Effect of Tetrafluoromethane Plasma Treatment of PMMA on MCF-7 Cell Proliferation

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Abstract: Polymethyl methacrylate (PMMA) was surface modified by tetrafluoromethane (CF_4) plasma and its effect on the human breast cancer cells (MCF-7) adhesion and proliferation were investigated. The promotion of MCF-7 cell growth was positively and quantitatively related to the CF_4 plasma modification as revealed by different characterizations. From ESCA analysis, it is revealed that CF_4 plasma treatment chemically alters the capability of PMMA to adsorb proteins from cell culture medium, which is about four times comparing to that of pristine PMMA. In addition to chemical alteration, CF_4 plasma also concurrently delivered physical modification by doubling the surface roughness, as observed by AFM analysis.

Keywords: Tetrafluoromethane plasma, surface modification, cell proliferation, MCF-7.

1. INTRODUCTION

Poly (methylmethacrylate) (PMMA) has been widely used as the material for substrates in biomedical application for its advantages of chemically inert, transparent, excellent mechanical properties, low cost, and ease of fabrication. It has been widely used in applications such as tissue engineering [1], microarrays [2], biosensors [3], and MEMS [4]. To tether the bioactive compounds to PMMA, surface modification methods, such as wet chemical method, ionized gas treatment, and UV radiation need to be conducted. Surface properties such as electrical charge, functional groups [5], and contact angle [6-7] play important roles on governing the response of biomolecules to the surface [6, 8-10].

Wet chemical method is a classical approach that can be performed at any laboratory without specialized equipment. It has been demonstrated that the PMMA surface can be activated by hydrolysis through sodium hydroxide or sulfuric acid [1, 11-13], followed by aminolysis using various diamines to introduce primary amines onto the PMMA surface. In recent years, plasma treatment has demonstrated to be an effective surface modification method. The desired functional groups were incorporated on the top layer of the PMMA surface by selecting appropriate plasma gases such as Ar, N_2, O_2, or CF_4. In addition, plasma polymerization can also be used in preparing thin films for coatings, immunosensors, and interfaces for biomaterials [14-16]. The advantages of plasma polymerized thin films include pin-hole free, great homogeneity, mechanical, chemical, and adhesion properties [16].

PMMA has been used in a variety of biomedical applications such intraocular and contact lenses. The modification of PMMA surface plays important roles on cell adhesion and attachment. Researchers in the past had demonstrated methods to control cell adhesion on PMMA by incorporating the surface with heparin [17], poly(ethylene glycol) [18], RGD peptide [19], implanting positive fluorine [20], and oxygen plasma treatment [21].

In this work, the effect of CF_4 plasma modified PMMA on cell proliferation was evaluated by complementary surface characterizations such as surface wettability, the Electron Spectroscopy for Chemical Analysis (ESCA), and atomic force microscopy (AFM). Human breast cancer cells (MCF-7) were used in this study in an effort to develop a microfluidic drug screening system.

2. MATERIALS AND METHODS

2.1. Surface Modification

The plasma treatment was carried out at the plasma chamber with constant pressure evacuated by a mechanical pump. Plasma was generated by applying power from a radio frequency generator (13.56 MHz). The PMMA surface modification was processed with pretreatment by using

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oxygen plasma with 10 sccm flow rate for 10 min at applied power of 20 Watt. CF_4 plasma treatment was then carried out by introducing gas flow at 10 sccm with the total pressure at 100 mTorr for 1 min.

In addition to CF_4 plasma modified PMMA, pristine PMMA and a tissue culture polystyrene (TCPS) dish (Nunclon™) were used as the reference materials in order to demonstrate the effect of CF_4 plasma treatment on MCF-7 cell proliferation.

2.2. Cell Culture

The MCF-7 human mammary epithelial cell line, available from ATCC, was used in cell culture experiments. The cells were incubated with DMEM/F-12 medium (Invitrogen) at 37°C in a humidified 5% CO_2 incubator for 24 h. A suspension of 6×10^4 cells/ml was prepared in this experiment.

2.3. Cell Proliferation Test

The number of living cells in culture was quantified using the CellTiter 96 aqueous one solution cell proliferation assay (Promega). Test wells were fabricated by attaching a PDMS block (with wells) to the modified PMMA surfaces. MCF-7 cell suspension in medium of 500 μL (3×10^4 cells) were placed in each well and incubated at 37°C with 5% CO_2 for 1, 4, 12, and 24 h. After incubation, the medium in each well was removed and a mixture of 10 μL MTS reagent and 100 μL of medium was added to each well and incubated for 15 min. An amount of 100 μL of solution in each well was withdrawn and injected into wells in a microtiter plate, respectively. Optical density (OD) in each well was measured at 492 nm using a Microplate reader (Triad, Dynex Technologies). In addition, the morphology of cells was also examined by using an inverse optical microscope (IX71, Olympus).

2.4. Surface Protein Content Analysis

The sample plates were prepared as 1 x 1 cm^2 and were washed by ethanol and deionized water, followed by nitrogen gas flow. The sample plates were immersed in cell culture medium for 24 h and then dried in laminar flow and kept in desiccator prior use. The surface characterizations were performed by water contact angle (WCA), ESCA, and AFM. The static contact angle measurement was carried out with a goniometer. At least five contact angle measurements were performed for each sample. Elemental and compositional analyses were carried out using X-ray photoelectron spectroscopy (Thermo VG Scientific Theta Probe) with Al (1486.6 eV) and Mg (1253.6 eV) as sources of X-rays. Morphological observations of protein adsorbed samples were carried out by Atomic Force Microscopy (Digital Instruments Nanoscope III Atomic Force Microscopy, Veeco Ltd.).

2.5. Statistical Analysis

The cell proliferation results were statistically analyzed by employing one-way analysis of variance (ANOVA) executed by Minitab® statistical software. Fisher's pairwise comparison test was applied to compare the effect of different surfaces (TCPS, pristine PMMA and CF_4-plasma-treated PMMA) utilized to grow MCF-7 cells in order to determine which sample yields higher cell number, indicated by: (*) = p <0.05; (**) = p <0.01; and (***) = p <0.001.

3. EXPERIMENTAL RESULTS

3.1. Cell Proliferation

MCF-7 cells were seeded and incubated for 24 h in the wells in which the surfaces of PMMA were modified. The proliferation of cells was measured by conducting MTS assay and the results were shown in Fig. (**1**). Compared to TCPS standard and the pristine PMMA, the CF_4 plasma modified PMMA exhibited the highest growth rate for MCF-7 cells. For the cells incubated for 12 h, PMMA surface modified by CF_4 plasma improved the growth of MCF-7 cells by 73.6%, and 89.3%, than that on TCPS and pristine PMMA, respectively. After 24 h incubation, although the increment was not as significant as that of 12 h incubation time, a moderate cell density enhancement for the MCF-7 cells on the CF_4 plasma modified PMMA was 9.9% and 31.7%, compared to TCPS and pristine PMMA, respectively. The statistic analyses clear demonstrate that the CF_4 plasma can improve the growth of MCF-7 cells than that on TCPS and pristine PMMA, for both incubation duration of 12 and 24h, as indicated by $p < 0.001$.

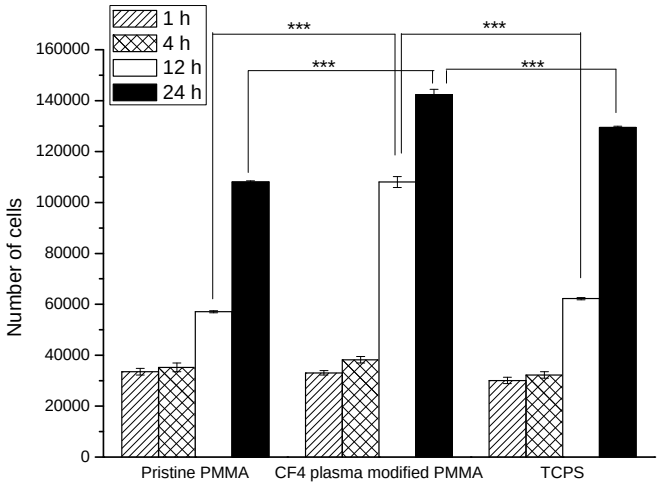

Fig. (1). MCF-7 cell count on various surfaces after incubation for 1, 4, 12, and 24 hours. (*) = p <0.05; (**) = p <0.01; (***) = p <0.001.

This analytical observation by MCF-7 cells proliferation test was also supported by physical appearance as evidenced by optical microscopy images (Fig. **2**). Superior coverage and adhesion of MCF-7 cells were revealed on CF_4 plasma treated PMMA, compared to that on pristine PMMA and TCPS surfaces. Different MCF-7 cell morphology was also perceived. On pristine PMMA, the cells were grown apart, with small amount of proliferated cells and mostly were in round shape, suggesting less active growth preference on pristine PMMA. Less round and more proliferated cells grown were observed on TCPS, the standard for cell culture substrate. Astounding proliferation was demonstrated on CF_4 plasma modified PMMA, with the least amount of round cells, and very intense proliferated MCF-7 cells that outperform pristine PMMA, and even TCPS.

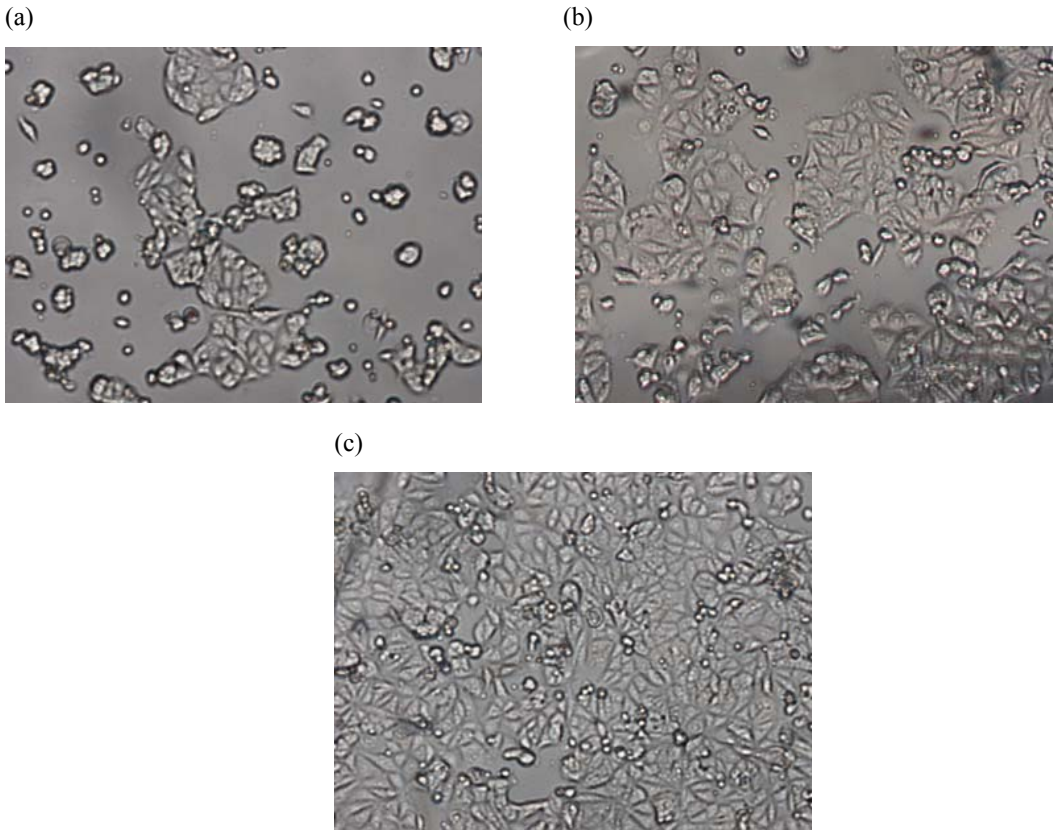

Fig. (2). Microscopic pictures of MCF-7 cells grown on various surfaces after incubation for 24 hours: (**a**) pristine PMMA; (**b**) TCPS; and (**c**) CF_4 plasma modified PMMA.

3.2. Surface Characterization

From the results of both cell proliferation and optical microscopy, it is obvious that after CF_4 plasma treatment, the MCF-7 cell proliferation on PMMA was greatly improved. To further explore this phenomenon, it is necessary to examine the effect of protein adsorption, which was originated from the cell culture medium, occurred on the modified surfaces.

Water contact angle measurements were used to physically examine the protein adsorption on different substrates. Pristine PMMA showed hydrophobic property with water contact angle of 96° (Table **1**). However, the samples showed very different water contact angles after immersed in cell culture medium (CCM). After the PMMA was immersed in CCM at 37 °C for 24 h, water contact angle was changed from 96° to 26°, which was resulted from the protein adsorption during the incubation in cell culture medium. Similar results were also observed for the CF_4 plasma treated PMMA. The water contact angle for the PMMA surface after CF_4 plasma treatment was more than 107°, indicating a hydrophobic surface was resulted. After incubation in CCM, the surface water contact angle decreased to about 29°. The results indicated that protein immobilization during CCM incubation brought similar water contact angle on different surfaces.

Because the water contact angle results indicated that the physical observations could not evidently describe the difference in protein adsorption capability caused by CF_4 plasma treatment. Additional analyses to evaluate the surface composition were therefore performed to confirm the effect of CF_4 plasma to the surface.

ESCA analyses were carried out for the pristine PMMA, and PMMA treated by CF_4 plasma. All samples were incubated in cell culture medium for 24 h to observe the protein adsorption. Since the pristine PMMA is composed by 74.5% C, 25.5% O and no nitrogen, N 1s content provided by ESCA analyses can be utilized as a useful indication for the incorporation of proteins from cell culture medium.

After immersed in CCM for 24 h, pristine PMMA showed 2.85 % of nitrogen (Table **2**), which indicated

Table 1. Water Contact Angle Measurements

	Pristine PMMA	PMMA Immersed in CCM	CF_4 Plasma Treated PMMA	CF_4 Plasma Treated PMMA After Immersed in CCM
Average water contact angle (degree)	95.92 ±2.26	25.86 ±9.70	107.23 ±1.81	28.58 ±5.69

Table 2. Total Surface Chemical Composition from ESCA Analyses Wide Scan

Sample \ Surface Composition	C 1s	O 1s	N 1s	F 1s
Pristine PMMA	74.50	25.50	0.00	0.00
Pristine PMMA + CCM	94.34	2.80	2.85	0.00
CF_4 plasma treated PMMA	43.37	14.37	0.00	42.26
CF_4 plasma treated PMMA + CCM	70.71	17.71	10.87	0.70

limited amount of protein was incorporated on the surfaces of pristine PMMA. On the other hand, the CF_4 plasma treatment altered the PMMA surface significantly that the surface composition revealed 43.37% of carbon, 14.37% of oxygen and 42.26% of fluorine. After immersed in CCM, the nitrogen component increased to 10.87% on the CF_4 plasma treated PMMA, almost four times than that of pristine PMMA, which indicated that protein adsorption was enhanced significantly by the fluorine containing surface.

Besides chemical analysis, nanometer-scale surface roughness measurement was also conducted to elucidate the effect of CF_4 plasma on the surface morphology of PMMA. AFM was utilized to analyze surface topography for pristine PMMA and CF_4 plasmas modified PMMA before and after incubation in culture medium for 24 h. The three-dimensional images of the surfaces were shown in Fig. (**3**) and the surface topography was quantified by the surface roughness (Table **3**). AFM results showed that the pristine PMMA have a relatively smooth surface, with a roughness of 2.28 nm (Fig. **3a**). After being immersed in cell culture medium, the roughness of pristine PMMA increased to 4.5 nm (Fig. **3b**), which seemed to indicate that the rougher surface was resulted from protein adsorption. On the other hand, the average roughness of the CF_4 plasma treated PMMA was 3.59 ± 0.40 nm (Fig. **3c**), which is close to pristine PMMA. This showed that CF_4 plasma treatment only modified the outermost layer of the surface without resulting in significant change of surface morphology. After incubated in cell culture medium, the surface roughness of the plasma treated PMMA increased to 9.40 ± 0.63 nm, which seemed to indicate that more protein was incorporated on the surface of the CF_4 plasma modified PMMA than the pristine PMMA.

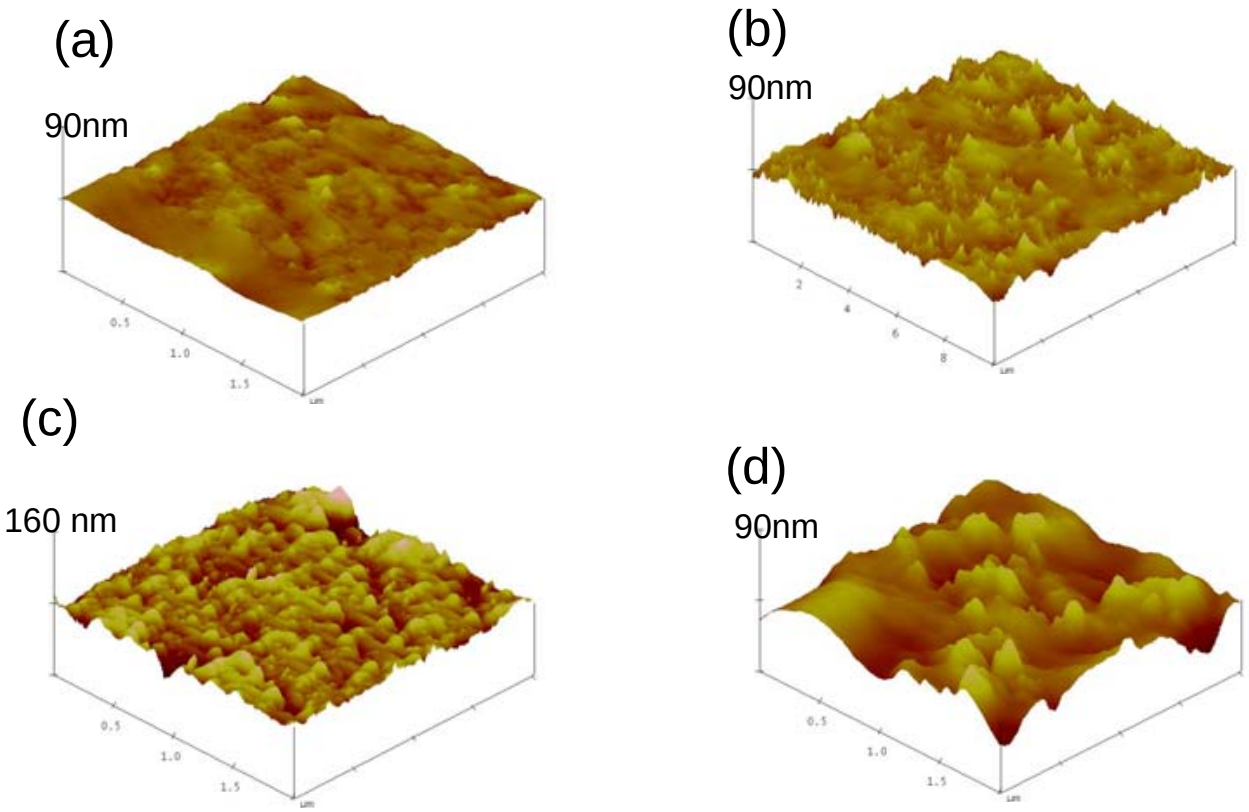

Fig. (3). AFM 3-dimensional images of the samples as follows: (**a**) pristine PMMA; (**b**) PMMA + cell culture medium; (**c**) CF_4 plasma modified PMMA; and (**d**) CF_4 plasma modified PMMA + cell culture medium.

Table 3. Surface Roughness Calculated by AFM Analyses

Sample	Pristine PMMA	PMMA + CCM	CF_4 Plasma Treated PMMA	CF_4 Plasma Treated PMMA + CCM
Average roughness (nm)	2.28 ± 0.25	4.50 ± 0.40	3.59 ± 0.40	9.40 ± 0.63

Table 4. Summary of Effect of CF_4 Plasma Treatment and CCM Addition onto PMMA Surface Towards its Physical, Chemical, and Cell Growth Properties

Surfaces	CF_4 Plasma	CCM Addition	N-Content (%)	WCA (Degree)	Roughness (nm)	MCF7 Cells/mL
Pristine PMMA	-1	+1	2.85	26	4.5	108000
CF_4 treated PMMA	+1	+1	10.87	29	9.4	142000

According to the results of contact angle, ESCA and AFM analyses, proteins in cell culture medium play key roles on promoting cell proliferation. The CF_4 plasma treatment greatly promoted the protein adsorption on PMMA surface, which was proved clearly by the reduction of water contact angle, the increment of nitrogen content, and the surface morphology. Bacakova *et al*. [22] also showed that fluorine ions implanted polyethylene improves adhesion and growth of both endothelial cells and vascular smooth muscle cells. The extracellular matrix adsorbed to the polymer from the culture medium serum contributed to the cell adhesion.

4. DISCUSSION

The improved capability of protein adsorption on CF_4 plasma treated PMMA revealed by ESCA explained the different growth rate of MCF-7 cells. The 2.85% protein adsorption capacity on pristine PMMA demonstrated MCF-7 growth of 10.8×10^4 cells/ml, whereas the growth on CF_4 plasma treated one, with 10.87% protein adsorption capacity, accounted for 14.2×10^4 cells/ml. The improvement in MCF-7 cell growth is about 4.2×10^3 cells for every 1% of increased amount of adsorbed protein onto the surface. Further experiment to explore and quantify the cell growth improvement as effect of different CF_4 plasma treatment parameters (power, flowrate, duration, etc.) are still undergoing.

Moreover, the effects of CF_4 plasma treatment and CCM addition towards the physical and chemical modification of PMMA and its influence to the MCF-7 cell growth were summarized in Table **4**. It is clearly revealed that although CF_4 plasma treatment did not alter the hydrophobicity of PMMA much, it promoted protein adsorption (as detected by ESCA) and enhanced the surface area (increased roughness measured by AFM), and showed progressed growth of MCF-7 cells. It is noted that the effect of protein from CCM addition also brought the same trend that of CF_4 plasma treatment which is currently a research topic ongoing in our group.

5. CONCLUSIONS

In this study, evaluation of the effect of CF_4 plasma treatment of PMMA on MCF-7 cell proliferation was conducted. We discovered that interactions between MCF-7 cells and PMMA substrates were significantly enhanced by CF_4 plasma treatment and proven statistically. The carbon-fluorine functionalities generated *via* CF_4 plasma treatments were shown to effectively promote cell proliferation after 24 h of cultivation.

The rationale of cell count enhancement was further identified to be highly associated with the adsorption of proteins in cell culture media onto PMMA surfaces by ESCA and AFM analyses. In summary, this study revealed that CF_4 plasma assisted the incorporation of protein from cell culture medium which later promoted the MCF-7 cell growth in both qualitative and quantitative manners.

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