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Adsorption Behavior of Antimicrobial Peptide Histatin 5 on PMMA

Masao Yoshinari[1], Tetsuo Kato[2], Kenichi Matsuzaka[3], Tohru Hayakawa[4], Takashi Inoue[3], Yutaka Oda[1], Katsuji Okuda[2], Masaki Shimono[5]

[1] Department of Dental Materials Science and Oral Health Science Center, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

[2] Department of Microbiology and Oral Health Science Center, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

[3] Department of Clinical Pathophysiology and Oral Health Science Center, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

[4] Department of Dental Biomaterials, Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, 2-870-1, Sakaecho-nishi, Matsudo, Chiba 271-8587, Japan

[5] Department of Pathology and Oral Health Science Center, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

Correspondence to: Masao Yoshinari, Department of Dental Materials Science and Oral Health Science Center, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan (e-mail: yosinari@tdc.ac.jp)

Running Heads: ADSORPTION of HISTATIN 5 on PMMA

Abstract: Adsorption of antimicrobial peptide histatin 5 on a poly(methyl methacrylate) denture base may serve to prevent biofilm formation, leading to a reduction of denture-induced stomatitis. This study focused on adsorption behavior of histatin 5 onto PMMA surfaces modified using a cold plasma technique and the effectiveness of histatin 5 adsorption for reducing *C. albicans* biofilm formation by the quartz crystal microbalance-dissipation (QCM-D) technique. PMMA spin-coated specimens were treated with oxygen (O_2) plasma using a plasma surface modification apparatus. The amount of histatin 5 adsorbed onto the PMMA treated with O_2 plasma is more than six times greater than that adsorbed onto untreated PMMA. The degree of histatin 5 adsorption had a negative correlation with the contact angle, whereas that of zeta-potential showed no significant correlation. XPS analysis revealed that the introduction of the carboxyl and O_2 functional groups were observable on the O_2 plasma treated PMMA. Increased surface hydrophilicity and the formation of the carboxyl could be responsible for histatin 5 adsorption on plasma-treated PMMA. There is no significant difference between histatin-adsorbed PMMA and control PMMA for *C. albicans* initially attached. On the contrary, the amount of *C. albicans* colonization on histatin-adsorbed PMMA was significantly less than the control.

Keywords:

antimicrobial, surface modification, glow discharge (RF/Plasma), histatin 5, PMMA

INTRODUCTION

Denture-induced stomatitis is a common intraoral disease, which is associated with high levels of *Candida albicans* (*C. albicans*) adhesion and biofilm formation to a poly(methyl methacrylate) (PMMA) denture base[1,2,3]. Candida biofilms on PMMA were mostly blastophores with very few hyphal forms. Above the layer of cells, profuse matrix was seen which consisted of extracellular matrix and hyphal elements[4]. Biofilm cells are reported to be more resistant to antimicrobial agents compared with the planktonic cells[5]. For that reason, various surface modifications of PMMA surfaces, such as changes in the physicochemical nature[6,7], treatment with antibiotics[8,9], and antifungal agents[10] have been attempted to reduce the adhesion of *C. albicans* on the denture surface.

The loading of antimicrobial peptides onto a PMMA denture is an important candidate for incorporating antimicrobial activity. Antimicrobial peptides are a new class of promising antimicrobial agents with a low tendency to induce resistance *in vitro*[11,12]. This type of peptide is expected to be used in place of antibiotics because there are no known antibiotic-resistant bacteria such as methicillin-resistant *S. aureus* (MRSA) and there are no known side-effects.[13,14] Clinical applications for antimicrobial peptides are currently under investigation, such as the saliva substitute xanthan loaded with antimicrobial peptides for treating oral candidosis[15]. Histatins, a family of basic peptides secreted by the major salivary glands in humans, possess antimicrobial activities. The antimicrobial activities are thought to be one means of regulating biofilm formation in the oral cavity[16]. Especially, histatin 5 is effective for its antimicrobial activity, and has fungicidal and fungistatic effects on *C. albicans* cells[15,16]. Though some antimicrobial peptides have some side effects such as hemolysis, histatins showed no or low hemolytic activity using human erythrocytes[17,18]. Consequently, surface loading of histatin 5 by either adsorption or chemical crosslinking provides a higher concentration of active molecules on the PMMA denture, leading to the reduction of *C.*

albicans biofilm formation. Edgerton M *et al.* showed that modification of PMMA at the surface by copolymerization of methyl methacrylic acid for introducing carboxyl groups resulted in a polymer that was capable of double the adsorption of the added amount of histatin 5 per surface area[16].

Cold plasma surface modification using various gases such as Ar, O_2, N_2, and SO_2 has been utilized to modify blood compatibility, to influence cell adhesion and growth, and to control protein adsorption[19-22]. Notably, O_2 plasma treatment was reported able to control hydrophilicity/hydrophobicity and to introduce several functional groups leading to applications such as humidity sensors, enzyme immobilization, and polymer bonding without the use of adhesive[23-25]. Our previous study showed that the O_2 plasma treatment enabled introduction of an O_2 functional group on the surface and promoted the adhesion of proteins such as fibronectin on a hexamethyldisiloxane polymer[26].

Therefore, this study focused on modifying the PMMA surface via cold plasma treatment in order to provide functional groups to which histatin 5 would be adsorbed. Additionally, the effectiveness of histatin 5 surface adsorption was evaluated for reducing *C. albicans* biofilm formation by the quartz crystal microbalance-dissipation technique, which is useful for evaluating protein adsorption.

MATERIALS AND METHODS

Cold Plasma Treatment

PMMA-coated specimens were prepared on various substrates using a spin-coating system. As substrates for spin coating, coverslips (f=13 mm, Nunc, Tokyo, Japan) were used for surface roughness measurement, contact angle measurement, zeta-potential measurement, XPS analysis, and SEM observation. CaF_2 crystals (f=20 mm, JASCO, Tokyo, Japan) were used for FT-IR analysis. QCM sensors (f=14 mm, Q-Sense AB, Göteborg, Sweden) were used for the

adsorption assay of histatin 5, and initial attachment and colonization assay of *C. albicans*. The PMMA polymer (Wako, Osaka, Japan) has a molecular weight of 80,000 and a density of 1.19-1.20g/cm^3 at 25℃. The polymer films were dissolved in a chloroform solution (mass fraction of 4%). The solution was spin coated onto various substrates at 4000 rpm for 20 s using a spin coater (1H-D7, Mikasa, Tokyo, Japan). The films were then dried under vacuum for 24 h and were stored in a desiccator at room temperature. The film thickness under these conditions was approximately 200 nm as determined by a QCM-D instrument (QCM-D300, Q-Sense AB, Göteborg, Sweden) that was mentioned in "Adsorption Assay of Histatin 5". Briefly, a frequency shift of spin-coated PMMA showed about 10,000 Hz was converted into the mass of 2.53 x 10,000 ng/cm^2, and then the mass was divided by a density of PMMA of 1.20g/cm^3 and was finally converted into film thickness of ~ 200 nm.

PMMA-coated specimens were treated with O_2 for 10 min at room temperature with a gas flow rate of 50 sccm (mL/min) and a chamber pressure of 1.5 Pa using a commercially available plasma surface modification apparatus (VEP-1000, ULVAC Inc., Kanagawa, Japan). The plasma was generated using a radiofrequency generator operating at 13.56 MHz at a power level of 200 W. Finally, as-spin-coated PMMA (PMMA) and O_2 plasma treated (PMMA-O2) were prepared.

Surface Characterization of Cold Plasma-treated Surfaces

The surface roughness of the PMMA and the PMMA-O2 were measured by a Handy Surf E-30A (Tokyo Seimitsu, Tokyo, Japan). Measurement was done on 3 randomly selected fields of each sample and the mean surface roughness (Ra) was calculated.

The contact angle with respect to double-distilled water was measured using a Contact Angle Meter (CA-P, Kyowa Interface Science Co. Ltd., Tokyo, Japan). Five measurements of 15 s each were made for each surface type; all analyses were performed at the same temperature and

humidity.

The zeta-potential of the surfaces were measured using an electrophoretic light scattering spectrophotometer (ELS-6000, Ohtsuka Electronics, Tokyo, Japan). Measurements were performed in 10 mM NaCl solution at 25°C using latex particles as a monitor in the cell for flat plate sample. Data presented are the mean of measurements of three samples, each of which were measured four times.

An X-ray photoelectron spectroscopy (XPS) analysis (ESCA-750, Shimadzu, Kyoto, Japan) was performed with Mg-Ka as an X-ray source at 8 kV and 30 mA for measuring the intensity of C1s and O1s. The binding energy of each spectrum was calibrated with C1s of 285.0 eV for charging correction. Resolution of the instrument was 1.15 eV of FWHM at Ag 3d 5/2. Baseline correction was done with a conventional Shirley method, and peak fitting and deconvolution were performed with reference to the database[27]. Fourier transform infrared absorption spectroscopy (FT-IR-430, Jasco Corp., Tokyo, Japan) analysis was conducted with PMMA spin coated on CaF_2 crystals at a resolution of 4-cm^{-1}. Each surface type was measured three times.

Adsorption Assay of Histatin 5

Schematic illustrations of PMMA spin coating, plasma treatment, and adsorption assay for QCM-D measurement are shown in Figure 1.

Commercially available histatin 5 (Asp-Ser-His-Ala-Lys-Arg-His-His-Gly-Tyr-Lys-Arg-Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr, Human, 4270-S, Peptide Institute, Japan) and QCM-D instrument as mentioned above were used for the adsorption assay. The QCM-D instrument was operated with AT-cut single-crystal quartz sensors with a resonant frequency of 5 MHz for the adsorption assay. The crystal resonant frequency (? f) and the dissipation factor (? D) of the oscillator were measured simultaneously at a fundamental resonant frequency (5 MHz) and at a number of overtones,

including 35 MHz. Monitoring the resonance behavior of piezoelectric oscillation enables measurements of mass adsorption at the surface in real time, usually as a function of the decrease in resonance frequency. The frequency shift is related to the adsorbed mass; the adsorbed mass was estimated by the Sauerbrey equation[28]. The adsorbed mass is calculated by the following equation as a function of frequency change (?f).

? m= (-C?f)/n

Here C = 17.7 ng Hz^{-1} cm^{-2} for a 5 MHz quartz crystal, n = 1,3,5,7 is the overtone number. At 35 MHz, a frequency shift of 1 Hz corresponds to a mass change of approximately 2.53 ng/cm^2.

A second measurement parameter, dissipation (D), gives qualitative information about the viscoelastic properties of the adsorbed layer. The increase in energy dissipation is proportional to the root of the product of viscosity and density. If the adsorbed layer is rigid, a low ? D value would be obtained.

A histatin 5 solution in PBS (-) (10μg/mL, pH = 7.4) was introduced into an axial flow chamber consisting of a T-loop in order to thermally equilibrate the sample at 25 ±0.05ºC. The sequence of injections into the QCM cell for an experimental run was as follows: 0.5 mL of PBS (-), 0.5 mL of histatin 5 solution, and 0.5 mL of PBS (-). The results were expressed as the mean ± SD of five specimens..

Initial Attachment and Colonization Assay against *C. albicans*

The initial attachment and colonization assay against *C. albicans* JCM 1542 (Riken, Saitama, Japan) was performed using the QCM-D instrument as mentioned above. PMMA spin coated (PMMA) as a control, PMMA-O2 and histatin-adsorbed specimens (PMMA-O2-His) were evaluated. Adsorption of histatin 5 was accomplished with the following steps: treatment with O_2 plasma for 10 min, immersion in 0.5mL of histatin 5 solution in PBS (-) (10μg/mL, pH =

7.4) for 2 hours at room temperature, and gentle washing with distilled water. A *C. albicans* strain was cultured in broth (glucose 10 g, peptone 5 g, and yeast extract 3g / 1L water) at room temperature for one day. A 0.3-mL sample including approximately 10^6/mL of *C. albicans* strain was directly introduced into the window chamber of the QCM-D instrument. After incubation for one day, the culture medium that included unattached cells was removed by the use of a micropipette and washed two times with culture medium. At this point, the crystal resonant frequency (? f) was measured as the amount of initial attachment of *C. albicans*. After that the measurement, 0.3 mL of the culture medium was added into the window chamber and *C. albicans* were cultured for one day. At this point, ? f was measured as the amount of colonized *C. albicans* as a biofilm formation. The results were expressed as the mean ± SD of three specimens.

Scanning Electron Microscopy

PMMA-coated coverslips were incubated with *C. albicans* in the same way as for the biofilm formation assay. The specimens were fixed in 1.0% glutaraldehyde in PBS (-) solution for 2 hours at room temperature, and then washed 3 times with PBS (-) solution and dehydrated through a series of graded ethanol solutions (70%, 80%, 90%, 95%, and 100%). The specimens were subsequently freeze-dried, sputter-coated with Au-Pd, and observed using a scanning electron microscope (JSM-6340F, JEOL, Japan).

Statistical Analysis

The data was analyzed for statistical significance using analysis of variance (ANOVA) followed by Scheffe's test for multiple comparisons.

RESULTS

Surface Characterization after Cold Plasma Treatment

Both control PMMA and the PMMA-O2 showed mirror-like surfaces with 0.06±0.01 μm and 0.07±0.02 μm of Ra, respectively, and there was no significant difference in Ra between specimens ($p>0.05$).

The contact angles were 68±4 degrees on the PMMA. These values decreased dramatically after the O_2 plasma treatment (PMMA-O2) with 14±2 degrees ($p<0.01$).

The zeta-potentials were -38 ± 5 mV on the PMMA and -28 ±7 mV on the PMMA-O2 surfaces, respectively, that were comparable with reported values[29]. There was no significant differences between the specimens ($p>0.05$).

The XPS spectra of the PMMA and PMMA-O2 specimens are shown in Figure 2. To identify the molecular species present in the C1s and O1s spectra, Gaussian model peaks were fitted in the spectra. In the C1s peak of the PMMA surface, C-H (C-C), C-OH (C-O), and C=O peaks appeared at around 285.0 eV, 286.6 eV, and 288.6 eV, which corresponded to C-CH_x, the carbonyl group, and the carboxyl group, respectively. The C=O peak increased on the PMMA-O2 specimen in comparison to the PMMA specimen. In the O1s peak, OH and C=O peaks appeared at around 532.6 eV and 534.2 eV, respectively. The intensity ratios of the O/C element and carboxyl group to the CH_x group (C=O/C-H) on the PMMA and PMMA-O2 surfaces are shown in Figure 3. Compared to that of the PMMA specimen, the intensity of O was increased on the PMMA-O2 specimen ($p<0.01$). The intensity ratio of C=O/C-H became higher in the sequence, PMMA-O2>PMMA ($p<0.01$).

The FT-IR spectra of the PMMA and PMMA-O2 specimens are shown in Figure 4. All spectra were identified as polymethylmethacrylate (BP #1455) by the Sadtler search system. No apparent changes were observable between the specimens.

Adsorption of Histatin 5

Figure 5 (a) shows ? f and ? D versus time and (b) the ?D-? f plot of histatin 5 adsorption on the PMMA-O2 specimen as a schematic example. The break point was observed in the ? D-? f plot, indicating change in rigidness and density of adsorbed histatin 5 before and after the break point.

The estimated amounts of histatin 5 adsorption on the QCM sensors are shown in Figure 6 with the measurement results of contact angle and zeta-potential. The amount of histatin 5 adsorption was estimated by the Sauerbrey equation[28] 1 h after injection of the histatin 5 solution. The amount of histatin 5 adsorption onto the PMMA-O2 sensors was more than six times greater than that onto the PMMA sensor ($p<0.01$). The amounts of histatin 5 adsorption showed a significant negative correlation with the contact angle, whereas that of the zeta-potential showed no significant correlation.

Initial Attachment and Colonization of *C. albicans*

Figure 7(a) plots a typical example of the shift in frequency and dissipation versus time for exposure of the PMMA specimen to *C. albicans* as obtained via QCM-D measurement. The SEM images are also shown in Figure 7(a). The starting point (1) of this graph is after one day of incubation, i.e., after the initial attachment of *C. albicans*. The frequency curve shows a decrease over time during the early stages of attachment until it reached a certain frequency after about 10 hrs. Fungal attachment was observed on the substrates at the initial stage (1) of SEM observation. Hyphae formation was observed after 3 hrs of incubation (2), and fungal colonization proceeded after 8 hrs of incubation (3). At 22 hrs of incubation (4), the *C. albicans* cells observed embedded in the extracellular polymeric material had an amorphous granular appearance[30]. A dissipation shift (? D) was observed to be almost the reverse tendency to the frequency shift. The ? D-? f plot for exposure of the PMMA specimen to *C. albicans* is shown in Figure 7(b) as a schematic example. The break point was observed in the ?D-? f plot (at ˜ 2 hrs),

showing change in rigidness of fungal attachment before and after the break point during the colonization of *C. albicans*.

The amounts of *C. albicans* initially attached (one day of incubation) and colonized (one day of incubation after initial attachment) are shown in Figure 8. There was no significant difference in the amount of initial attachment at point (1) of *C. albicans* among the specimens ($p>0.05$). However, there was a significant difference in the amount of *C. albicans* fungal colonization at point (4) between specimens with and without histatin-5 adsorption ($p<0.01$).

DISCUSSION

The purpose of the present study was to evaluate the adsorption behavior of histatin 5 onto plasma surface modified PMMA by using the quartz crystal microbalance-dissipation (QCM-D) technique as well as surface characterization. This study also determined the effectiveness of histatin 5 adsorption for reducing *C. albicans* biofilm formation.

Recent studies have confirmed that the quartz crystal microbalance-dissipation (QCM-D) technique is useful for evaluating surface-related processes with a real-time measurement in liquids, including protein adsorption, complementary activity on biomaterials, and analysis of DNA hybridization[31-36]. Höök et al. reported the effectiveness of the QCM-D technique for analyzing the adsorption kinetics of three model proteins on titanium oxide surfaces compared with ellipsometry and optical waveguide lightmode spectroscopy[34]. QCM-D measurement was performed to obtain more detailed information about the quantity of protein adsorption, and the QCM system offered advantages of short response times compared to conventional immunoassay systems[36]. They also pointed out that the mass calculated from the resonance frequency shift included both protein mass and water that was bound or hydrodynamically coupled to the protein adlayer. The results in this study showed a clear difference of frequency shift among the conditions on both the histatin adsorption assay and the colonization assay of

the *C. albicans.* These results indicate that the QCM-D technique is effective in the evaluation of the adsorption behavior of peptides such as histatin 5 and the biofilm formation behavior of fungi with comparatively high molecular weights.

The ? D (dissipation factor) - ? f (frequency factor) plot of histatin 5 adsorption in Figure 5 (b) showed the break point in the plot. This phenomenon demonstrates that the frequency change after the first adsorption process (before the break point in the ? D-? f plot) was consistent with the approximate formation of a monolayer. In contrast, after the second process (at ~ 5 min), the slope of the ? D-? f plot becomes steep, indicating formation of a multilayer[37]. The break point of ? D-? f plot for exposure of the PMMA specimen to *C. albicans* was also observed in Figure 7(b) at around 2 hrs, suggesting the shift from fungal attachment to hyphae formation.

This study showed that the change in surface functional groups in the experimentally modified PMMA polymer permitted superior adsorption properties for histatin 5. The amount of histatin 5 adsorption onto O_2 treated sensor coated with PMMA was more than six times greater than that onto sensors without modification. The amount of histatin 5 adsorption had a negative correlation with the contact angle, whereas that of zeta-potential showed no significant correlation. In addition, XPS analysis revealed that the introduction of the carboxyl group and O_2-functional group onto the PMMA surface by O_2 plasma treatment. On FT-IR analysis, all spectra were identified as polymethylmethacrylate, but no apparent changes were observed among the specimens. This is due to the ultra-thin modified layers compared to thickness of spin-coated PMMA.

Histatin 5 molecules have cationic ions (pI>9, positively charged at pH = 7.4) and are amphipathic with both hydrophilic and hydrophobic domains[38]. Therefore, it was expected that histatin 5 would easily be absorbed on the untreated PMMA that was negatively charged (zeta potential = -38 mV) through electrostatic force. However, small amounts of histatin 5 were

absorbed on PMMA surface compared to those on O_2 plasma treated PMMA surfaces. Accordingly, it is considered that there are two possible explanations for increasing the histatin 5 adsorption by O_2 plasma treatment. On one hand, the increase of surface energy by plasma treatment increases the van der Waals force. On the other hand, the hydrogen bond mediated by the carboxyl group for the PMMA-O2 specimens could be responsible for this mechanism[16, 39]. Further investigation should be preformed to clarify bonding mechanism more accurately.

There is no significant difference in the amount of initial attachment of *C. albicans* among the control (PMMA), O_2-treated PMMA and the histatin-adsorbed PMMA. However, the amount of *C. albicans* biofilm formation on the histatin-adsorbed PMMA significantly decreased compared to that on the other specimens. These results indicate that histatin-adsorption does not prevent or reduce adhesion of the microorganism to the denture surface, but that direct candidacidal activity of the adsorbed molecules is responsible for reducing *C. albicans* biofilm formation on the denture surface. In addition, the facts that O2-treated PMMA did not reduce the biofilm formation proved that the reduction of fungal growth was attributed to the histatin 5 and not the change in surface properties of PMMA.

In summary, histatin 5 adsorption increased onto PMMA by oxygen plasma treatment, and *C. albicans* colonization on PMMA was decreased significantly by histatin 5 adsorption. In addition, increasing surface hydrophilicity and the formation of a carboxyl group could be responsible for histatin 5 adsorption on plasma-treated PMMA. The limitations of this study are that the temperature was not physiological and the stability of adsorbed histatin 5 was not evaluated under oral environment. However, this study mainly focused on the adsorption behavior of histatin 5 onto PMMA using plasma treatment under controlled conditions[40]. Further study is necessary to investigate the stability of adsorbed histatins by exposing to simulated saliva, and to establish the method for direct loading of histatins from saliva in oral environment.

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Legend for figures

Figure 1 Schematic illustrations of (1) PMMA spin coating, (2) plasma treatment, and (3) adsorption assay for QCM-D measurement.

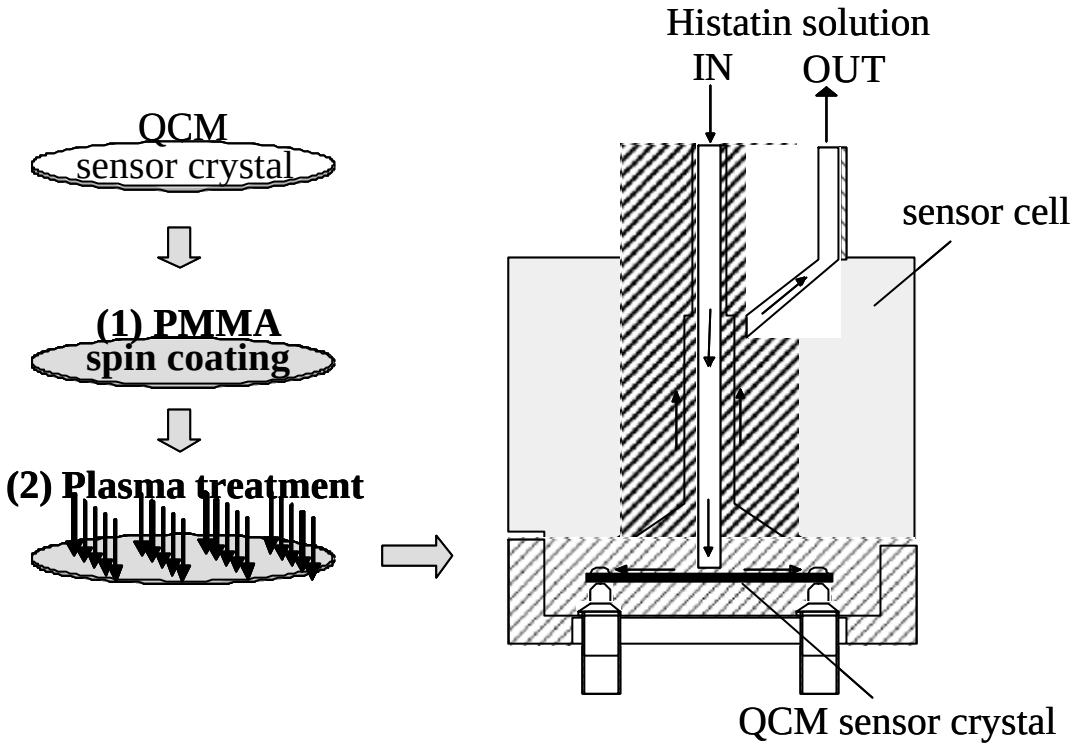


Figure 2 XPS spectra of PMMA and PMMA-O2 specimens.

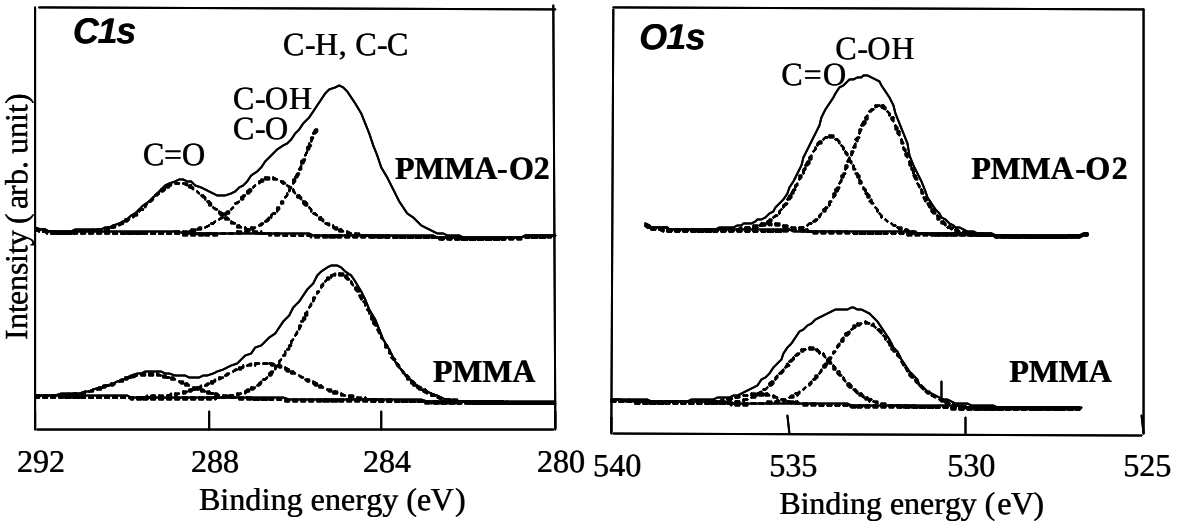


Figure 3 The intensity ratio of O /C element and carboxyl group to CHx group (C=O / C-H) on PMMA and PMMA-O2 surfaces. Identical letters indicate no significant difference ($p>0.05$).

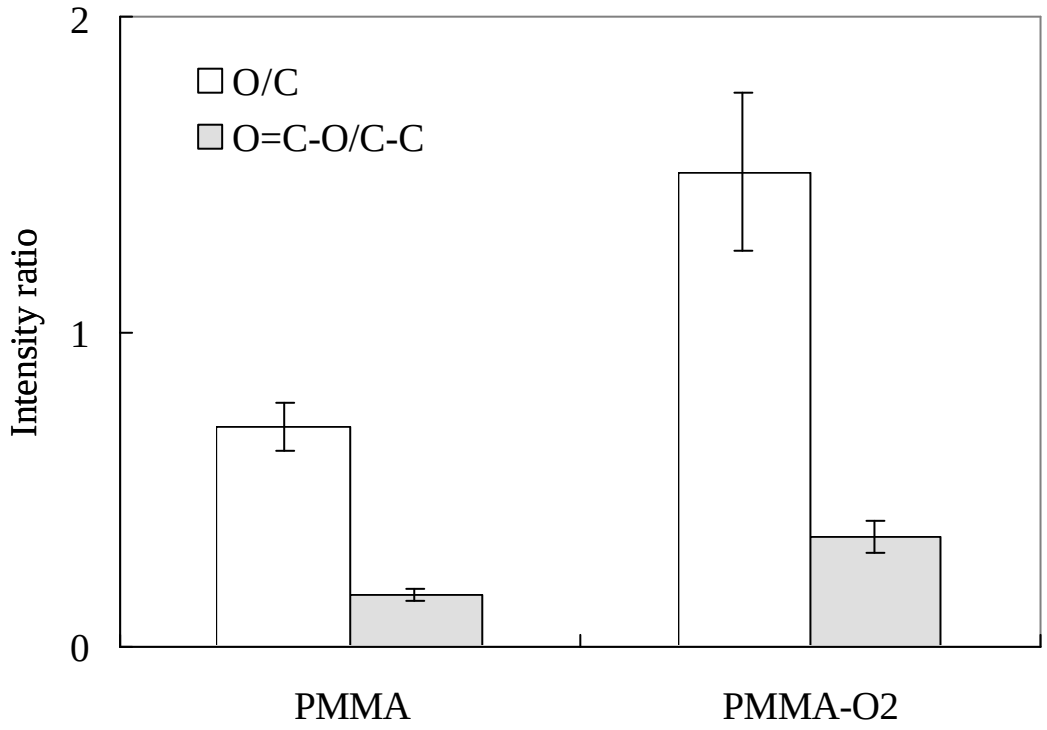


Figure 4 FT-IR spectra of the PMMA and PMMA-O2 specimens.

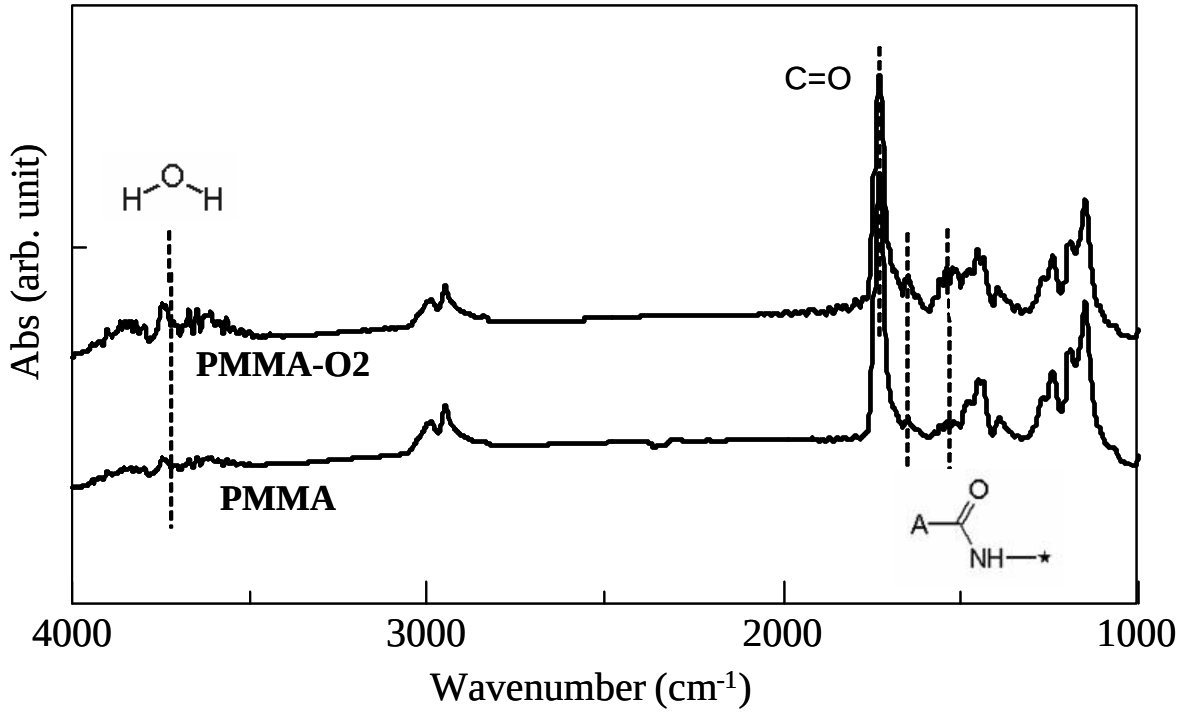


Figure5 (a) Δ f and Δ D versus time, and (b) the Δ D- Δ f plot of histatin-5 adsorption on PMMA-O2 specimen as a schematic example.

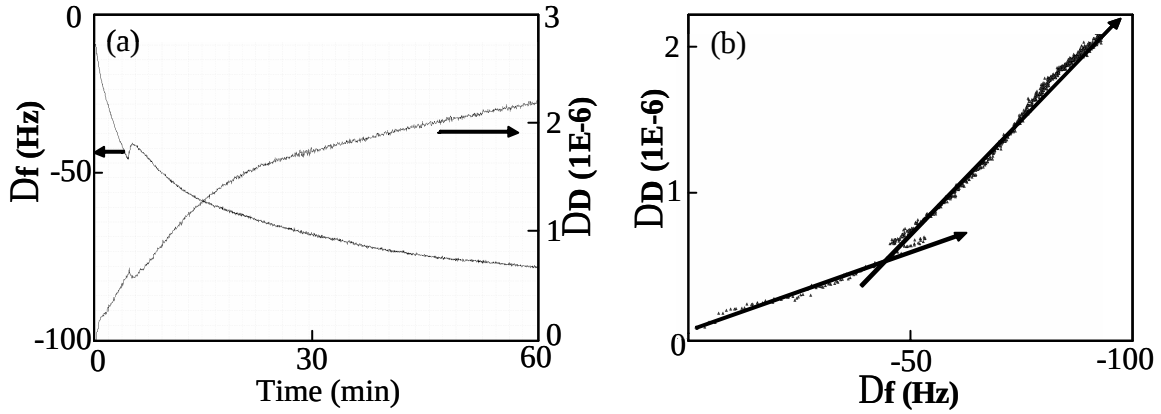
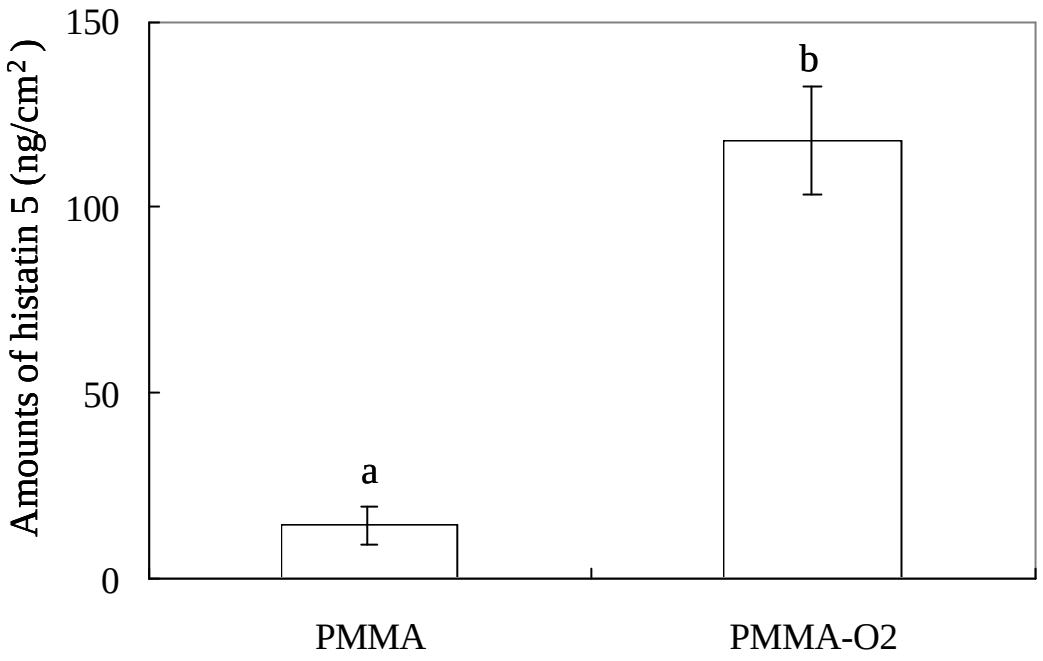


Figure 6 Estimated amounts of histatin 5 adsorption on QCM sensors. The contact angle and zeta-potential are also shown in this figure. Identical letters indicate no significant difference ($p>0.05$).



Contact angle(°)	68 ± 4^{a}	14 ± 2^{b}
zeta-potential (mV)	$-38\pm5^{a'}$	$-28\pm7^{a'}$

Figure 7 (a) Typical example of shift in frequency and dissipation against time for exposure of PMMA specimen to *C. albicans*. The SEM images are also shown in (a).

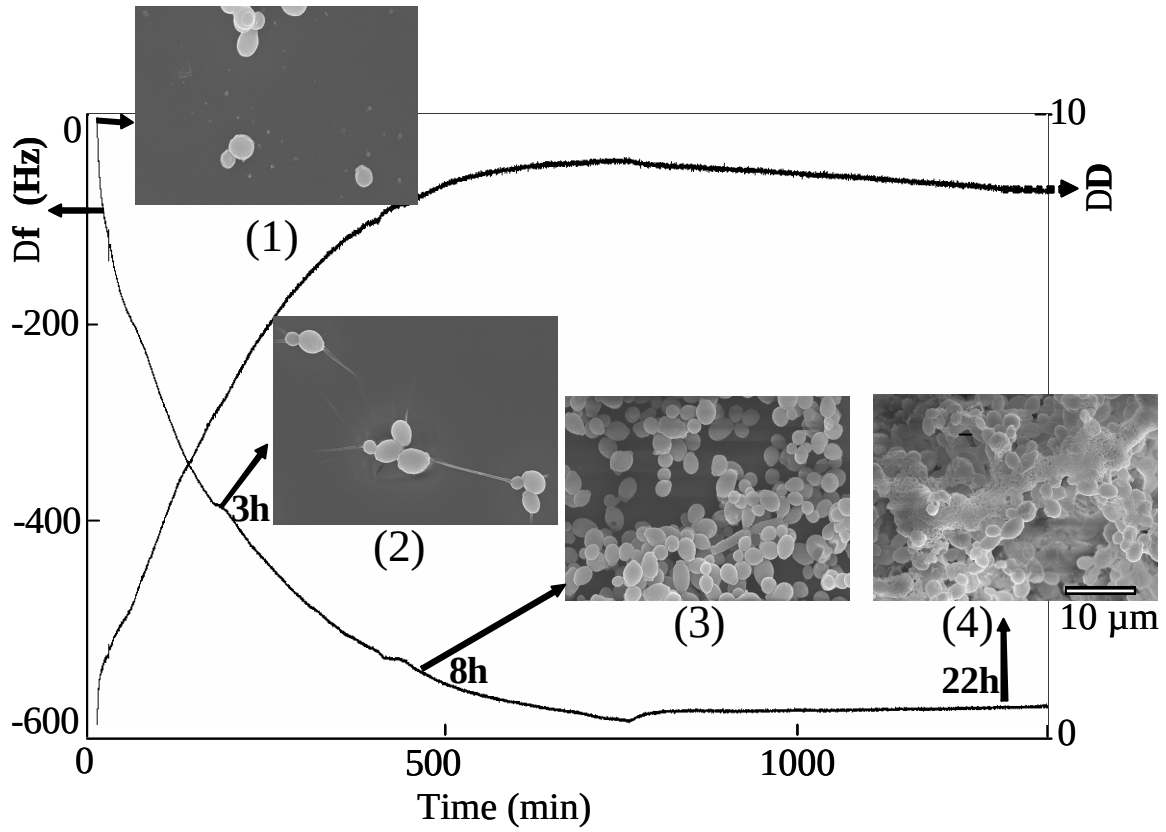


Figure 7 (b) Δ D- Δ f plot of (a) as a schematic example.

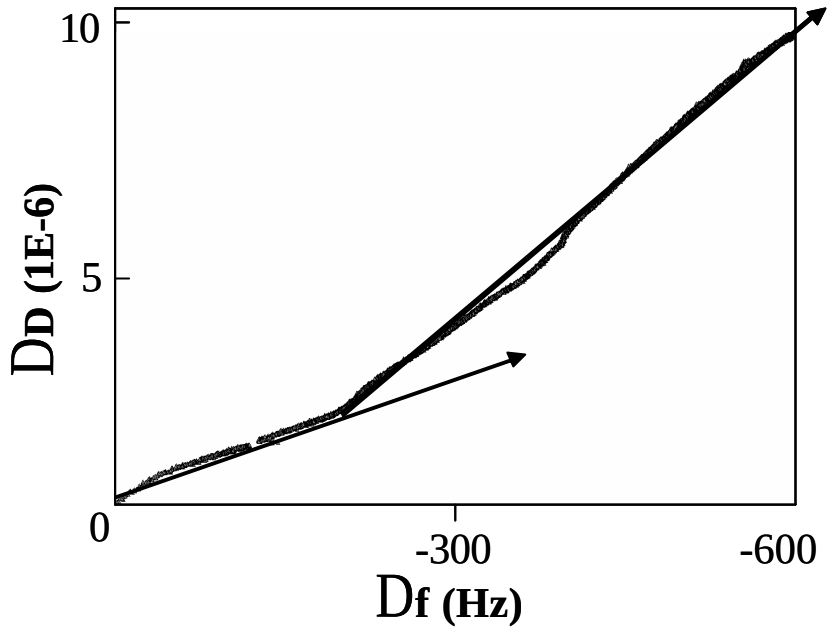


Figure 8 Amounts of *C. albicans* initially attached and colonized. Identical letters indicate no significant difference ($p>0.05$).