Silicon-induced DNA damage pathway and its modulation by titanium plasma immersion ion implantation

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Abstract

Micronuclei tests (MNT) using the Chinese Hamster Ovary (CHO) cells and Chinese Hamster Ovary Mutant cells (XRS5) have been conducted to evaluate the biocompatibility of silicon and titanium plasma-implanted silicon. Pure Si induces high MN ratios of the two cell lines and thus has poor biocompatibility. The MN ratio of CHO cells is higher than background by about 44% and the MN ratio of XRS5 cells is even higher by about 180%, suggesting that most of the cellular DNA damages on the Si wafer are DNA double-strand breaks (DSB) and are efficiently repaired by the nonhomologous end-joining (NHEJ) pathway. The surface biocompatibility of Si can be enhanced by Ti plasma immersion ion implantation (PIII). The altered oxidized species on the Ti plasma-implanted surface block cellular DSB repaired by the NHEJ pathway and decrease the MN ratio of XRS5 cells. By increasing the Ti implantation time and consequently the Ti implant fluence, the oxygen binding energy shifts toward a lower energy and the intensity of the Si peaks corresponding to SiO2 continually diminishes and even disappear. At the same time, the MN ratios of the two cell lines decrease. Our results suggest that the rest of the DNA damages which cannot be repaired by the NHEJ pathway may be blocked because the surface bonding changes from predominantly Si–O on the 10 min Ti-implanted Si to Ti–O on the 120 min Ti-implanted Si. Our results also suggest that the genotoxicity of cell assay such as MNT and DSB is a valid method to investigate biocompatibility.

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1. Introduction

Silicon is an important semiconductor that is widely used in the microelectronics industry. In recent years, there have been increasing studies on silicon and silicon-based materials due to their potential applications to biomedical devices and medical implants such as biosensors and bio-microelectro-mechanical systems (BioMEMS) [1–6]. With regard to in vitro applications, good bioactivity and biocompatibility are crucial. Silicon is generally considered to have poor biocompatibility [7] which may limit the applications of Si-based biosensors or BioMEMS inside the human body. Therefore, it is desirable to improve the bioactivity and biocompatibility of silicon. Plasma immersion ion implantation (PIII) is a good technique to improve the surface biocompatibility of materials by introducing non-indigenous species to alter the surface structure, composition, and chemistry. Our previous studies have shown that by plasma implanting hydrogen, oxygen, or sodium, the biocompatibility of Si can be enhanced [7–10]. These investigations have been conducted using cytotoxicity assay methods such as cellular lactate dehydrogenase (LDH), alkaline phosphatase (ALP) [11,12], as well as apatite growth [7,10].
Genotoxicity is an important factor when evaluating the bioactivity and biocompatibility of materials. In conventional radiation biology research, the genotoxicity assay is conducted by means of the cellular micronuclei test (MNT) because micronuclei (MN) can embody DNA damages including base damage, DNA double strand breaks (DSB), and phosphodiester backbone damage [13]. DSBs are generally considered to be the most relevant lesion in radiation-induced killing of cells [13]. Recent research on Nijmegen breakage syndrome, which predisposes patients to cancer, suggests a direct link between activation of cell-cycle checkpoints and DSB repair [14]. In vertebrates, DSBs are primarily repaired by Ku-dependent nonhomologous end-joining (NHEJ).

In this paper, MNT of the two cell lines are adopted to evaluate the biocompatibility of Si with and without undergoing Ti PIII [15]. Our results reveal that pure Si induces a high level of MN ratio, and so it is not a biocompatible material, which is consistent with results obtained by other methods [16–18]. However, the cellular MN ratio on Si can be lowered by Ti PIII that alters the surface structure or chemistry of the Si surface. It is found to be able to block cellular DNA damage repaired by NHEJ pathway. In addition, the MN ratio can be further pared by increasing the Ti implant fluence, and it may be attributed to blocking of the other four DNA repair pathways including reversal repair, base excision repair, nucleotide excision repair, and mismatch repair.

2. Experimental methods

2.1. Ion implantation and materials characterization

Single crystal p-type (1 0 0) silicon wafers were used in our experiments. Prior to PIII, the Si wafers were cleaned with HF and deionized water. Ti was plasma-implanted into the polished side of the wafers using a plasma immersion ion implanter equipped with a cathodic arc metal ion source [19,20]. The cathodic arc was ignited using a pulse duration of 300 ps, repetition rate of 30 Hz, and arc current of 1 A. PIII was conducted by applying a pulsed voltage of 5 or 10 kV to the silicon samples. The main treatment conditions are summarized in Table 1.

The surface composition and depth profiles were determined by X-ray photoelectron spectroscopy (XPS) with argon sputtering and the Al K$_\alpha$ excitation line. Contact mode atomic force microscopy (AFM) was conducted on a Park Scientific Instrument (PSI) Autoprobe Research System to evaluate the surface morphology and roughness. The static contact angles on the samples were measured by the sessile drop method on a Ramé-Hart instrument at ambient humidity and temperature using distilled water as the medium.

2.2. Cells and culture medium

Chinese Hamster Ovary (CHO ATCC@ number: CRL-9618) cells and DSB repair-deficient Chinese Hamster Ovary mutant (XRS5 ATCC@ number: CRL-2348) cells were purchased from ATCC. XRS5 belongs to the X-ray complementation group 5 and mutant in the p86 subunit of the Ku autoantigen. Ku has been shown to be involved in DSB repair and V(D)J recombination. The CHO and XPS5 cells were maintained in a complete medium made of a mixture of 45% Dulbecco’s Modified Eagle Medium (D-MEM) (Invitrogen Cat no. 11995-040), 45% F-12 (Invitrogen Cat no. 11765-047) and 10% Fetal Calf Serum (FCS, Hyclone Cat no. SV30087.02). The cultures were maintained at 37°C under 95% air and 5% CO$_2$. The subconfluent monolayers were dissociated with a 0.01% solution of trypsin and resuspended into a fresh complete medium.

2.3. Cytoskeleton staining and MN scoring

A 100 µl complete culture medium containing about 5.0 × 10$^4$ cells (CHO or XRS5) was seeded on each sample for 2 h after sterilization in 75% ethanol and equilibration in a phosphate buffered saline (PBS). After the cells attached onto the surface of the sample, the culture medium was replaced by 4 ml of the fresh medium in a 60 mm dish. The CHO cells were seeded on the samples at a concentration of 25,000 cells/cm$^2$ in the complete medium for 2 days. Afterwards, the samples were rinsed with PBS, fixed with 2% paraformaldehyde, and immunofluorescently stained for the cytoskeleton protein f-actin with phalloidin-fluorescein isothiocyanate (Sigma). The nuclei were counterstained with hoechst33342. Pictures were then taken with a digital camera (Carl Zeiss Axioplan 2). The morphometrics of the cells on the different samples are shown in Fig. 1.

The cytokinesis block (CB) MN technique developed by Fenech and Morley [21] was employed to evaluate the formation of MN. The cells seeded on the samples for 2 days were trypsinized and inoculated in 35 mm tissue culture dishes. The culture medium was then replaced by one containing 2.5 µg/ml CB. After further incubation for 12 h, the cells were rinsed by PBS and fixed in methanol/acetic acid [9:1(v/v)] for 20 min. The air-dried cells were stained with 10 µg/ml acridine orange and observed under a fluorescence microscope. The MN in the binucleated cells were scored and classified according to standard criteria [22] and Y$_{MN}$ was calculated as the ratio of the number of MN to the scored number of binucleated cells. At least 1000 binucleated cells were scored at each sample for the MN measurement in three separate experiments.

2.4. Statistical analyses

Means were obtained from three independent experiments. Three replicates were counted for each experimental point and in each

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Table 1

<table>
<thead>
<tr>
<th>Samples</th>
<th>PIII conditions</th>
<th>Treatment conditions</th>
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<tbody>
<tr>
<td></td>
<td>Working pressure (Pa)</td>
<td>Implantation voltage (kV)</td>
</tr>
<tr>
<td>Ti</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Si</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Ti10</td>
<td>3.4 × 10$^{-2}$</td>
<td>10</td>
</tr>
<tr>
<td>Ti40</td>
<td>3.4 × 10$^{-2}$</td>
<td>10</td>
</tr>
<tr>
<td>Ti60</td>
<td>5 × 10$^{-2}$</td>
<td>5</td>
</tr>
<tr>
<td>Ti120</td>
<td>5 × 10$^{-2}$</td>
<td>5</td>
</tr>
</tbody>
</table>
experiments to determine $Y_{MN}$. All of the results are presented as means with standard deviations. Comparison of $Y_{MN}$ among different samples was based on the Student’s $t$-test.

3. Result and discussion

Figs. 1a–d display the morphometrical assay by immunofluorescent staining of the cytoskeleton protein f-actin (phalloidin-FITC, green). The nuclei counterstained with hoechst33342 (blue) show that cellular cytoskeleton protein f-actin on the pure Si, pure Ti, and Si implanted with Ti for 60 min are the same as those on the culture dish under fluoroscope (Carl Zeiss Axioplan 2). The cells on the different samples cannot be distinguished by the morphmetrical assay under fluorescence microscopy. Therefore, MN studies were performed in order to investigate potential genotoxicity. Fig. 2 shows one of the typical CHO MN seeded in the complete medium for 2 days. Some of the CHO become binucleated cells (indicated by white arrows) after further culturing for 12 h in the complete medium containing $2.5 \mu g/ml$ CB. In addition, some of binucleated cells produce MN as shown by the red arrow. The cytoplasm is stained red whereas the nuclei and MN are stained green and yellow.

The MN rate of the CHO and XRS5 cells seeded on tissue culture dishes (BD Falcon Cat no. 353002) are defined as the background data. Figs. 3a and b show that the MN background values obtained from the CHO and XRS5 cells are $2.033 \pm 0.088\%$ and $2.933 \pm 0.066\%$, and the pure Si wafer induces a relatively high MN ratio whereas the Ti wafer does not introduce changes in the MN ratio for the two cell lines. These results indicate that pure Si produces high genotoxicity, and so it is not a biocompatible material.

DNA damages can be recovered or bypassed by several pathways according to DNA repair mechanisms such as reversal repair, base excision repair, nucleotide excision repair, mismatch repair and DSBs repair [23]. DSBs potentially induce cell death [24–26]. In higher eukaryotes, inactivating an essential gene of a single nonrepaired DSB can be sufficient to cause cell death via apoptosis [27]. There are two pathways for DSB repair, homologous recombination (HR) and NHEJ. In a simple eukaryote like yeast, HR is the main pathway whereas in mammals, the NHEJ pathway predominates [28,29]. Here, NHEJ damage refers to the damage repaired by NHEJ pathways.
The DNA damage can be embodied by the increase in the MN ratio [30,31]. CHO is the wild-type Chinese Hamster Ovary cell line. XRS5 is a DSB repair-deficient Chinese Hamster Ovary mutant cell line which mutates in the p86 subunit of the Ku autoantigen. Ku is the sensor that detects DNA damages by binding to the broken ends and bringing them together by binding two DNA molecules [32]. XRS5 belongs to the Ku autoantigen and DSBs of this cell cannot be repaired by the NHEJ pathways. In other words, the MN ratios of the CHO and XRS5 cells should be at the same level on the surface treated similarly if DNA damages are not repaired by Ku-dependent NHEJ.

The MN ratios of the two cell lines are normalized to background as 100% as shown in Fig. 3c. The MN ratios of CHO and XRS5 on pure Ti are similar, suggesting that Ti does not induce NHEJ damage. However, the MN ratio of CHO is higher than the background value by about 44% and that of the XRS5 cells is even higher by about 180% revealing that 77% of the silicon induced DNA damage is repaired by the NHEJ pathway.

It is well known that the surface composition and morphology of biomaterials influence the cell behavior [33–36]. As shown in Figs. 3a and b, the Ti-implanted Si sample has low MN ratios. The MN ratio of the XRS5 on the 10 min Ti-implanted Si is smaller than that on pure silicon by 122%. However, the MN ratios of CHO on pure Si and 10 min Ti-implanted Si are about the same as shown in Fig. 3c. With regard to the 120 min Ti-implanted silicon sample, the MN ratios of the CHO and XRS5 are the same as those on the dish and pure Ti (Fig. 3c). The MN ratios of the CHO and XRS5 measured from the other samples decrease with longer Ti PIII time. The depth profiles of Ti and Si in the Ti PIII samples versus implantation time are depicted in Fig. 4. The Si intensity decreases continuously but the Ti intensity increases with implantation time. An intense oxygen peak is observed from the 10 min Ti PIII Si. In addition, the Si signal corresponding to SiO$_2$ is enhanced, indicating that a large amount of SiO$_2$ exists on the surface of the Ti PIII Si. In comparison, the oxygen signal and Si signal corresponding to SiO$_2$ measured from pure Si are relatively weak (Fig. 5a). It is thus believed that the cellular NHEJ damage caused by pure Si may be blocked by different oxide species on the surface. In fact, with longer implantation time, the oxygen binding energy shifts toward a lower energy (Fig. 5b), the Si content corresponding to SiO$_2$ is lower, and the Ti content increases. The surface bonding states change from dominantly Si–O to Ti–O. Figs. 3a and b also show that the MN ratios of both the CHO and XRS5 cells decrease with longer Ti implantation time. Our results suggest that DNA damages may be caused by the presence of Si–O.

The AFM images of the pure silicon and Si plasma-implanted with Ti for 10 min and 60 min are shown in Figs. 6a–c, respectively. The pure silicon surface is quite smooth with a mean roughness of 0.058 nm and the Ti-implanted Si wafers are rougher with mean roughness.
values of 0.288 and 12.2 nm for the 10 and 60 min Ti-implanted Si samples. The surface contact angles measured from the tissue culture dishes (BD Falcon Cat no. 353002), Si wafer, and Ti-implanted Si (10, 40, 60, and 120 min) are shown in Fig. 6d. The contact angles on the surface increase with Ti PIII time (from 10 to 60 min), but the contact angles drop after 60 min Ti PIII. This can be attributed to the change in the physical and chemical properties as well as morphology of the surface. The influence of the wetting properties and morphology on the change of the MN ratios needs further studies and new findings will be reported in due course.

4. Conclusion

CHO and XRS5 micronuclei tests (MNT) are employed to evaluate the potential biocompatibility of Si and Ti-implanted Si. Pure Si induces a high MN ratio which is mainly caused by DSB and so may possess a poor biocompatibility. Cellular DNA damages on the Si wafer are mainly repaired by the NHEJ pathways. The MN ratio on Si can be lowered by Ti plasma immersion ion implantation (PIII) for 10 min. The higher oxide content on the surface blocks the cellular DNA damage repaired by NHEJ pathway and decreases the MN ratio for XRS5. By increasing the Ti implantation time and consequently the Ti fluence, the oxygen binding energy shifts to a lower value. The intensity of the Si peak corresponding to SiO2 diminishes and at the same time, the MN ratios of the two cell lines decrease. Thus, it can be reasonably concluded that the rest of DNA damages which cannot be repaired by the NHEJ pathway may be blocked because of the change from dominantly Si–O to Ti–O bonding states on the Ti-implanted Si wafers. Hence, the MN ratios are lower.
with longer Ti implantation time. The results demonstrate that the genetic toxicity of cell assays such as MNT and DSB assay is a valid method to investigate biocompatibility.

Acknowledgments

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