Grafting of Biocompatible Polymers on DLC Thin Films by Plasma Irradiation-Post Polymerization Technique for Application of Biomedical Devices and Cell Microarrays

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Two-step surface modification of metal or glass substrates has been developed for the long-term reliability and safety of biomedical devices (stents, catheters, blood contacting parts) and cell microarrays for cell diagnosis and cell recovery. First step, carboxylic group (COOH)s containing DLC (DLC/OOH) were coated on an aluminum surface by a plasma enhanced chemical vapor deposition (PECVD). Second step, an anti-thrombogenic, poly(N-α-(methacrylamide-L-lysine) (PLysMA) or poly[2-(meth-acryloyloxy)ethyl phosphorylcholine] (PMPC) was grafted on the DLC/OOH surface by plasma irradiation-post polymerization technique.

Furthermore, to develop novel cell microarrays having regulatory-arranged glass spots and coated the surface with DLC/OOH around the spots for evaluation of cell function and selection of targeting cells by immobilization of single cells on the spots, the microarrays were also grafted with PMPC on the DLC/OOH by the plasma irradiation-post polymerization technique. Surface biocompatibility and characterization were examined by SPM, XPS, and adsorption of plasma proteins.

Keywords: plasma irradiation-post polymerization, poly(N-α-(acrylamide-L-lysine), poly[2-methacryloyloxy)ethyl phosphorylcholine], DLC, cell microarray

1. Introduction
Physically stable diamond like carbon (DLC) coatings give many potential applications on biomedical fields. Metallic biomedical devices show long-term shape stability and durability, however their biocompatibility of untreated surfaces was usually very low. Drugs coating for improving the biocompatibility on the untreated metallic devices easily peel off to lost rapidly their functionality with no attractive interaction between drugs and metal surfaces. The newly developed biomimetics DLC thin film showed a high cyto compatibility on the surface of stent by plasma treatment [1, 2]. Thus the development of biomimetic DLC thin films with a higher biocompatibility is expected [3].

On the other hand, the grafting of biocompatible polymers has promised an effective improvement of the biocompatibility of medical devices in the long periods. For example, the surface grafting of zwitterionic poly[2-(methacryloyloxy)ethyl phosphorylcholine] (PMPC) provides both biocompatibility and wear resistance of tibial polyethylene insert in artificial knee joints [4]. We have also developed the low-temperature
plasma irradiation-post polymerization technique as a surface treatment method [5]. Therefore, the biocompatibility of DLC coated metallic or inorganic biomedical devices are considered to be improved by the plasma irradiation-post polymerization of biocompatible PMPC, zwitter-ionic poly(o-methacryloyl-L-serine) [6], poly[N-α-(meth)acrylamide-L-lysine] [7], and poly[N-ε-(meth)acrylamide-L-lysine] [7], etc.

An embryonic stem (ES) cell or an induced pluripotent stem (iPS) cell has attracted much attention as a potential cell source for regenerative medicine. One of key technology of clinical application of their cells is the diagnosis and the selection of induced differentiated cells [8, 9]. Microarrays are recognized as one of major tools in the assessment of gene expression via cDNA or RNA and are now accepted as a powerful experimental tool for high-throughput screening of a large number of samples [8, 10]. Hence, single cell attached microarray (cell microarray)s have a potential function for high-throughput screening of candidate cells for specific ES or iPS cells differentiation. For this purpose, surface treatments are needed to fabricate a cell microarray with spontaneously regulatory-arranged cells after be seeded the candidate cells.

In this study, we attempted low temperature plasma surface treatment on DLC coated substrates such as an aluminum substrate or a glass to improve the biocompatibility of metallic or inorganic materials and to develop a novel cell diagnosis tool. The grafting of biocompatible poly[N-α-methacrylamide-L-lysine (LysMA)] (PLysMA) or PMPC was carried out on DLC coated an aluminum or a microarray with regulatory-patterned micro-sized spots on a glass substrate by the plasma irradiation-post polymerization technique. The surface characterization was also examined (Figure 1).

![Chemical structure of biocompatible polymer](image)

Figure 1. Chemical structure of biocompatible polymer.

2. Experimental
2.1. Materials
LysMA [7] and MPC [11] were synthesized according to previous reports. Bovine serum fibrinogen (Fib), human serum plasminogen (Plg), human serum tissue-type plasminogen activator (t-PA), and human serum thrombin (Th) were obtained from Sigma Chemical Co. without further purification. 2,2-diphenyl-1-picrylhydrazyl (DPPH: Wako Pure Chemical Industries Ltd.) and Fib (Wako Pure Chemical Industries Ltd.) from bovine plasma Fib containing Plg for fibrin plate assay, 4-amino-fluorescein (4-AFL: Tokyo Kasei Industry Co.) were used without further purification.

2.2. Preparation of DLC thin film
DLC was deposited on an aluminum substrate with ionized-assisted deposition of plasma enhanced chemical vapor deposition (PECVD) [1-3]. To introduce COOH groups into the DLC (DLC/COOH) surface, DLC coated an aluminum substrate was treated by using capacitive coupled plasma (CCP) with the radio frequency (RF) power of 100 W, with O₂ gas introduced into the chamber under 4 Pa, and an irradiation time for 15 s.

2.3. Preparation of DLC microarray
DLC was deposited on a glass substrate (10 mm × 10 mm) with PECVD using CCP [12] with C₂H₂ gas introduced. DLC microarray was prepared with technique of photolithography. A photo resist was spread out on the DLC coated glass substrate by using a spin coater, and precisely patterned photo mask (7 mm × 7 mm) made from quartz glass and chrome was subsequently casted on the DLC coated substrate. After the resulting substrate was exposed to ultra violet light, the photo resist was stripped with developer. Then, the DLC films were etched by reactive ion etching (RIE) with O₂ plasma. The process chamber was connected to an RF power supply. The RF power of 100 W was applied to generate CCP under 4 Pa, for 180 s. During RIE process, the DLC coated glass substrate was applied with self-bias (Vdc) of -630 volt. Finally, remaining photo resist was stripped with acetone. In this experiment, each spot with 50 µm diameter was arrayed in matrix at a low and a line spacing of 100 µm on the microarray (10 mm × 10 mm in size). The DLC microarray was also treated with O₂ plasma to introduce COOH groups into the DLC (DLC/COOH) surface.

2.4. Plasma irradiation-post polymerization on DLC coated aluminum
A strip of DLC coated an aluminum film (10 mm × 10 mm, thickness=50 μm) was washed with methanol, dried in a vacuum at room temperature. After the film was irradiated with Ar plasma [13], a known amount of LysMA or MPC was post-graft polymerized in the presence of ammonium peroxodisulfate in phosphate buffer saline (PBS: pH=7.4) or ethanol solution at 70°C for 20 h to give the PLysMA or PMPC grafted DLC/(COOH) [PLysMA/PMPC-g-DLC/(COOH)].

2.5. Plasma irradiation-post polymerization on DLC/(COOH) microarray
PMPC grafted on microarray (10 mm × 10 mm) was prepared by a manner similar with above 2.4 term.

2.6. Surface characterization
Surface characterization of g-DLC/(COOH) was analyzed from contact angle to water [13], scanning probe microscope (SPM: Shimizu SPM-9500J3) by phase imaging mode, and X-ray photoelectron spectroscopy (XPS: Jeol JPS9010). XPS measurements were carried out with non-monochromatized AlKα rays (1486.3 eV) as a X-ray source, at an acceleration voltage of 10 kV, an emission current of 10 mA. The detection angle of photoelectron was 0 degree for DLC/(COOH) and 70 degree for g-DLC/(COOH), respectively. The pass energy was 10 eV for DLC/(COOH) and 30 eV for g-DLC/(COOH), respectively. Fluorescence microscopy was observed using an Olympus inverted microscope (type: CK X 31).

2.7. Protein adsorption on g-DLC/(COOH)s
The biocompatibility of the g-DLC/(COOH)s was evaluated by protein adsorption. The adsorption protein was detected by antigen-antibody test [14]. Primary antibodies [anti/t-PA (rabbit) IgG, anti/Plg (rabbit) IgG, anti/Fib (Goat) IgG; MP Biomedicals] were used after be 10,000 times diluted with PBS solution containing 10 wt% skim milk and 0.1 wt% tween20. Horseradish peroxidase labeled secondary antibody (anti/rabbit IgG and anti/Goat IgG, MP Biomedicals) were used after be 20,000 times diluted with PBS solution containing 0.5 wt% tween20. The detection of immobilized specific antigens (Plg, t-PA, and Fib) was used with a ECL Plus Western blotting detection reagent (Amersham Bioscience) after 25 μL of Stock Acridan solution in dioxane/ethanol was diluted with 1000 μL of Tris buffer (pH 7.5) solution.

2.8. Fibrin plate assay [15]
Four milliliter of bovine serum Fib containing 72.1 wt% Fig and 27.9 wt% Plg was poured into a petri dish (φ=6cm). The g-DLC/COOH film was placed on the bottom of the dish. One hundred microliter of the mixed solution with a concentration of 1 : 3 (10 IU/mL of Th in physiological salt solution; 300 μg/mL of CaCl2 in aqueous solution in volume ratio) was added and allowed to stand for 1 h at 37°C to give a fibrin plate. Fibrinolytic activity was evaluated from a lysis area of the fibrin plate with a constant interval (30 min) after be dropped vertically with 60 μL of PBS (pH 7.4) containing t-PA (0.25 μg/mL) on the center of the g-DLC/COOH film.

3. Results and Discussion
3.1. Grafting of biocompatible polymers on DLC/(COOH) surface
The use of low-temperature plasma has been studied to modify surface properties. In the case of plasma irradiation-post polymerization, the graft polymerization is initiated with peroxides generated by oxidation when the organic surface is brought into contact with air after exposure to Ar plasma [13]. The generated peroxides on the surface of DLC/(COOH) were detected with the DPPH as a radical scavenger according to the previous report [13]. Figure 2 shows the concentration of free radicals on the DLC film calculated from the DPPH consumption as a function of Ar plasma irradiation time. The concentrations of free radicals of DLC

![Figure 2. Concentration of peroxides produced on (A) DLC/COOH, (B) DLC as a function of time, Ar plasma irradiation condition; RF power=20 W, Ar flow =10 sccm, initial pressure=1.3 Pa, post treatment condition; air exposure time=60 sec, at 70°C, for 20 h, [DPPH]=1.0×10^-4 mol/L, the film immersed in a 3mL of benzene solution.](image)
/COOH treated with O₂ plasma were higher than that of DLC treated with Ar plasma. From the XPS measurements, the amount of COOH groups of the DLC/COOH is 4 times higher than that of the DLC. The amount of COOH groups was calculated with 1.2 μmol/cm² on the DLC/COOH by using titration method [16]. The results of Ar plasma-post polymerization are shown in Table 1. From this table, the amount of graft polymers on the DLC/COOHs was higher than that of the DLCs. The contact angle (θ)s of biocompatible polymer grafted DLC/COOH (g-DLC/COOH) were also lower than those of DLC (g-DLC)s. As a result, the surface of COOH group introduced DLC provides an effective generation of free radicals. Hence, it is considered that the amount of hydrophilic graft polymers on the DLC/COOHs increased comparing with the DLCs and the contact angle (θ)s of the g-DLC/COOHs also decreased comparing with those of the g-DLCs.

3.2. Characterization of g-DLC/COOH
The contact angles (θ)s of g-DLC/COOHs and comparing with those on the DLC/COOHs. The

Table 1 Characterization of g-DLC and g-DLC/COOH

<table>
<thead>
<tr>
<th>g-DLC</th>
<th>g-DLC/COOH</th>
<th>Amount of graft polymer (mg/cm²)</th>
<th>Contact Angle (°)</th>
<th>Mn (g-DLC/COOH)</th>
<th>Mn (g-DLC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLysMA-g-DLC</td>
<td>0.020</td>
<td>45</td>
<td>8.3 x 10³</td>
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<tr>
<td>PMPC-g-DLC</td>
<td>0.007</td>
<td>20</td>
<td>2.9 x 10³</td>
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<td></td>
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<tr>
<td>PLysMA-g-DLC/COOH</td>
<td>0.038</td>
<td>25</td>
<td>8.0 x 10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMPC-g-DLC/COOH</td>
<td>0.030</td>
<td>7</td>
<td>6.3 x 10³</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) value of untreated DLC/COOH (64 degree), DLC (77 degree).
b) Number average of molecular weight (Mn) of the grafting polymer on g-DLC/COOH or g-DLC, calculated from the amount of graft polymer and the amount of free radicals on DLC/COOH or DLC surface by radical scavenger method using DPPH.

Figure 3. SPM images of DLC/(COOH) and g-DLC/(COOH)s by phase imaging mode; (A) DLC, (B) PLysMA-g-DLC, (C) PMPC-g-DLC, (D) DLC/COOH, (E) PLysMA-g-DLC/COOH, and (F) PMPC-g-DLC/COOH (measured area; 500 nm x 500 nm).
patible polymers were considered to be coated with a uniform manner on the DLC/(COOH)s. The signal of N\textsubscript{1s} (BE: 402.7 eV) on the PLysMA-g-DLC/(COOH)s and the signals of P\textsubscript{2p} [Binding energy (BE): 133.6 eV] and N\textsubscript{1s} (BE: 399.9 eV) on the PMPC-g-DLC/(COOH)s were also detected by XPS measurements in contrast to no N\textsubscript{1s} or P\textsubscript{2p} signal detection on the untreated DLC/(COOH). Therefore, the immobilization of biocompatible PLysMA or PMPC was confirmed on DLC/(COOH) surfaces by the plasma irradiation-post polymerization technique.

3.3. Adsorption of serum proteins on g-DLC/(COOH)s and fibrinolytic activity on PLysMA-g-DLC/(COOH)

The plasma protein adsorption is the initial event in blood-material interaction. The low adsorption of blood coagulation proteins shows normally a high biocompatibility [17]. On the other hand, the selective adsorption of fibrinolytic protein of Plg and t-PA serves as the anti-thrombogenic surface [7]. The low adsorption of blood coagulation protein, Fib was also observed on the PLysMA-g-DLC/(COOH) and the PMPC-g-DLC/(COOH) surfaces comparing with the untreated DLC/(COOH)s (Figure 4). The low Fib adsorption on the PMPC-g-DLC/(COOH) and the PLysMA-g-DLC/(COOH) was considered to be attributed to a hydrogel layer formed with the zwitterionic biocompatible graft polymers. For example, a hydrophobic interaction between proteins and g-DLC/(COOH) suppressed by a hydrogel structure [18]. The enhancement of fibrinolytic activity on the PLysMA-g-DLC/(COOH) was observed comparing with that on the PMPC-g-DLC/(COOH) or the untreated DLC/(COOH) based on Plg and t-PA selective adsorption on the PLysMA-g-DLC/(COOH) by using a fibrin plate assay (Figure 5). The authors reported that the selective adsorption of Plg on the PLysMA immobilized surface in contrast to the low adsorption for Fib or γ-globulin on the same surface was observed by binding assay using resonant mirror biosensor and the fibrinolytic activity also enhanced in the presence of PLysMA [7, 19].

![Figure 5. Fibrinolytic activity test on (A) PLysMA-g-DLC/(COOH), (B) PMPC-g-DLC/(COOH), and (C) untreated DLC/(COOH).](image)

As a result, the higher biocompatibility of PLysMA-g-DLC/(COOH)s or PMPC-g-DLC/(COOH)s showed than untreated DLC/(COOH)s. The biocompatibility of DLC/(COOH) surfaces was improved to graft the biocompatible polymer by plasma irradiation-post polymerization technique.

3.4. Grafting of PMPC on DLC/(COOH) microarray

To attach spontaneously single cell on every spot fabricated on a cell microarray after be seeded cells, PMPC was grafted on the DLC/(COOH) region around glass spots on the microarray for precise cell diagnosis, because cell attachment on the PMPC-g-DLC/(COOH) is reduced by the formation of hydrogel layer based on the biocompatible PMPC segments.

The contact angle (θ=5 degree) of microarray after PMPC grafting decreased that (θ=50 degree) of original one. From the XPS measurements, P\textsubscript{2p} signal was detected on the PMPC grafted DLC/
COOH microarray. Figure 6 shows the fluorescence microscope images of the microarray after being immersed in an ethanol solution containing 0.1 mM of 4-AFL as a model of fluorescence probe-labeled cell at room temperature for 1 hr. The fluorescence from 4-AFL on the glass spots was specifically detected despite low fluorescence from the PMPC grafted DLC/COOH region around the glass spots. The low 4-AFL adsorption on the grafted PMPC surface was considered to be attributed to the suppression of hydrophobic interaction between 4-AFL and the PMPC grafted surface to give the selective adsorption of 4-AFL to glass spots on the DLC/COOH microarray.

![Figure 6. Fluorescence microscope images of the DLC/COOH microarray after adsorption with 4-AFL, (A) magnification ×40, (B) magnification ×100.](image)

4. Conclusion

The immobilization of biocompatible PLysMA or PMPC on the DLC/COOH was confirmed by plasma irradiation-post polymerization technique. The amount of graft polymers on the DLC/COOH increased compared with the DLC. The biocompatibility of DLC/COOH mediated metallic devices was drastically improved by immobilizing PLysMA or PMPC.

To prepare cell non-attached region for single cell attached microarray, the selective grafting of PMPC on the DLC/COOH around the glass spots on the cell microarray was successfully by the plasma irradiation-post polymerization.

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