Article

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Analysis of Nanoscale Protein Film Consisting of Lactoferrin/11-MUA Bilayers for Bioelectronic Device

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We fabricated and analyzed a nanoscale biofilm of human lactoferrin making use of 11-mercapto-undecanoic acid (11-MUA) as chemical linker. The fabrication of the bimolecular/organic hetero monolayer (lactoferrin/11-MUA) on gold substrate was confirmed with Raman spectroscopy. Cyclic voltammetry (CV) was carried out to observe the electrochemical properties of the nanoscaled biofilm under various pH conditions and at different time intervals. The well-defined redox properties were observed, even in certain harsh pH conditions and after a long time, proving the stabilities of this biofilm. Atomic force microscopy (AFM) was further employed to confirm the retention time by investigating the morphology variety of the biofilm over time. All these results proved that, the proposed nanoscaled thin film composed of lactoferrin and 11-MUA is a powerful alternative for making bioelectronics devices.

KEYWORDS: *Biofilm, Lactoferrin, Raman Spectroscopy, Atomic Force Microscopy, Cyclic Voltammetry, Bioelectronics Devices.*

INTRODUCTION

Biosystems are described as the most delicate and complex electronic "factory" in the world, having unique accuracy and sensitivity. In this "factory", an arsenal of genes, proteins, ion channels, pumps, and signaling molecules are employed and electron transference was involved in various bioprocesses. This inspires scientists of investigating these natural phenomena, introducing them into biotechnology and applying them for researches in medicine, agriculture, and bioengineering.[1–5] The most important advances in biotechnology of late years have been reviewed by Baker and DeFrancesco.[6]

Recently, the development of electronic devices got hampered due to physical and technical limitations of conventional silicon substrate. A breakthrough in electronics can be realized by integrating biological components into artificial devices to perform multiple functions that either reconstruct the biological circuitry on a chip or extend that functionality.[7–9] Several principles have been proposed, conducting polymer memory, all-optical flip-flop, and logic gate for example.[10–13]

In previous studies in Choi's group, biomolecular hetero Langmuir-Blodgett (LB) was proposed as a shift register memory having simple electronic functions, such as the molecular diode, switching with photocurrent generation and a rectifying property. Various electronic devices have been proposed based on these studies.[14–18]

Lactoferrin (LF), a metalloprotein, has two or three specific binding sites to ferric irons $(Fe)^{3+}$. Lactoferrin has higher affinity to Fe^{3+} compared to other transferrin, which endues well-defined redox properties and thermal stability to lactoferrin.[19–21] Altogether, ultra small size, low drive potential and great stability make lactoferrin an eligible material for nanoscaled electronic devices, meeting the demands of miniaturation, low energy cost and stability. Only if we can control over the properties of lactoferrin layer on some kind of solid support can we fabricate a new electronic device.

The electrochemical method is a method to bind proteins and phospholipids on solid substrate applying self-assembled monolayers (SAMs) of alkanethiol since unlike charges attract. In this work, 11-MUA acted as the alkanethiol linker, protein lactoferrin was immobilized on it.

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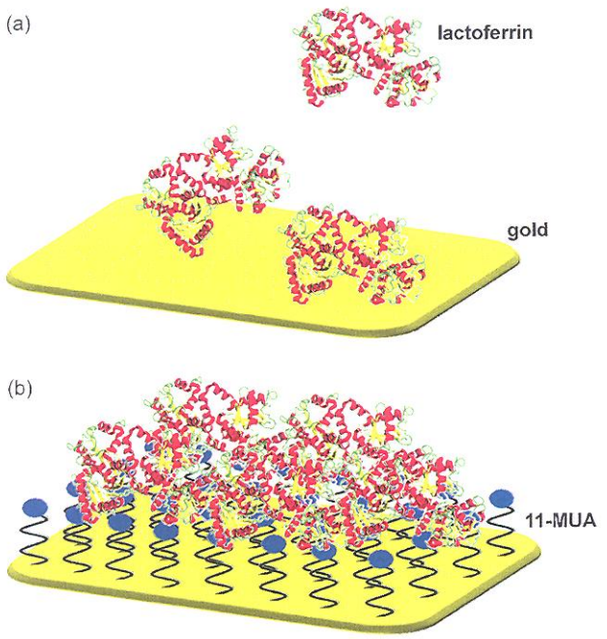


Figure 1. Schematic diagram of lactoferrin protein layer on gold surface. (a) Lactoferrin adsorbed on au without 11-MUA; (b) Lactoferrin immobilized with chemical linker 11-MUA.

Figure 1 shows the schematic diagram of gold surfaces with lactoferrin physically adsorbed and covantly adsorbed using chemical linker 11-MUA.

The fabrication of lactoferrin layer on Au with 11-MUA was confirmed with Raman spectroscopy. The redox characteristic investigation, the pH resistance testification and the durability test were carried out by the electrochemical analysis approach cyclic voltammetry (CV). The degradation of the biofilm over time was studied by observing the morphology transformation using AFM.

These data showed that lactoferrin had been self assembled into thin monolayer on solid surpport successfully with chemical linker and the biofilm has advantages of stability, including harsh condition resistance and long lifetime. The redox characteristics illustrated the potential usage of this biofilm as bioelectric device.

EXPERIMENTAL DETAILS

Fabrication of Thin Protein Film Using Linker

To fabricate gold (Au) substrate, Chromium (Cr) was sputtered onto the silicon (100) substrate initially with the thickness of 20 Å, followed by Au with that of 430 Å. After fabrication, the Au substrate was cleaned using piranha solution composed of 30 vol% H_2O_2 (Duksan Chemical, Korea) and 70 vol% H_2SO_4 (Duksan Chemical, Korea) at 70 °C for 3 min, washed with ethanol and DI water repeatedly and dried under ultra pure nitrogen gas.

The cleaned Au substrate was immersed in the 11-MUA solution (150 mM, in 5:5 of ethanol and glycerol) overnight at ambient condition for surface modification.

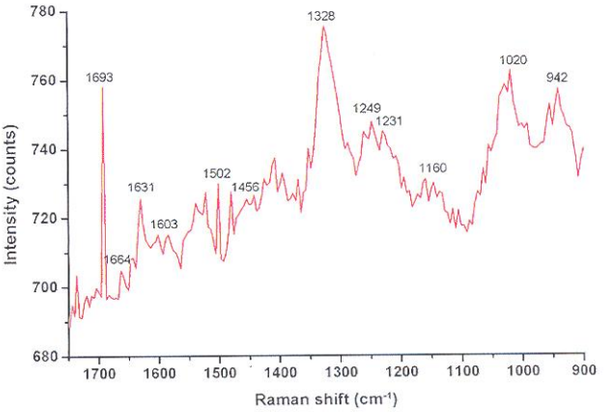


Figure 2. The Raman spectrum of the gold surface with lactoferrin immobilized via 11-MUA. The Raman shift provides structural and activity information.

Then get it washed by ethanol and DI water repeatedly and dried under ultrapure N_2 gas.

The lactoferrin film can be formed by immersing 11-MUA modified Au substrate in lactoferrin solution (0.1 mg/ml in 10 mM HEPES solution, pH 7.0) for at least 6 hours, ensuring the electrostatic interaction between 11-MUA and lactoferrin to be processed completely. Then the substrate was rinsed with DI water and dried under ultrapure N_2 gas. In this way, the biofilm consisting of lactoferrin and 11-MUA was fabricated.[17, 18, 22]

Confirming the Fabrication of the Biofilm by Raman Spectroscopy

The machine used for Raman spectroscopy was a NTEGRA Spectra (Scanning Confocal Raman Spectrometer, NTMDT, Russia) equipped with a liquid nitrogen-cooled CCD detector and an inverted optical microscope (Olympus IX71); The XYZ three dimensional scanning range was 100 μm × 100 μm × 6 μm; The resolution of the spectrometer was 200 nm in the XY plane while 500 nm along the Z axis. The incident laser used for

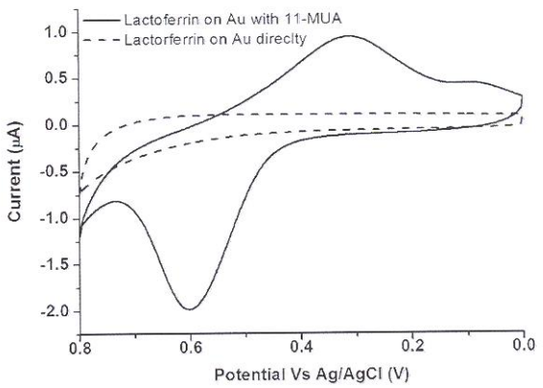
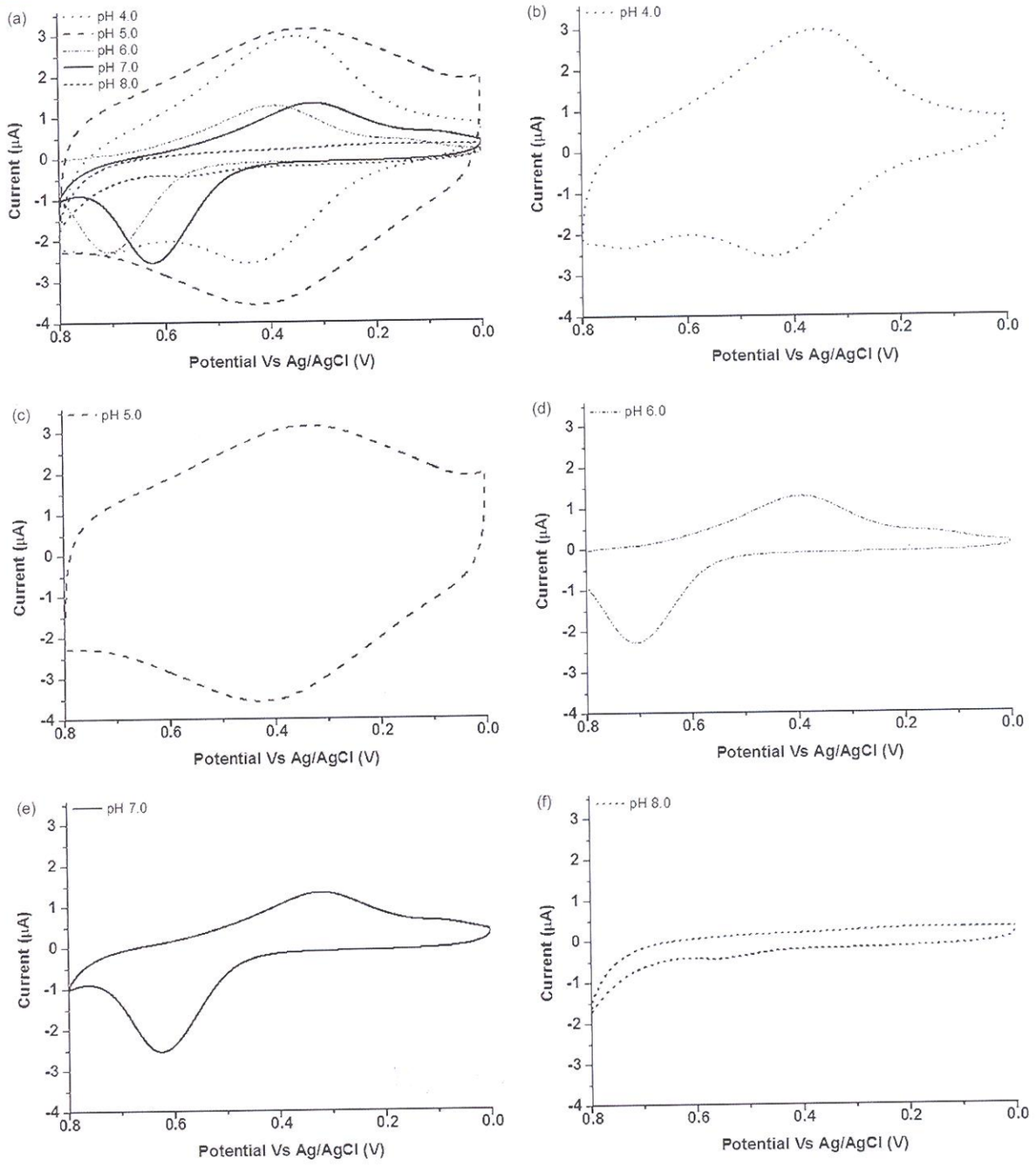


Figure 3. Voltammogram of lactoferrin immobilized on gold substrate with and without chemical linker 11-MUA. The scan rate is 50 mV/s.



(g) Redox values of lactoferrin/11-MUA under different pH conditions

E(V) \ pH	4	5	6	7	8
Oxidation	0.435	0.432	0.707	0.624	0.563
Reduction	0.359	0.335	0.392	0.318	0.184
△E	0.076	0.097	0.315	0.306	0.379

Figure 4. (a) The stress test of lactoferrin/11-MUA by CV under different pH conditions. (b–f) The CV results of the biofilm under different pH conditions respectively. (g) The voltage values of reduction and oxidation abstracted from (b–f). The scan rate is 50 mV/s.

obtaining Raman spectra was an infrared laser that emits light at the wavelength of 785 nm. Five scans of 30 s from 200–1000 cm^{-1} were recorded and the mean intensity was used as the Raman signals.

Electrochemical Measurement

The electrochemical analyzer (CHI 660, USA) was run by general purpose electrochemical analysis software and was used for redox properties analysis. The electrochemical cell made up of quartz of volume 5 ml was used. The three electrode system used for CV was a platinum counter electrode; an Ag/AgCl double junction reference electrode and the newly fabricated protein layer modified gold substrate of area 0.25 cm^2 as working electrode. The electrolyte was 10 mM HEPES buffer (pH 7.0) and all experiments were carried out at room temperature. For pH dependent research, the CV test was carried out under different pH conditions.

Surface Analysis

The surface morphology of the lactoferrin/11-MUA biofilm on Au substrate was investigated using AFM (Digital instruments Nanoscope (R) IV, USA). The mode chose for it was the contact mode equipped with 1–10 Ω-cm Phosphorous (n) doped (Si) tip with a spring constant of 20–80 N/m, which has resonant frequencies between 230–305 kHz. The scanning size was 300 nm× 300 nm and the scan rate was 1 Hz and 3 Hz for bare gold and lactoferrin/11-MUA immobilized Au surface respectively.

RESULTS AND DISCUSSION

Raman Investigation of Lactoferrin Immobilized on Au with Linker 11-MUA

The Raman spectrum of the newly fabricated lactoferrin/11-MUA biofilm was shown in Figure 2. Then bands can be assigned by referring to literatures.[23–26] It's easy to find that the two most intense bands were at 1693 cm^{-1} and 1328 cm^{-1}. The band at 1693 cm^{-1} was assigned to the symmetric C=O stretching vibration of –COOH group, while 1328 cm^{-1}, together with 1249 and 1231 cm^{-1} were assigned to a broad amide III band. The bands at 1664 and 1631 cm^{-1} and the shoulder band at 1644 cm^{-1}, were assigned to the amide I mode. These amide bands provided information about the secondary structure of lactoferrin, that's, it was a mix of α-helix, β-sheet and random coil arrangement, whereas α-helix was dominant.

The peaks approximately at 1603, 1502, 1262 and 1160 cm^{-1} were the characteristic vibration modes of tyrosinate coordination to the metal ions, and they are assigned as follows. The band at 1603 cm^{-1} was the doublet bands of ring stretches; the one at 1502 cm^{-1} was asymmetric ring stretch; 1262 cm^{-1} and 1160 cm^{-1} were assigned to C–O stretching and C–O bending of the phenolate ligand respectively. The peaks 1456 cm^{-1} and 942 cm^{-1} were CH_2 deformation mode and the backbone C–C stretching respectively.

These characteristic protein bands indicated that lactoferrin has been immobilized successfully on Au. Moreover, the secondary structural information provided in the Raman spectrum showed that the activity of lactoferrin stands a good chance of being well maintained.

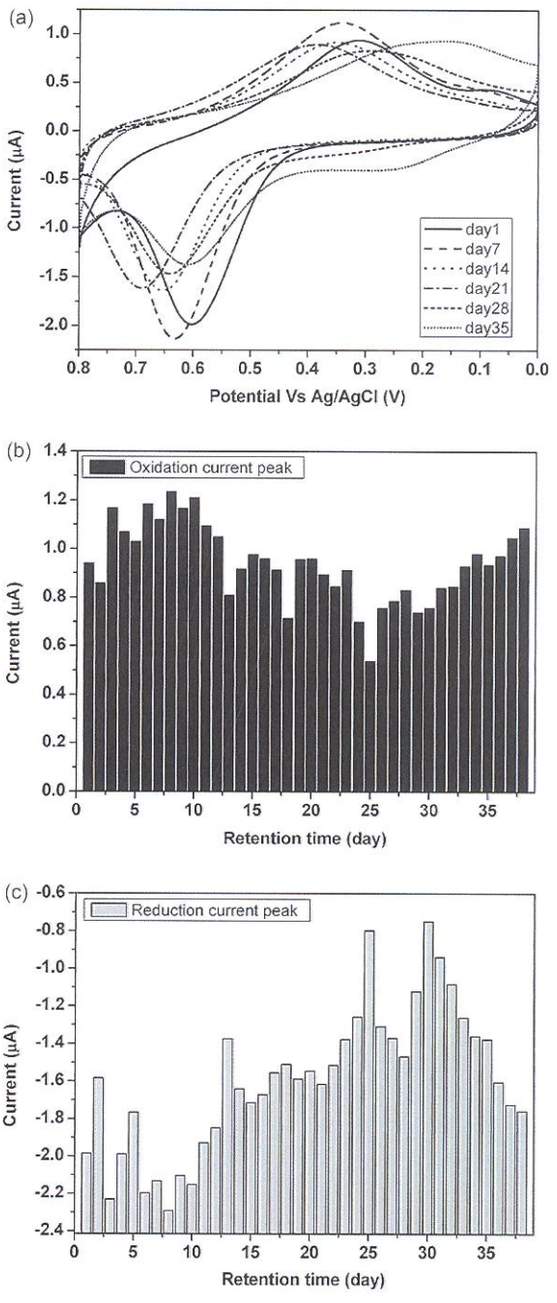


Figure 5. The durability test of the newly fabricated nanodevice processed by CV. (a) Cyclic voltammogram obtained in different time. (b, c) The oxidation and the reduction current peaks versus time (day).

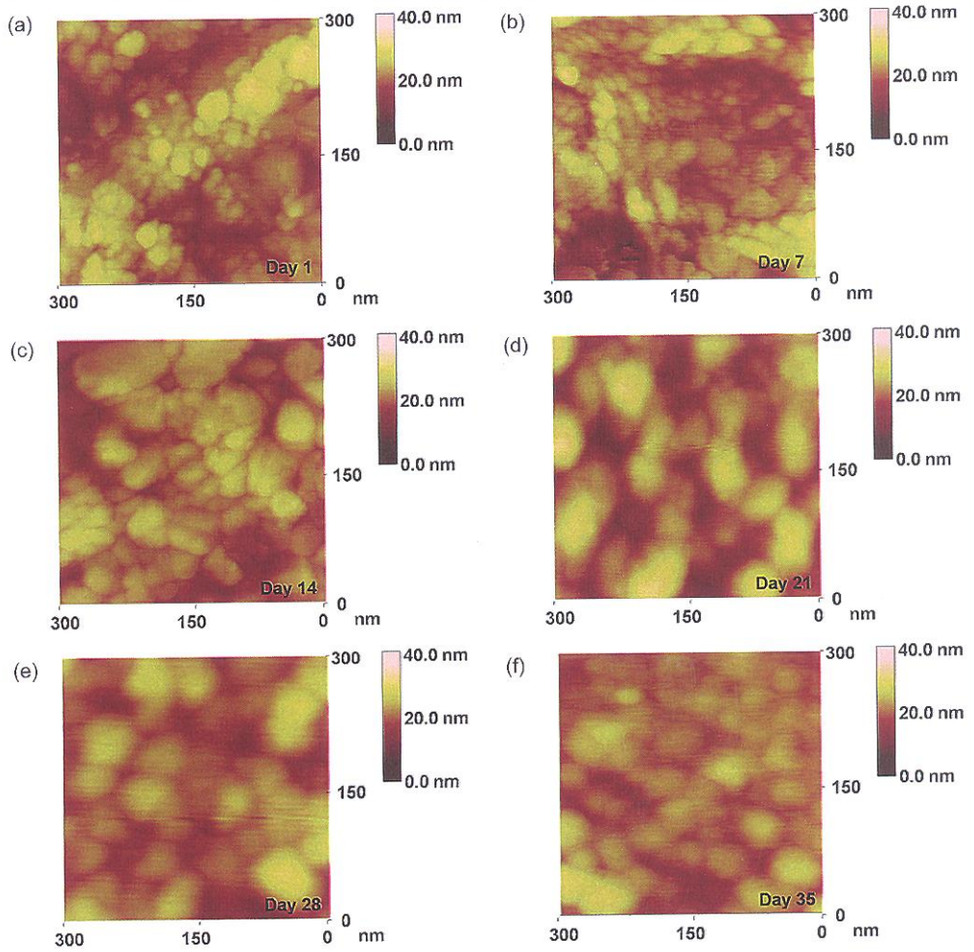


Figure 6. **The morphology of the biofilm lactoferrin/11-MUA on gold substrate was investigated with AFM right after fabrication. (a), 7 days later (b), 14 days later (c), 21 days later (d), 28 days later (e) and 35 days later (f).**

Redox Characterizations

Figure 3 was the cyclic voltammogram displaying the electrochemical properties of the lactoferrin film adsorbed on gold with and without linker. The redox peaks of lactoferrin covalently immobilized on Au were much more well-defined and stable compared to that of immobilized directly, indicating the efficient adsorption of lactoferrin on Au with the help of 11-MUA. The oxidation and the reduction potential of lactoferrin on 11-MUA/Au surface were 624 mV and 318 mV respectively, having stable redox potential of 471 mV. The voltage difference was 300 mV, which indicated the extremely fast electron transfer process in the system. These stable electrical properties of lactoferrin layer on Au with linker implied that the immobilization technology can offer quite a stable and well-oriented protein layer for bioelectronic device application.

The Stability Tests Based on CV

The stress test was carried out by performing CV under various pH conditions while the durability test was carried out by performing CV at certain time intervals.

Figure 4 shows that the redox peaks shifted as pH value varied. The difference between oxidation potential and reduction potential decreased with the decrease of pH value. This indicated a faster electron-transfer process, which was attributed to the degradation of partial lactoferrin under harsh condition. The electrochemical properties of the biofilm were well preserved under acidic condition, even when the pH value was decreased to 4.0, while they were severely diminished under alkaline condition. Since, the resistance to harsh acidic condition can be expected from the lactoferrin biofilm. Taking the stable redox potential into account, the optimum working condition for the fabricated bioflim is pH 7.0.

Figure 5 showed that the redox properties could be activated for 38 days. Since the stability of bimolecular is much fragile, to obtain the linear signal of the redox signals is difficult. And no-completed immobilized proteins might exist in the process of immobilization and seem to be detached under a harsh condition. This may contribute to the instability of the potentials as well. Overall, the interval of the oxidation and the reduction potential

decreased over time, indicating lower electron transfer resistance and a more reversible electrochemical reaction system. Even though the redox current varied, the redox peaks of the protein layer stayed well-defined and the current stayed high after 38 days. The lifetime of this biofilm could be expected to be long.

These tests demonstrated that this newly fabricated biofilm had advantages of stabilities, including harsh condition resistance and long-term usage.

Morphology Analysis Using Atomic Force Microscopy (AFM)

The surface morphologies of the lactoferrin/11-MUA heterolayers with the passage of time were investigated by AFM.

Figure 6 displayed the AFM images of lactoferrin self-assembled on gold with 11-MUA at defined times (Day 1, Day 7, Day 14, Day 21, Day 28, Day 35). From day 1 to day 14, the lactoferrin was well oriented the 15–25 nm size of small lumps approximately. Figures 6(a)–(c) depicts these phenomena. However, from the fourteenth day on, these lumps became blend and blurred, as shown in Figure 6(c), implying the processing degradation of lactoferrin lumps. Though the degradation sustained, as shown in Figures 6(d)–(f), lactoferrin layer could be identified as well-stayed 35 days later. This result, in accordance of the durability test taken by CV, confirmed the fatigue of this device. These results indicated that the lactoferrin/11-MUA heterolayers were well arranged and organized on Au surface with chemical linker.

CONCLUSION

In the present research, with thiol group (–SH), 11-MUA got covalently attached on the gold surface providing high-oriented carboxyl group (–COOH) terminating groups for electrostatic interaction with lactoferrin. The lactoferrin self-assembled on 11-MUA surface provided a stable and well-oriented platform, which could be used as bioinformatics device. The fabrication of lactoferrin on 11-MUA was confirmed by Raman spectroscopy. The cyclic voltammetry experiment characterized the activity, pH resistance, and lifetime of this biofilm. The durability of lactoferrin film was further confirmed by AFM analysis. Not only the stress test but also the durability tests of the fabricated biofilm confirmed the stability of this newly fabricated biofilm. All these results proved that lactoferrin immobilized on gold substrate via chemical linker 11-MUA can be applied for bioelectronic device.

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