Studies on visual sensor from self-assembled polypeptides

Muthusamy Sivakumara, Ryojiro Tominaga, Tomoyuki Koga, Takatoshi Kinoshita, Mineo Sugiyama, Kenji Yamaguchi

Graduate School of Engineering, Department of Materials Science and Engineering, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya 466-8555, Japan
Pokka Corporation, Nishikasugai-gun, Aichi 481-8510, Japan

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Abstract

A silane terminated poly (ε-benzylxoycarbonyl-L-lysine) was synthesized and deposited as a self-assembled monolayer on the pre-colored silica substrate which was fabricated by Langmuir–Blodgett (LB) method. The monolayer was covalently attached to silica surface through terminal silane groups. The secondary structure of polymer, α-helix, was confirmed by circular dichroism spectroscopy. Furthermore, the conversion of the monolayer surface from non-ionic to cationic was accomplished by removal of ε-benzylxoycarbonyl group (Z group). The uniform monolayer on silica substrate and the changes occurring in the tethered monolayer were analyzed by atomic force microscopy (AFM). RNA aptamer with 72 sequences of nucleotides could be immobilized on the poly (L-lysine) cationic surface through electrostatic interaction. The superior recognition capability of RNA aptamer with specific microorganism is becoming a promising candidate in detection of bacteria. In this study, the binding of target bacteria, Sphingobium yanoikuyae, to the resulting RNA probe leads to a color change at the surface which is visually observed by real-time and confirmed through UV-reflective spectrophotometer. The RNA probes can be used as a high efficiency and versatile tool to visually sense the bacterial contamination in the food products.

Keywords: Structural color; LB method; RNA aptamer; Bacteria; Visual sensor

1. Introduction

Research on biosensors focused on the development of new synthetic nano-materials that truly mimic the structural color formation of butterfly wings. The elegant use of polypeptides in this surface and/or interface studies plays a key role for biosensor applications [1]. The development of chameleon-type display system is an impressive accomplishment in our group [2]. In recent years, the silicon wafer is a promising candidate for biosensor applications [3–5] which is developed from hybrid materials. Generally, the surface-immobilized probes are consisting of self-assembled monolayers (SAMs) of polypeptides or oligonucleotides. Moreover, the covalent attachment of organic moieties to silica surface allow for a significant extension in the use of silica as a substrate in biomimetic to biosensor applications. Based on the above concept, the polypeptide is hybridized with pre-colored silica (SiO₂) substrate. In order to bind the oligonucleotides on the silica substrate, a suitable polypeptide has been selected, i.e. poly (L-lysine) (PLL) for this report. In this approach, the pre-colored silica surface is first derivatized with PLL in order to have a stable surface charge [6].

The graft polymerization on solid surface can produce a stable and densely packed organic layer, but they are too harsh to allow using labile bioactive materials [7]. So, we conceived of different way to fix a bioactive probe on the substrate. First, we attached monolayer of silane terminated poly (ε-benzylxoycarbonyl-L-lysine) (PBCL) by Longmuir–Blodgett (LB) method. In the first report [8] of this series, we described the SAMs of silane terminated PBCL deposited through LB method. Secondly, the PBCL was converted to cationic monolayer onto the silica surface for the immobilization of RNA aptamers. This will open up a new platform for biosensing materials including selective recognition of microorganism. RNA aptamers were
originated from in vitro selection experiments [9], which start from random sequences libraries for high affinity of binding. The RNA aptamer is very useful because of excellent binding selectivity, the structural stability and used as superior and inexpensive substitutes for antibodies [10]. Recently there has been increasing interest in the detection of specific microorganisms using methods which do not require the use of labels such as radioisotopes, enzymes and fluorescence groups. Indeed, we are reporting here, the first biochip developed by structural color.

2. Experimental procedure

2.1. Materials

ε-Benzylxocarbonyl-lysine was obtained from Kokusan Chemical Co. Ltd, Tokyo, Japan. The following chemicals and solvents (Nacalai Tesque, Inc., Kyoto, Japan) were used without further purification. Triphosgene, THF, ethyl acetate, n-hexane, N,N-dimethylformamide (DMF) and diethyl ether (all solvents are dried with molecular sieves) are used for polymer synthesis and aceton, toluene, ethanol and MilliQ water used for cleaning purpose. Silicon wafer and 3-aminopropyltrimethoxysilane (KBM-903), the initiator/coupling agent, were procured from The Nilaco Co., Tokyo, Japan and Shin-Etsu Chemical Co. Ltd, Tokyo, Japan, respectively.

2.2. Methods

ε-Benzylxocarbonyl-lysine anhydride (NCA) monomer was synthesized by usual NCA synthesis method [11] and preserved from moisture before polymerization. 3-Aminopropyltrimethoxysilane (KBM-903), a silane coupling agent, was selected as an initiator. As a result, the silane-terminated poly (ε-benzylxocarbonyl-L-lysine) (PBCL) was synthesized from its NCA in DMF at 25 °C for 24 h. The molar ratio of monomer to initiator was 50:1. The confirmation of polymerization was examined by FT-IR spectra using Perkin–Elmer Spectrum 2000 FT-IR with the resolution of 1 cm⁻¹ in the range of 400–2000 cm⁻¹.

The degree of polymerization of silane terminated PBCL was evaluated to be 14 from high-resolution ¹H NMR (Bruker, AVANCE200 FT-NMR spectrometer) in trifluoroacetic acid. The silane terminated PBCL was dissolved in trifluoroethanol (TFE) and the secondary structure of the polypeptide [12] was estimated from J-820 K Spectropolarimeter (JASCO) at 25 °C in N₂ atmosphere.

The LB method was performed by use of LB film deposition apparatus NL-B1040-MWCT (Nippon Laser and Electronics Lab., Japan). The silane terminated PBCL was dissolved in a mixed solvent of DMF/benzene (3:7 vol/vol) and subjected to sonication for a few minutes. A 25 µl of the polymer solution (45 µmol/l) was spread over on pure water by micropipette at 25 °C and after evaporation of the solvent mixture, the monolayer was transferred by upward drawing LB method onto the pre-colored silica substrate at a suitable surface pressure. In this case, before the LB film deposition, the silicon wafer has been sintered at high temperature (~1060 °C for 3 h) to obtain the pre-colored silica substrate. Further, the monolayer of silane terminated PBCL on the silica substrate was annealed for fixation [13] (at 110 °C for 20 min).

And then, the PBCL monolayer fixed on the substrate was treated with HBr/CH₃COOH and benzene mixture (25% HBr/CH₃COOH:Benzene, 1:3 vol/vol) to remove ε-benzylxocarbonyl group (Z group) from the monolayer of PBCL yielding cationic poly (L-lysine) (PLL). The PLL monolayer on the substrate was rinsed continuously with toluene, acetone and MilliQ water, and finally dried in N₂ stream at 25 °C.

RNA aptamer was kindly provided by Prof. Kikuchi, Toyohashi University of Technology, Japan. The RNA aptamer suspension in aqueous solution of 70% ethanol was centrifuged at 15,000 rpm for 15 min at 4 °C. After discarding the supernatant, the RNA aptamer was pellitized by air drying (stored at −85 °C). The required concentration of RNA aptamer solution was prepared in DEPC (diethyl pyro-carbonate) treated MilliQ water. A suitable amount of the solution was put on the PLL fixed substrate (~10 mm²) to get biochips coated with RNA aptamer. Sphingobium yanoikuyae was kindly offered by Prof. A. Hiraishi, Toyohashi University of Technology, Japan. Binding studies were carried out by immersing the RNA chip obtained into the diluted solution of S. yanoikuyae [14] (1 × 10⁵ cfu/ml) at 25 °C for 30 min. The biochip was air dried, then subjected to topography and UV-reflective spectrophotometer analysis.

UV-reflective measurements were carried out by use of UV/Vis Spectrophotometer V-500 (JASCO) together with the attachment of ARV-474 (JASCO) at an incident angle of 10°. The surface topography was analyzed with Atomic Force Microscopy (AFM) at ambient temperature by use of a Nanoscope IV (Digital Instruments Inc.) in tapping mode.

3. Results and discussion

Scheme 1 shows a step-by-step process of biochip preparation and application.

(1) A monolayer was derived from PBCL and it was covalently attached to a pre-colored silica surface through silane coupling agent which is located at the terminal (Scheme 1a).
(2) Modification of PBCL to PLL on the silica surface for the formation of cationic charge (Scheme 1b).
(3) Immobilization of RNA aptamer, which is called RNA chip (Scheme 1c).
(4) Finally, the RNA chip can detect target bacteria as a visual sensor (Scheme 1d).
First, the silane terminated PBCL was analyzed by FT-IR. The amide I and amide II adsorption peaks at 1652 and 1543 cm$^{-1}$, respectively, indicated $\alpha$-helix formation. The $\alpha$-helical content in TFE was estimated from Fig. 1 (dashed line) and is found to be 60% ($\Theta_{222} = -20,183$). In addition, the secondary structure of the deposited monolayer on quartz plate instead of silica surface was also confirmed. Fig. 1 (solid line) shows a typical $\alpha$-helix CD pattern. These results have demonstrated the formation of highly ordered monolayers in which the $\alpha$-helical rods are laid on the surface of silica substrate.

3.1. Formation of monolayer

A $\pi$-$A$ isotherm of the silane terminated PBCL monolayer at the air–water interface was shown in Fig. 2. In general, $\pi$-$A$ isotherms of the $\alpha$-helical polypeptide monolayer display a steep rise followed by a more or less horizontal plateau. But, in the present case, there are two transitions, one gradual and the other steep, indicating an unusual $\pi$-$A$ isotherm. The specialty of the polypeptide is that silane coupling agent is positioned at the terminal (Scheme 1). So, the anomalous $\pi$-$A$ isotherm may be owing to the flexible silane coupling moiety at the peptide terminal. Also, we have tried to compare the actual limiting area of both the stages (332 and 174 Å$^2$/molecule) with the calculated value (336 Å$^2$/molecule) when the PBCL rods are laid on the surface. It is clear that the first stage of gradual rise is approximately consistent with the calculated value. The above trend has also been verified with AFM analysis, where the first stage showed a good uniform surface morphology (Fig. 2A) rather than second stage (Fig. 2B). Accordingly the lower pressure (≈5 mN/m) region was optimized for monolayer deposition. The intermolecular interaction based on the flexible PBCL side chains and the terminal resulted in the formation of particular class of $\alpha$-helical molecular membranes which in turn leads to produce a partly bilayered membrane (Fig. 2B).

3.2. Formation of cationic surface

The removal of Z group is a significant part of this study, leading to the formation of cationic charges on the silica surface (Scheme 1). The wettability of the surface was changed significantly when PBCL was converted to PLL.
cationic surface [15]. The monolayer of silane terminated PLL does not strip off from the pre-colored silica substrate against the AFM probe scratching. We can interpret this stability of monolayer on the silica substrate due to covalent bonding of silane coupling at the terminal of polypeptide chain after LB film deposition. In addition, the stability of the PLL monolayer was also good when the resulting chip was rinsed with different solvents such as toluene, acetone and ethyl alcohol continuously during the cleaning process of debenzylation method. This was also monitored and confirmed by AFM topology (Fig. 3B) that the polymer chain was not physically adsorbed but chemically anchored on to the silica substrate.

3.3. Immobilization of RNA aptamer

The interaction of RNA aptamer molecules in the aqueous solution with the organized cationic surface of the tethered monolayer of PLL is evident from the AFM topography image shown in Fig. 3C. The RNA aptamer is bound to cationic surface owing to the effective neutralization at the monolayer surface. The aptamer are found to cover an entire surface of cationic charge, providing good evidence for the successful immobilization. This has demonstrated that the RNA aptamer could be fixed at the top of the surface which is suitable for sensing the microorganisms.

3.4. Binding of bacteria

The color of the resultant chip changes which can be visually observed when a bacterium is bound on its substrate. Monitoring the color change permits a real-time observation of bacteria detection. The RNA aptamer-modified probes showed the color change from yellow to orange in the presence of target Sphingobium bacteria when the concentration is 1000 ppm. This shift of $\lambda_{\text{min}}$ from 463 to 481 nm observed in the reflectance spectra is shown in Fig. 4. In order to estimate the size and thickness of the bacteria, a rare binding portion of the chip, around top edge of the immersed side of the substrate into the bacteria solution, was directly observed by AFM (Fig. 5(i)). As a result, the number of isolated bacteria can be seen. The shape is almost spherical whose diameter is 100–200 nm.

The thickness from the aptamer binding level is estimated to be ca. 30 nm as a maximum.

The structural color of a layer whose refractive index is $n$ can be explained by the following equations of interference of light [16].

$$
\lambda_{\text{max}} = \frac{2h}{m} \sqrt{n^2 - \sin^2 \alpha} \quad (1)
$$

$$
\lambda_{\text{min}} = \frac{4h}{2m - 1} \sqrt{n^2 - \sin^2 \alpha} \quad (2)
$$

where $\alpha$ is the incident angle, $h$ is the thickness of the layer, and $m$ is a natural number ($m = 1, 2, \ldots$). From Eq. (2),

$$
\Delta \lambda_{\text{min}} = \frac{4\sqrt{n^2 - \sin^2 \alpha}}{2m - 1} \Delta h \quad (3)
$$

can be derived. The experimental condition $\alpha = 10^\circ$, a general $n$ value of organic materials = 1.5 and the average observed value of $\Delta h = 15$ nm (Fig. 5(i)) were inserted in Eq. (3) yielding $\Delta \lambda_{\text{min}} = 30$ nm ($m = 2$) and 18 nm ($m = 3$). The observed $\Delta \lambda_{\text{min}} = 18$ nm can be comparable to these calculated $\Delta \lambda_{\text{min}}$. So, it may be said, therefore, that the shift
of $\lambda_{\text{min}}$ is owing to the thickness increase based on the bacteria binding.

This RNA chip behaves as a color indicator of an environment, especially when introduced into the bacterial contaminated solution.

The stability of bacterial binding was verified by sonication of the RNA chip for 10 min. The AFM image which shows that some bacteria were still remaining indicates rather strong binding between the RNA aptamer and bacteria (Fig. 5(ii)).

In short, we have demonstrated biologically hybrid silicon probe that are sensitive to bacteria and their detection process is suitable for making a visual sensor.

4. Conclusion

We have successfully synthesized a silane functionalized PBCL. FT-IR and CD measurements revealed the $\alpha$-helical conformation and $\alpha$-helical content, respectively. Further, the silane terminated PBCL was transferred to the surface of silica substrate as self-assembled monolayers (SAMs) by LB method, which plays a key role for the preparation of template in biochip preparation.

The topology of self-assembled monolayers (SAMs), the subsequent protonated surface of silane terminated PLL and RNA aptamer biochip was characterized by AFM measurements. The results proved that the well-arranged $\alpha$-helical rods gives a smooth surface. It was also confirmed that the debenzylation/protonation of PBCL and immobilization of the RNA aptamer on the surface resulted in some degree of roughness on the silica substrate.

The RNA aptamer probe can be proved to be particularly useful in the area of food contamination for the identification of microorganisms including bacteria. The biochip demonstrated in this report opens a new category of stimulus-responsive system.

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