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Research Article

Hepatocyte Adhesion Behavior on Modified Hydroxyapatite Nanocrystals with Quartz Crystal Microbalance

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Abstract The initial adhesion, spreading and cytoskeleton changes of hepatocyte-like cells on hydroxyapatite (HAp) and oxidized poly(styrene) (PSox) sensors pre-adsorbed fetal bovine serum (FBS), collagen (Col) and fibronectin (FN) were analyzed by using a quartz crystal microbalance with dissipation technique and a confocal laser scanning microscope (CLSM). The ΔD-Δf plots of the cell adhesion behavior on HAp nanocrystals clearly depended on the pre-adsorbed proteins, while that on PSox showed almost same behavior irrespective of the proteins. The different adhesion behavior depended on the substrate surfaces was attributed to the cell-surface interactions. CLSM images showed that the morphology of the cultured cells depended on the surfaces and the cells on the HAp and PSox adsorbed Col or FN had sometimes pseudopods. The different cellular morphology indicated that the cytoskeleton changes and the rearrangement of the extracellular matrix at the interface caused the species of pre-adsorbed proteins.

Keywords hydroxyapatite; QCM-D; hepatocyte; cell adhesion; protein-modification

1 Introduction

Cell adhesion to extracellular matrix (ECM) plays important roles in cellular behaviors such as proliferation, migration, differentiation, and survival [1]. It has been described that the biomaterial surfaces modified with the ECM affect the cellular behaviors [3]. Control of cell-matrix interactions is indispensable for designing superior biomaterials in tissue engineering. There are many types of the ECM with arginine-glycine-asparagine (RGD) peptide such as fibronectin (Fn), collagens (Col), vitronectin, laminin and etc. which have an ability to adhere cells via integrin receptor. Therefore, the interfacial phenomena of cell and biomaterial surfaces modified with the ECM should be clarified for controlling cell functions.

To detect the interfacial phenomena, a quartz crystal microbalance with dissipation (QCM-D) technique is one of excellent *in situ* analytical methods. Hydroxyapatite (HAp) sensor applicable for the QCM-D technique was recently fabricated with an electrophoretic deposition method in our group to analyze protein adsorption [2,5]. The cell adhesion behavior on the HAp surface with the QCM-D technique has not been fully investigated [4].

In the present study, we investigated the initial adhesion of hepatocyte-like cells on the adlayer of fetal bovine serum (FBS), FN and Col on the HAp sensor and the oxidized poly(styrene) (PSox) sensor. The morphological differences of hepatocyte-like cells on the modified surfaces were observed with a confocal laser scanning microscope (CLSM).

2 Materials and methods

Gold (QSX-301) and poly(styrene) sensors (QSX-305, film thickness: 40 nm) were purchased from Q-Sense Inc.. Fetal bovine serum (FBS, Model number: 12603C, JRH biosciences Co. Ltd.), Porcine dermis type I collagen dissolved in HCl solution (Col: Nitta gelatin Co. Ltd.), fibronectin (FN: Cat. No. 341631: Calbobiochem Co. Ltd.), Dulbecco's minimum essential medium (DMEM: No. D5796, Aldrich-Sigma Co. Ltd.), phosphate buffer saline (PBS: Dullbecco Co. Ltd.), HCl (Special grade, Wako Co. Ltd.) , 0.05 w/v% trypsin-0.053M-EDTA (No. 204-16935, Wako Co. Ltd.), a 35 mm culture dish (No. 3000-035, Iwaki Co. Ltd.) and formaldehyde (37%, Wako Co. Ltd.) were used. Human liver carcinoma cell line (hepatocytes: RCB1648) were provided by Riken BioResource Center.

The HAp sensor was fabricated by the electrophoretic deposition method based on our previous reports [2,5]. The poly(styrene) sensor oxidized by a UV/OZONE treatment (PSox) was used as a reference. The hepatocyte cells were cultured in the culture dish at 37 °C in a humidified atmosphere of 5% CO_2. The cells washed with 15 mL of PBS and treated with 1 mL of trypsin-EDTA for 5 min were dispersed into 10 vol% FBS/DMEM. The cell suspensions in the 10 vol% FBS/DMEM were adjusted at 2.5×10^4 cells/mL.

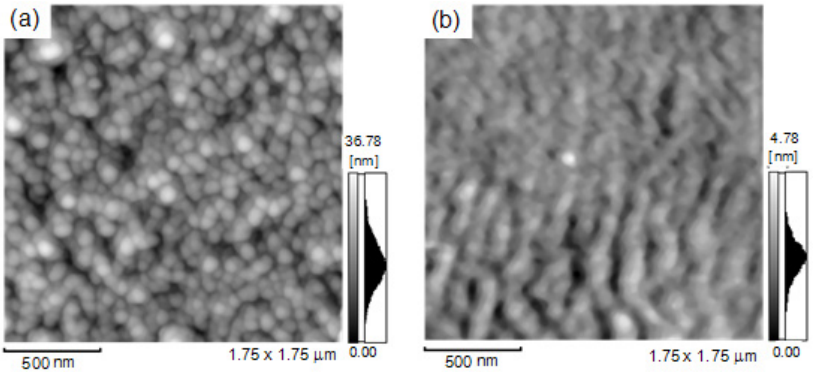

Figure 1: AFM topographic images of (a) HAp and (b) PSox sensors.

QCM-D (D300, Q-Sense AB) measurements were performed at 37.0 ± 0.05 °C by real time *in situ* monitoring of Δf and ΔD at 15 MHz. The measured Δf was divided by harmonic overtone (n = 3) as a fundamental frequency of 5 MHz. The viscoelastic property of the FBS adlayers was evaluated by a saturated $\Delta D/(\Delta f)$ value from ΔD-Δf plots. The pre-adsorption of 100 μg/mL of FN or 40 μg/mL Col dispersed in PBS was measured to stabilize the Δf and ΔD curves, rinsed with 0.5 mL of PBS, and subsequently exchanged the buffer as DMEM (0.5 mL), and the adsorption of FBS dispersed into DMEM at 10 vol% was then monitored for 1 h. The HAp and PSox pre-adsorbed FBS, FN or Col were abbreviated as FBS-, FN- or Col-HAp and FBS-, FN- or Col-PSox. The cell suspension was seeded at 0.5 mL on the adlayer on HAp or PSox sensors, and cultured for 2 h in air, and rinsed with 0.5 mL of 10 vol% FBS/DMEM. The cultured cells on the sensors were fixed with 3.7 vol% formaldehyde in PBS. The cells fixed were soaked into 1 mL of ethanol/ultrapure water series at 50, 60, 70, 80, 90, 100 vol% for each 5 min, and were into 1 mL of t-butyl alcohol three times at 37 °C. The samples were kept at 4 °C for 0.5 h and then freeze-dried at 4 °C for 4–5 h.

The sensor surfaces were observed with an atomic force microscope (AFM: SPM-9500, Shimazu Inc.). Silicon probe mounted on cantilever (OMCL-AC160TS, OLYMPUS Inc.) was employed for the dynamic mode. The surface roughness was calculated by root mean squares (RMS) in the Z-range images. The morphology of the cells cultured on the sensors was observed with a confocal laser scanning microscope (CLSM: OLS-3000, OLYMPUS Inc.). The number, area, and volume of the adherent cells were calculated from the 2-D and the 3-D images (n = 10) obtained with scanning Z-range at a z-step of 10 nm.

3 Results and discussion

Figure 1 shows AFM topographic images of the HAp and PSox sensors. The HAp surface deposited on the gold sensor and the PSox surface have a RMS value of 4.4 ± 0.4 nm and 0.4 ± 0.2 nm. The HAp sensor showed rough surface compared with PSox sensor.

The FBS adsorption at 60 min caused the Δf shifts at −39.5 ± 3.3 Hz on HAp and at −76.5 ± 9.9 Hz on PSox. The saturated $\Delta D/\Delta f$ was $-3.2 \pm 1.8 \times 10^{-8}$ on HAp and $-3.9 \pm 1.7 \times 10^{-8}$ on PSox. While the amount of FBS adsorbed on HAp was smaller than that on PSox, the viscoelastic property from the saturated $\Delta D/\Delta f$ values was almost same on HAp and PSox.

The amount (Δf = −45.2 Hz) of FN adsorbed on HAp was three times lower than those on PSox (Δf = −125.3 Hz). The saturated $\Delta D/\Delta f$ value on HAp at $-10.5 \pm 2.1 \times 10^{-8}$ was higher than that on PSox at $-4.5 \pm 1.2 \times 10^{-8}$. The subsequent FBS adsorption showed that the Δf and ΔD on HAp were −32 Hz and $+1.8 \times 10^{-6}$, and those on PSox were −15 Hz and $+1.7 \times 10^{-6}$, respectively. On the other hand, the Col adsorption changed the Δf of −273.2 ± 14.1 Hz on HAp and −340.5 ± 17.5 Hz on PSox, and showed higher viscoelasticity compared with FN adsorption, judging from the saturated $\Delta D/\Delta f$ value of $-39.7 \pm 6.9 \times 10^{-8}$ on HAp and $-26.3 \pm 3.5 \times 10^{-8}$ on PSox. The subsequent FBS adsorption behavior on PSox was completely different from that on HAp; the Δf and ΔD values on HAp were +19 Hz and -7.8×10^{-6} and those on PSox were −31 Hz and -4.3×10^{-6}.

Figure 2 shows the ΔD-Δf plots of hepatocyte-like cells onto HAp and PSox sensors modified with the FBS, FN and Col for 2 h. The Δf, ΔD and the saturated $\Delta D/\Delta f$ values of the cell adhesion at 2 h onto FBS-HAp were −20.9 Hz, $+5.1 \times 10^{-6}$ and -10.8×10^{-8}, those onto FN-HAp were −8.1 Hz, -1.2×10^{-6} and $+11.3 \times 10^{-8}$, and those onto Col-HAp were +59.1 Hz, -19.1×10^{-6} and -31.9×10^{-8}. The number of adherent cells at 2 h, counted with the light microscopy, was 2.8×10^2, 5.0×10^2 and 6.3×10^2 cells/cm^2 on FBS-HAp, FN-HAp and Col-HAp. These results suggested the different adhesion process occurred on the modified surfaces. Particularly, the

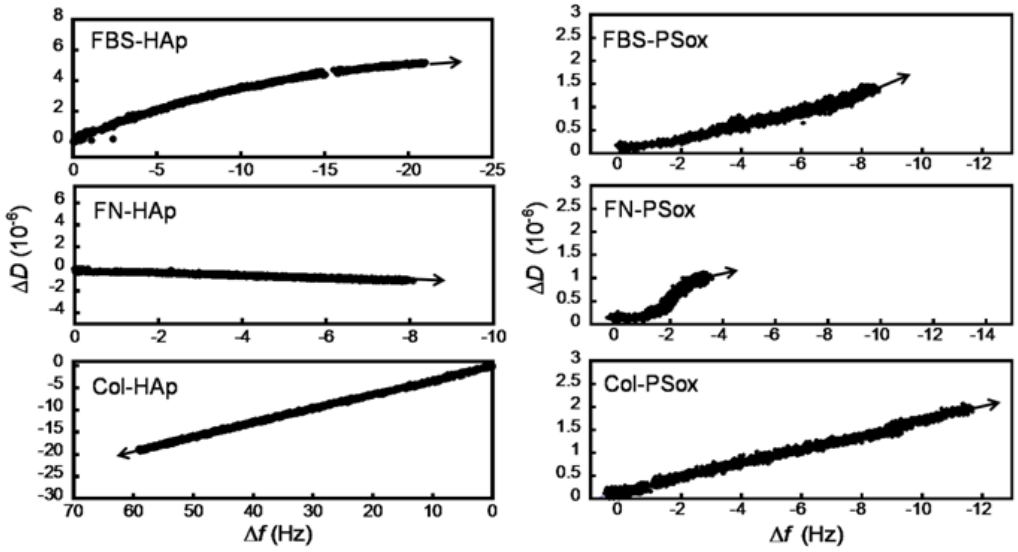

Figure 2: ΔD-Δf plots of hepatocyte-like cells onto the HAp and PSox sensors modified with FBS, FN and Col proteins for 2 h.

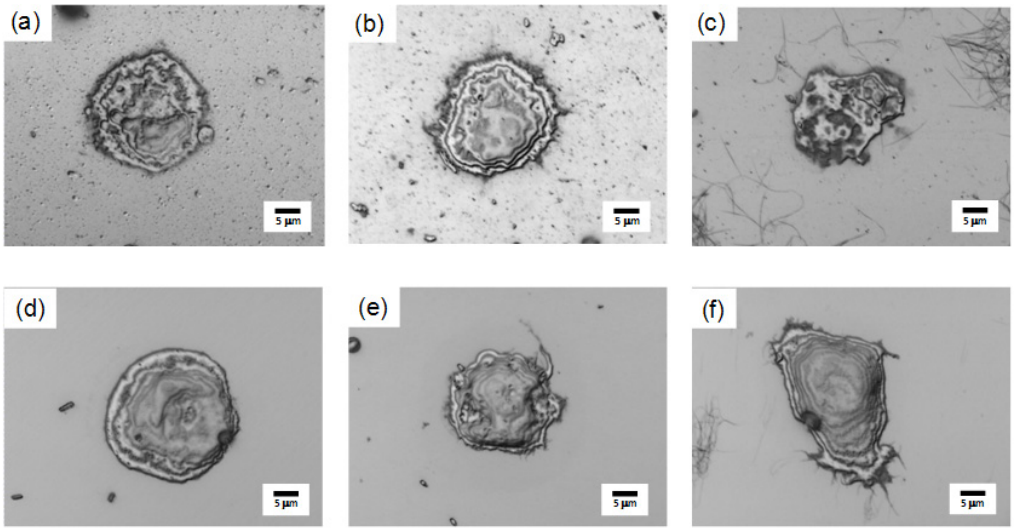

Figure 3: CLSM images of the cultured cell on (a) FBS-HAp, (b) FN-HAp, (c) Col-HAp, (d) FBS-PSox, (e) FN-PSox and (f) Col-PSox at 2 h.

cell adhesion onto Col-HAp clearly showed the increase in Δf with the decrease of ΔD, indicating the pre-adsorption of Col effectively affects the cell-surface interactions.

The Δf, ΔD and the saturated $\Delta D/\Delta f$ values of the cell adhesion at 2 h onto PSox were -8.5 Hz, $+1.4 \times 10^{-6}$ and -15.9×10^{-8}, those on FN-PSox were -3.4 Hz, $+1.1 \times 10^{-6}$ and -34.8×10^{-8} and those onto Col-PSox were -11.5 Hz, $+1.9 \times 10^{-6}$ and -15.4×10^{-8}. The number of adherent cells at 2 h was 6.9×10^2, 7.4×10^2 and 7.6×10^2 cells/cm^2 on FBS-PSox, FN-PSox and Col-PSox. These results indicate the adhesion process onto PSox was almost same irrespective of the modified surfaces. The different cell adhesion process depending on the surface pre-adsorbed proteins was successfully *in situ* monitored by the QCM-D technique.

Figure 3 shows the CLSM images of the cultured cell on (a) FBS-HAp, (b) FN-HAp, (c) Col-HAp, (d) FBS-PSox, (e) FN-PSox and (f) Col-PSox. The cell volume at 2 h was $45 \pm 21\,\mu$m^3, $70 \pm 13\,\mu$m^3 and $23 \pm 11\,\mu$m^3 on FBS-HAp, FN-HAp and Col-HAp, and $64 \pm 23\,\mu$m^3, $35 \pm 14\,\mu$m^3 and $73 \pm 15\,\mu$m^3 on FBS-PSox, FN-PSox and Col-PSox. The CLSM images clearly showed that the Col-modified surfaces had anisotropic morphologies, while the other surfaces had the round morphologies. Particularly,

the cells adhered on the surfaces modified with Col and FN had the pseudopods; the cells on the modified HAp expanded the planular, while those on the modified PSox expanded the fibrous. The different structures depended on the cell adhesion points, indicating that the cytoskeleton changes and the rearrangement of extracellular matrix at the interfaces caused the different binding behavior.

4 Conclusions

In this study, the adsorption behavior of FBS, FN and Col, and subsequent adhesion of hepatocyte-like cells dispersed into FBS/DMEM onto the HAp and PSox surfaces modified with proteins were investigated with the QCM-D technique. The ΔD-Δf plots of the protein adsorption and the subsequent cell adhesion showed the different behavior on the surfaces, clearly indicating the adhesion process affected to the cell-surface interactions through the protein adsorption. The CLSM images showed the different morphology and pseudopod dependent on the cell adhesion places, indicating that the cytoskeleton changes and the rearrangement of extracellular matrix at the interfaces.

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