (Fig. 1a). Among these chemicals, 16 mM Spd exhibited the strongest inhibitory activity on tungsten-induced DNA digestion; pBI221 ccDNA persisted after 24 h of incubation, although most of the ccDNA had been converted to ocDNA. However, this conversion was also observed in the absence of tungsten, suggesting the nicking activity of Spd. Calcium chloride (1 M) was found to have the second strongest inhibitory activity. The other two chemicals; 20% glycerol and 0.4T0.04E buffer, also exhibited the weak inhibitory activities. We also measured pH values in these solutions containing tungsten particles (Fig. 4b). The pH value declined in 1 M $CaCl_2$, 0.4T0.04E buffer, and 20% glycerol, in a manner similar to that seen in water (Fig. 1b). In contrast, the pH in the Spd solution remained at around 11.0, irrespective of the presence or absence of tungsten particles.

Tungsten-Induced DNA Degradation is Suppressed in TE Buffer

Tungsten-induced plasmid DNA degradation was affected by the pH values (Fig. 2) and concentrations of salts—such as NaCl and $CaCl_2$—in the solution (Figs. 3 and 4). We examined the effect of TE buffers on tungsten-induced DNA cleavage, because Tris–HCl can act both as a buffer and as a salt. The TE buffers used consisted of different concentrations of Tris–HCl buffer (pH 8.0) and 1 mM

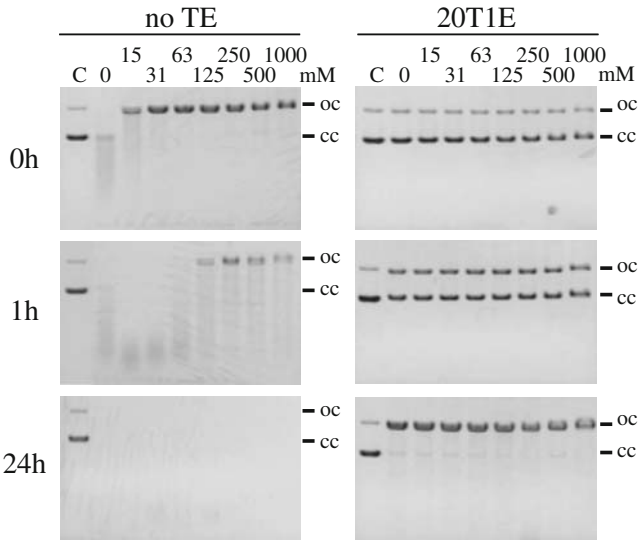

Fig. 3 Effect of sodium chloride on tungsten-induced DNA degradation. Plasmid pBI221 ccDNA was incubated with tungsten particles for the indicated periods at 25°C in water (no TE) or 20T1E containing different concentrations of NaCl. The method and presentation style follow those of Fig. 1a

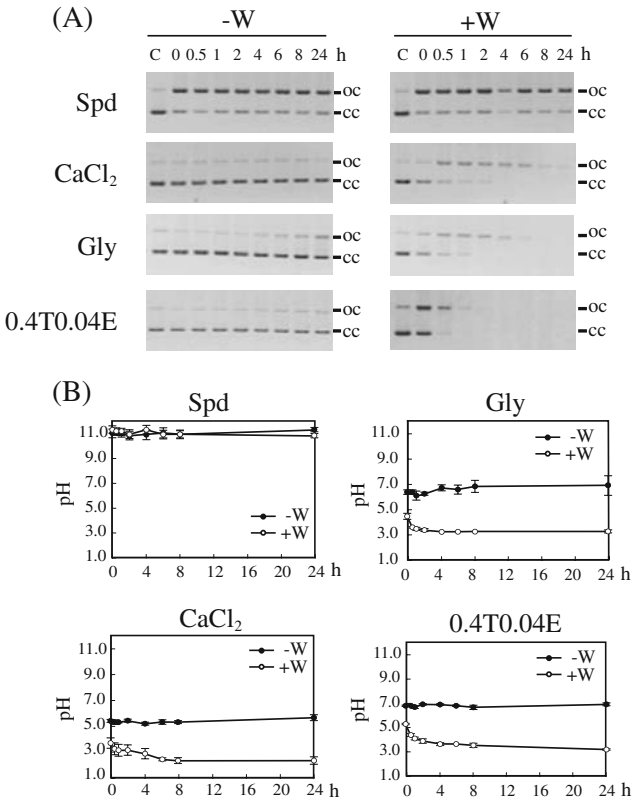

Fig. 4 Effects of chemicals used for DNA/metal particle adsorption on tungsten-induced DNA degradation and acidification. **a** Plasmid pBI221 ccDNA was incubated with (+W) or without (−W) tungsten particles for the indicated periods at 25°C, in each solution of the chemicals indicated. **b** The pH of each solution was measured at the times indicated and its value is shown as an average with SE of three independent experiments: *Spd* 16 mM spermidine-free base, *$CaCl_2$* 1 M calcium chloride, *Gly* 20% glycerol, *0.4T0.04E* 0.4 mM Tris–HCl buffer (pH 8.0) containing 0.04 mM EDTA. The method and presentation style follow those of Fig. 1a

EDTA (except in the case of 0.4T0.04E buffer containing 0.04 mM EDTA). The pBI221 ccDNA was rapidly degraded within 1 h when incubated at 25°C in 0.4T0.04E buffer (Fig. 5), although the rate of DNA degradation fluctuated among the replicated experiments (compare Fig.4a to Fig. 5). The degradation of ccDNA was, however, delayed when incubated at 4°C; ccDNA persisted for 4 to 6 h.

In the three other TE buffers containing >10 mM Tris–HCl, the plasmid ccDNA was more stable than that in the buffer containing 0.4 mM Tris–HCl. Throughout the incubation period at both temperatures, most of the plasmid ccDNA was retained in these TE buffers suspending the tungsten particles, although a fraction of the ccDNA was converted gradually to its ocDNA. The pH values in 0.4T0.04E were more acidic after 24 h of incubation (Table 1); in contrast, the pH of the other three TE buffers remained weakly alkaline throughout the incubation period.

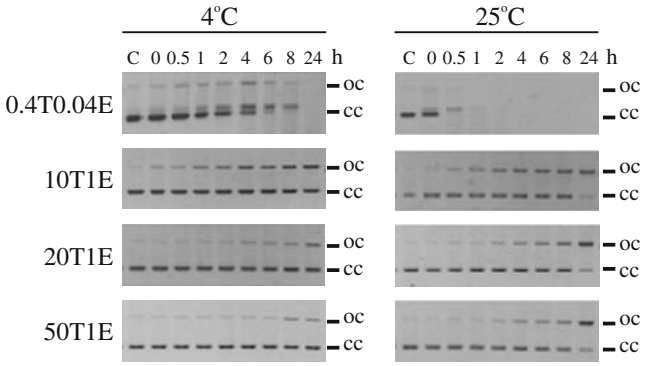

Fig. 5 Effects of TE buffer on tungsten-induced DNA degradation. Plasmid pBI221 ccDNA was incubated with tungsten particles for the indicated periods at 4°C or 25°C in the four TE buffers (pH 8.0), with different concentrations of Tris–HCl salt. The method and presentation style follow those of Fig. 1a

The TE Buffer Affects the Binding of DNA to Tungsten Particles and Structural Integrity of the Bound DNA

We next examined whether TE buffer influences DNA/tungsten particle adsorption and whether the structure of plasmid DNA remains stable on the particle if it is bound. As depicted in Fig. 6, most of the plasmid DNA was recovered from tungsten particles when the reaction was conducted in a coating mixture containing either 0.4T0.04E, 10T1E, or 20T1E; however, when adsorbed in the mixture including 50T1E, more than one-half of the plasmid DNA was recovered from the unbound fraction and the plastic tube surface, although a small fraction of the DNA remained bound to the particles. We next measured the pH of DNA adsorption mixture because it is influenced by added TE stock buffer (2.5×, adjusted to pH 8.0) as well as Spd and tungsten particle (Fig. 4b and Table 1). Among four tested samples, 0.4T0.04E-containing mixture showed the highest pH value (around 9.8). The pH value declined inverse-proportionally to Tris–HCl dose and was the lowest (around 8.5) in 50T1E-containing mixture. The pH value of the mixture was not affected largely by reaction temperature (4°C or 25°C).

Table 1 Changes in pH by tungsten of DNA-containing TE buffers

		0.4T0.04E	10TE	20TE	50TE
−W	0 h	6.8±0.03	7.9±0.06	7.9±0.03	7.9±0.03
	24 h	6.9±0.09	7.8±0.06	7.8±0.10	7.9±0.12
+W	0 h	5.3±0.10	7.7±0.03	7.8±0.10	7.8±0.06
	24 h	3.4±0.07	6.4±0.30	7.2±0.22	7.6±0.15

The TE buffers were adjusted to pH 8.0 before use. The pH was measured after a specified period (h) of incubation at 25°C and its value is shown as an average with SE of three independent experiments

Plasmid ccDNA changed mostly to ocDNA on the tungsten particles when the reaction was conducted at 25°C in the mixture containing 0.4T0.04E, which is the closest to the conventional DNA-binding condition (Sanford et al. 1993). However, the cc:oc ratio of the bound DNA increased, depending on the Tris–HCl salt concentration; the cc:oc ratio was higher in the mixture containing 20T1E or 10T1E than in 0.4T0.04E. Moreover, the cc:oc ratio in 20T1E was indistinguishable between 25°C and 4°C, whereas the ratios in both 10T1E and 0.4T0.04E were temperature dependent.

Alkaline pH of Adsorption Mixture is Critical for DNA/Metal Particle Binding

As mentioned above, DNA binding was inhibited in the presence of 50T1E (Fig. 6). Two possible explanations are at least considered for this phenomenon. One is that the binding efficiency between DNA and tungsten particles is determined by the final pH value of the DNA adsorption mixture, which is balanced by three pH-affecting components: Tris–HCl, Spd, and tungsten particles (Fig. 4b and Table 1). Actually, considerable difference in pH values was observed between the mixtures containing different kinds of TE buffers (Fig. 6). The other is that DNA binding is suppressed by the existence at greater-than-threshold levels of Tris-base ions, chloride ions, or both.

To explore the feasibility of the first explanation, we evaluated the relationship between the pH value of the adsorption mixture and the proportion of DNA bound to the particles (Table 2). Only a small portion of plasmid DNA (<20%) was bound to the particles, when the reaction was conducted in the 50T1E-containing adsorption mixture with final pH values <8.9; pH values of the 2.5× stock buffers added were adjusted to from 8.0 to 8.2. In contrast, most of the DNA (>98%) was bound to tungsten particles in the same mixtures with final pH values >9.4; pH values of the stock buffers were 8.4 and 8.5 in this case. However, the cc to oc conversion of tungsten-bound plasmid DNA was observed in these conditions, as well (data not shown).

Next, we conducted the same experiment using the adsorption mixture containing borate buffer instead of TE to test the second aforementioned explanation (Table 2). Similar results were observed in the case of borate buffer. The pH-dependent DNA adsorption was also observed when substituting gold particles for tungsten particles (data not shown).

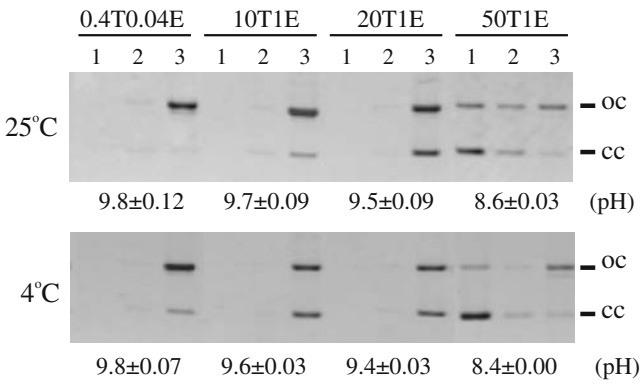

Fig. 6 Dose-dependent effects of TE buffer on DNA adsorption to tungsten particles and integrity of the bound DNA. Plasmid pBI221 ccDNA was bound to tungsten particles at 4°C or 25°C in the DNA adsorption mixture containing each of four kinds of TE buffer whose 2.5× TE stock buffer had been adjusted to pH 8.0 (see details in Materials and Methods). After the coating reaction, supernatant containing the unbound DNA (*1*) were separated from tungsten particles by centrifugation. The resulting supernatant was divided into two aliquots. Each aliquot was used for either DNA analysis or pH measurement. Tungsten particles were transferred to a new tube after preparing suspension in 100% ethanol, and then re-precipitated by centrifugation. DNA adsorbed to the vessel (*2*) and to the particles (*3*) was eluted in 50T1E buffer; it was then ethanol-precipitated, air-dried, and finally dissolved in 10T1E buffer. The methods of DNA handling and presentation style follow those of Fig. 1a. The pH value in DNA adsorption mixture is shown as an average with SE of three independent experiments

Plasmid ocDNA Exhibits Similar Performance to that of the Corresponding ccDNA on Transient Gene Expression

Tungsten-bound plasmid DNA was partially converted to its corresponding ocDNA in all coating conditions tested in this study. Therefore, to evaluate the value of tungsten-generated ocDNA on transformation efficiency, we compared the ocDNA with the corresponding ccDNA or *Eco*RI-digested linear DNA using transient gene expression system. We used chemically inert gold particles as microcarriers instead of tungsten particles to prevent further structural change of the prepared DNA during bombardment. As shown in Table 3, the number of GUS spots derived from the tungsten-generated ocDNA of pBI221 was comparable to that from the corresponding ccDNA, but it was approximately half of that from the linear DNA of the same plasmid. Moreover, a similar tendency was observed using the topological isomers of plasmid DNA, when tungsten particles were used as microcarriers.

Discussion

Tungsten-based biolistic bombardment has long been used to introduce genes into various organisms, although tungsten particles have been pointed out to cause DNA cleavage (Sanford et al. 1993). Herein, we present the characterization of tungsten-triggered plasmid DNA degradation and the betterment of the DNA/tungsten particle adsorption.

Table 2 Effect of pH in the adsorption mixture on the binding of DNA to tungsten particle

Buffer name	pH		DNA bound/DNA recovered (%)[a,b]
	2.5× stock buffer	Adsorption[a]	
50T1E buffer	8.0	8.6±0.18	13±1.3
	8.1	8.8±0.18	21±5.2
	8.2	8.9±0.17	20±4.2
	8.3	9.2±0.19	73±23.9
	8.4	9.4±0.17	98±1.7
	8.5	9.4±0.17	98±1.2
Borate buffer (Carmody 1961)	7.0	7.8±0.03	12±5.5
	7.5	8.0±0.07	9±2.5
	8.0	8.3±0.00	14±0.2
	8.5	8.8±0.10	14±0.4
	9.0	9.4±0.03	89±3.0
	9.5	9.9±0.00	98±1.6

[a] Results are indicated as means±standard errors of triplicate experiments

[b] Numbers indicate total fluorescence estimated from the ccDNA and ocDNA

Plasmid DNA disappeared immediately in water with suspended tungsten particles (Fig. 1) and DNA degradation was observed even in the tungsten suspension containing the chemicals used in the DNA coating method of Sanford et al. (1993), except Spd, when the incubation was prolonged (Fig. 4). Our results apparently conflict with those of Buchowicz's group; their results show that tungsten particles cause only relaxation and linearization, but not fragmentation of plasmid ccDNA (Krysiak et al. 1999a; Krysiak et al. 1999b; Mazuś et al. 2000). Although the causes to explain the different results have not been found yet, it might be due to the experimental conditions of the two laboratories: for instance, tungsten:DNA ratio (*w/w*; 5–50 in their study vs. 600 in our study), the buffer (TE and citrate buffers vs. TE), the incubation time (20 min vs. 30 min to 24 h), and incubation temperature (21°C vs. 4°C or 25°C).

Tungsten particles caused acidification of water (Fig. 1) and solutions containing any of ingredients used in the DNA adsorption, except Spd (Fig. 4b). The rate of tungsten-promoted DNA degradation accelerated at lower pH values and decelerated at relatively higher pH values although tungsten degraded DNA molecules still occurred regardless of pH conditions (Figs. 2 and 4). This finding is consistent with the previously reported pH dependency of tungsten-induced DNA nicking (Mazuś et al. 2000). Furthermore, plasmid DNA degraded at pH of <3.0 without tungsten particles (Fig. 2), xconcurring with the hydrolysis of DNA double helix under acidic pH conditions (i.e., <3.0), which finally generates pyrimidine oligonucleotides (Shapiro 1967). Therefore, severe DNA degradation observed under low pH conditions is likely caused by the concurrent effects of the DNA degrading ability of tungsten itself and tungsten-induced liquid acidification. These results indicate that tungsten-induced DNA degradation is highly dependent on pH and can be most efficiently suppressed when DNA is incubated with tungsten particles in an alkaline solution.

Thus far, tungsten has been reported to mediate various reactions as a metal catalyst, i.e. alkane metathesis (Thomas et al. 1980), hydrocarbon cracking (Grenoble et al. 1983), etc. In addition to pH, incubation temperature (Figs. 1, 5, and 6) as well as salt concentration of the solutions (Figs. 3, 4, and 5) affected tungsten-induced DNA degradation although they bear a lesser influence on this phenomenon than pH value. Taken together, these results suggest that tungsten-induced DNA degradation is another example of the catalytic reactions. Sanford et al. (1993) referred previously to catalysis of tungsten on DNA cleavage, but the data for that was not presented in their article. The mechanism of tungsten catalysis regarding DNA cleavage has not been proven yet; however, free radical species are not likely involved in this phenomenon because radical scavengers such as EDTA and ascorbate do not affect DNA

Table 3 Effect of plasmid DNA topology on transient gene expression

Topology of pBI221 DNA	Spot number/cm^2	Gold particle	Tungsten particle
cc	GUS	124.11±27.95	100.22±17.45
	GFP	677.83±80.56	297.59±54.70
	Efficiency	182.03±32.02b	340.42±35.89a
oc	GUS	70.51±4.53	112.10±28.78
	GFP	319.83±27.53	344.06±17.33
	Efficiency	221.34±5.2b	319.33±64.94a
linear	GUS	195.49±22.24	151.53±51.07
	GFP	407.02±47.07	213.41±26.13
	Efficiency	507.59±118.02c	689.17±199.34c

The efficiency of transient gene expression was presented as the number of GUS spots per one thousand GFP spots. Different letters at the right of the numbers indicate a statistically significant difference of values at $P<0.1$, using a Student's t test

cleavage by tungsten (Krysiak et al. 1999a; Buchowicz and Krysiak 2003).

Concurring with the inhibition of tungsten-induced DNA degradation by alkaline pH, plasmid DNA degradation was strongly suppressed in tungsten-suspended TE buffers (Fig. 5). In addition, these TE buffers were found to suppress degradation of tungsten-bound DNA in a dose-dependent manner (Fig. 6). Namely, a half amount of plasmid DNA was retained as ccDNA on the tungsten particle when the coating reaction was conducted in the mixture containing 20T1E. In contrast, plasmid ccDNA converted mostly to the ocDNA when the reaction was done at 25°C under 0.4T0.04E, which is closest to the conventional DNA binding condition (Sanford et al. 1993). These results suggest that TE buffer with a high dose of Tris–HCl is one of the most effective agents to protect plasmid DNA from tungsten-induced degradation.

To date, the mechanism of DNA/metal adsorption has not been studied in detail, so that the nature of this adhesion is poorly understood (Buchowicz and Krysiak 2003). We disclosed that DNA/tungsten adsorption occurred when the pH value of DNA coating mixture was maintained at 9.4 or more, regardless of a kind of buffer (Fig. 6 and Table 2). The similar behavior was observed when gold particles were substituted for tungsten particles (data not shown). These results suggest that an alkaline pH (greater than about 9.4) of the mixture is essential for DNA binding to metal particles. This is the first report showing the significance of alkaline pH on DNA/metal particle adsorption, to our knowledge.

It is worth discussing the structure of ocDNA made by tungsten and its value in biolistic bombardment. Linear forms of plasmid DNA were never detected in any solutions suspending tungsten particles (Figs. 1, 3, 4, and 5) except in borate–citrate–phosphate buffer with tungsten (Fig. 2). Thus, the results indicate that tungsten-generated ocDNA likely has multiple nicks in its double helical strands. Moreover, tungsten-generated ocDNA was found to be as effective as its ccDNA, but less so than the linear DNA on transient gene expression (Table 3). The evidence explains partly why tungsten particles have survived for a long time in biolistic bombardment experiments even if they mostly convert plasmid ccDNA to its ocDNA, because that tungsten-generated ocDNA is available for at least transient gene expression. However, it has to be examined whether tungsten-generated ocDNA is compatible, as with the cc- and linear forms of plasmid DNA (Nandadeva et al. 1999; Breitler et al. 2002), for stable transformation to estimate its true value in biolistic bombardment.

In conclusion, we disclosed that tungsten has two distinct effects on DNA degradation; it catalyzes DNA digestion with the acidic and neutral pH preference and assists auto-hydrolysis of DNA through liquid acidification (Shapiro 1967). Therefore, alkaline pH is effective in suppressing plasmid DNA breakdown by tungsten. We also found that alkaline pH >9.4 is crucial in accomplishing DNA/metal particle adsorption. Thus, it is important to keep an alkaline pH of the adsorption mixture, especially in the case of DNA/tungsten particle adhesion. However, at the same time, we have to pay attention to tungsten-independent nicking of plasmid DNA occurred at extremely high pH (Figs. 2 and 4). Based on these findings, we could effectively diminish relaxation of tungsten-bound ccDNA using TE buffers with a high dose of Tris–HCl. At the moment, we recommend to keep alkaline pH (>9.4) of the DNA adsorption mixture by using TE buffers containing >20 mM Tris–HCl, to prevent most effectively the cleavage of tungsten-bound DNA in the method of Sanford et al. (1993), in which a half portion of input plasmid ccDNA can be maintained as the same form, regardless of the reaction temperature (4°C and 25°C). Although the problem concerning DNA/tungsten particle adsorption has been partly solved by our study, more efforts to overcome the remaining tungsten's drawbacks (Sanford et al. 1993) are still demanded for the continuous usage of cost-effective tungsten particle in biolistic bombardment.

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