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Surface immobilization of antibody on silk fibroin through conformational transition

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ABSTRACT

In recent studies silk fibroin has been explored as a new material platform for biosensors. Based on these developments, a procedure for the immobilization of antibodies on silk fibroin substrates was developed as a route to functionalizing these biosensor systems. By controlling the conformational transition of the silk fibroin, a primary antibody was immobilized and enriched at the surface of silk fibroin substrates under mild reaction conditions to maintain antibody function. Compared to chemical crosslinking, the immobilization efficiency in the present approach was increased significantly. This method, achieving high loading of antibody while retaining function, improves the feasibility of silk fibroin as a platform material for biosensor applications.

1. Introduction

Biosensors have been widely applied in various fields such as medical diagnostics, environmental monitoring and as a defense against biohazards [1,2]. Recent reviews highlight four basic elements that are important to achieve a state-of-the art biosensor: (i) the biosensor itself; (ii) the surface chemistry and architecture; (iii) the method of protein immobilization; and (iv) the method for signal detection [3–5]. Immobilization strategies have been extensively developed based on different substrates and methods. These approaches are regarded as the key to high performance in biosensor platforms [6,7]. A critically important factor to be considered for these strategies is efficient immobilization of an antibody onto the solid surface in a manner that achieves high loading with retention of affinity [8].

To date, most biosensors have been fabricated using silicon, metal, glass and polymers as substrate materials [9,10]. However, a number of problematic issues such as low biocompatabity and high cost may hinder the widespread use of these systems and especially for biomedical applications. Recently, silk fibroin has been explored as a useful multifunctional material platform for the development of sensor devices, due to its excellent chemical and mechanical stability, biocompatibility, and optical properties [11,12]. Combined with the favorable optical properties of silk, new nanoimprinting techniques have been developed that suggest useful options for the fabrication of all-organic biophotonic components on the nanoscale that can be readily functionalized and employed as a new material platform [13]. Based on these developments, the study of antibody immobilization on silk fibroin substrates can further enhance the feasibility and thus utility of silk fibroin as a biosensor platform.

In previous studies, regenerated silk-based materials were normally stabilized by the induction of silk II formation through the use of solvents [14,15] or by physical stretching [16,17]. Recently, water-insoluble silk films mainly composed of silk I rather than silk II have been prepared by controlling the material curing process [18,19]. Compared to films mainly composed of silk II structure, silk films containing higher silk I content have better hydrophilic property and increased degradation rates [19], which leads to an extended range of biomedical applications. In the present study, new ways to prepare antibody-immobilized silk fibroin films were studied through the use of these different curing methods. The approaches studied are all based on noncovalent interactions with the antibody, in contrast to the common chemical approaches used in the field. Our goal was to exploit the ability to control conformational transitions of the silk protein to achieve stable antibody–silk interfaces.

2. Experimental methods

2.1. Materials

Cocoons of *Bombyx mori* silkworm were kindly supplied by Tajima Shoji Co. (Yokohama, Japan). The primary antibody (normal

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mouse IgG) and secondary antibody (Alexa Fluor 594 rabbit anti-mouse IgG(H + L)) were purchased from Invitrogen (Carlsbad, CA). 2-[N-Morpholino] ethane sulfonic acid (MES) buffer, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were from Sigma–Aldrich (St. Louis, MO).

2.2. Preparation of aqueous-derived silk solutions

B. mori silk fibroin solutions were prepared according to our previous published procedures [20]. Cocoons were boiled for 20 min in an aqueous solution of 0.02 M Na_2CO_3 and then rinsed thoroughly with distilled water to extract the sericin proteins. After drying, the extracted silk fibroin was dissolved in 9.3 M LiBr solution at 60 °C for 4 h, yielding a 20% (w/v) solution. This solution was dialyzed against distilled water using Slide-a-Lyzer dialysis cassettes (Pierce, molecular weight cut-off 3500) for 72 h to remove the salt. The solution was optically clear after dialysis and then centrifuged to remove the small amount of silk aggregates that formed during the process. The final concentration of silk in water was about 7.5%, determined by weighing the remaining solid after drying.

2.3. Film formation

Water-insoluble silk fibroin films were prepared according to our previous published procedures [18]. A 1.5 ml of silk solution was cast on polystyrene Petri dishes (diameter 30 mm). A lid with holes was placed over the dishes to control the drying area by changing the number of holes. When the hood airflow was maintained at 0.20 m s^{-1} and the drying area was adjusted to 3% of the total surface area of the dish, water-insoluble silk films formed with a drying time of 72 h at room temperature. These films had a thickness of about 100 μm and were used as control and termed SF-N.

2.4. Immobilization of antibody on silk films

As above, a 1.5 ml of silk solution (7.5%) was cast on polystyrene Petri dishes (diameter 30 mm). The hood airflow was maintained at 0.20 m s^{-1} and the drying area was adjusted to 3% of the total surface area of the dish to make sure that water-insoluble silk films formed with a drying time of 72 h. When the silk solution was concentrated for 70 h and transformed into a semisolid state, a 0.1 ml water solution containing 48 μg of primary antibody was cast on the surface of this semisolid silk fibroin film. Water-insoluble silk fibroin films with immobilized antibody were then formed when the semisolid silk fibroin continued to dry for another 2 h under the same drying conditions as described above. The films were then washed five times with distilled water to remove unbound antibody. These films were termed SF-70. As a control, a 0.1 ml water solution containing 48 μg of primary antibody was blended directly with 1.5 ml silk solution (7.5%) and then the blend solution was cast on polystyrene Petri dishes (diameter 30 mm). The water-insoluble silk fibroin films containing antibody were prepared after drying for 72 h under the above drying conditions and termed SF-B.

2.5. Immobilization of antibody via EDC coupling

Following incubation in MES conjugation buffer (pH 6.25) for 1 h, the water-insoluble silk fibroin films were immersed in MES buffer containing EDC and NHS to activate the acidic amino acid side chains for 45 min. The activated fibroin films were then transferred to primary antibody solutions (0.48 mg ml^{-1}) for 2 h to couple the antibody on the films. After washing with MES buffer three times and distilled water five times, the fibroin films containing surface bound antibody were characterized (termed SF-C).

2.6. Fourier transform infrared (FTIR) analysis

FTIR analysis was performed with a Bruker Equinox 55/S FTIR spectrometer (Billerica, MA), equipped with a deuterated triglycine sulfate detector and an attenuated total reflectance (ATR) cell. For each measurement, 32 scans were coded with resolution 4 cm^{-1}, with the wavenumbers ranging from 400 to 4000 cm^{-1}.

2.7. Fluorescence microscopy

The different films containing primary antibody were incubated with secondary antibody solution (Alexa Fluor 594 rabbit anti-mouse IgG(H + L), Invitrogen) at a concentration of 2 μg ml^{-1} for 2 h. After washing several times with distilled water to remove unbound secondary antibody, the films were measured by fluorescence microscopy (BX-60, Olympus, Japan) with single interference filter sets of red (Texas red) to determine the location of the antibody.

2.8. Immobilized antibody density

It is critical for antibody immobilization onto the solid surface to achieve high loading with retention of affinity. In order to study the density of active immobilized antibody, the content of secondary antibody adhering on the silk films was determined fluorometrically at excitation wavelength of 590 nm and emission wavelength of 617 nm using a Fluoroskan Ascent FL spectrofluorometer (Thermo Life Sciences, Basingstoke, UK). Based on fluorescence intensity, a standard curve relating fluorescence to antibody density was first prepared using the secondary antibody over a range of concentrations (data not shown). The fluorescence intensity of the samples was then measured and the amount of secondary antibody calculated by interpolation from the standard curve.

2.9. Confocal laser scanning microscopy

To determine the overall antibody distribution on the films, the primary antibody was replaced by the secondary antibody (Alexa Fluor 594 rabbit anti-mouse IgG(H + L)). Through the preparation processes described above, the secondary antibody was enriched on the surface. Based on our previous method [21], the samples were placed on a culture dish (3.5 cm diameter with glass bottoms) and cultured in phosphate-buffered saline solution for 2 h. The samples were then scanned from bottom to top using a confocal laser scanning microscope (TCS Leica SP2, Welzlar, Germany). The average fluorescence intensity in the scanned area was plotted against the scanning distance (film thickness).

2.10. Antibody stability

To determine the antibody stability immobilized on the surface of silk fibroin, the samples were stored at 4 or 25 °C. At different time points, the active antibody density was determined fluorometrically at an emission wavelength of 594 nm using a Fluoroskan Ascent FL spectrofluorometer (Thermo Life Sciences, Basingstoke, UK).

3. Results and discussion

3.1. FTIR

Changes in the structure of the antibody immobilized silk fibroin films after the different processes were determined by FTIR–ATR. The infrared spectral region within 1700–1500 cm^{-1} is assigned to absorption by the peptide backbones of amide I

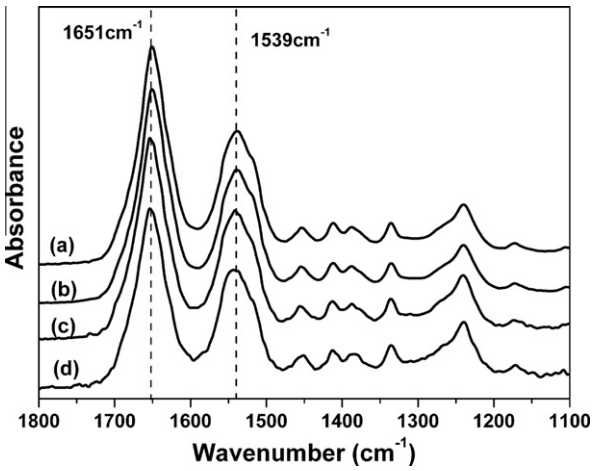

Fig. 1. FTIR spectra of water insoluble silk fibroin films with immobilized primary antibody. (a) SF-N, without antibody; (b) SF-B, antibody directly blended with silk fibroin solution; (c) SF-70, antibody cast on the half-solid silk fibroin that has dried for 70 h; and (d) SF-C, antibody immobilized by EDC crosslinking.

(1700–1600 cm^{-1}), amide II (1600–1500 cm^{-1}) and amide III (1210–1280 cm^{-1}), which are used for the analysis of different secondary structures of silk fibroin. The peaks at 1610–1630 cm^{-1} (amide I), 1510–1520 cm^{-1} (amide II) and 1250–1270 cm^{-1} (amide III) are characteristic of the silk II secondary structure [22]. Although some researchers have suggested that absorption at 1648–1654 cm^{-1} (amide I) and 1535–1542 cm^{-1} are indicative of random coil structure [23], other studies have revealed that these peaks correspond to the silk I conformation [24,25]. Recently, it has been further confirmed that the peaks at 1648–1654 cm^{-1} (amide I) and 1535–1542 cm^{-1} are characteristic of silk I secondary structure by wide-angle X-ray scattering results [18]. As shown in Fig. 1a, through the same preparation processes as in our recent study [18], the water insoluble silk fibroin film prepared in this study was mainly composed of silk I structure. After the antibody was immobilized on the films with the different methods, the films maintained the silk I structure (Fig. 1b–d).

3.2. Immobilized antibody

The fluorescence microscopy images were assessed after the antibody-immobilized silk fibroin films were incubated with the secondary antibody. The amount of fluorescence signal was dependent on the amount of the secondary antibody bound on the top layer of the samples, reflecting the concentration of the primary antibody immobilized on the surface of the films. As shown in Fig. 2a, SF-N had no significant fluorescence signal. SF-70 had the strongest signal intensity, much higher than those of SF-B and SF-C (Fig. 2b–d). The results were further confirmed in the spectrofluorimetry study. As shown in Fig. 3, the secondary antibody levels on the surface of SF-B and SF-C films were 0.16 and 0.27 μg cm^{-2}, respectively, while the density on the surface of SF-70 increased to 0.4 μg cm^{-2}. The results indicate that active primary antibody was enriched on the surface. Covalent binding has been widely used in the immobilization of antibodies. Compared to covalent methods, more antibody was immobilized on the surface of silk fibroin by controlling the conformational changes during the drying process. Importantly, 0.1 ml antibody solution was enough to cover the silk fibroin substrate with a diameter of 3 cm using the present method, while at least 10 ml of antibody solution would be needed if the antibody was immobilized on the silk fibroin substrate of the same size using the crosslinking method. Although other methods have been used to improve the efficiency of the antibody for covalent attachment [26], the present results indicate that the efficiency of surface coupling was increased significantly by controlling the silk structure when compared to the crosslinking approach.

It is difficult to strike a balance between an interaction sufficiently strong to prevent leaching but weak enough to allow the antibody enough flexibility to maintain its activity [27]. The antibody immobilized by physical adsorption such as electrostatic and hydrophobic/hydrophilic interactions generally maintains higher activity than that immobilized by covalent interactions,

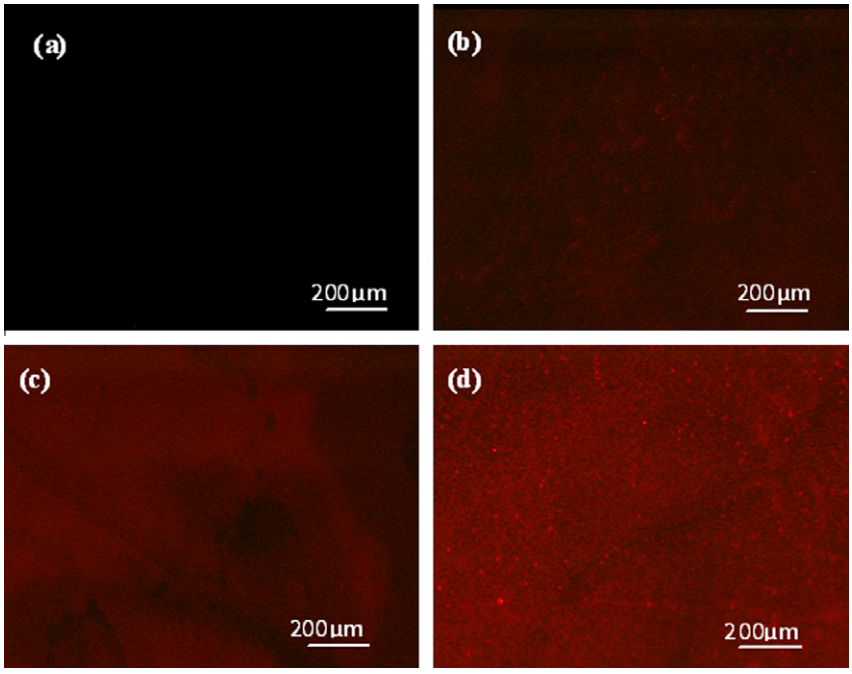

Fig. 2. Fluorescence microscopy images of primary antibody immobilized silk films treated with secondary antibody: (a) SF-N, without antibody; (b) SF-B, antibody directly blended with silk fibroin solution; (c) SF-C, antibody immobilized by EDC crosslinking; and (d) SF-70, antibody cast on the half-solid silk fibroin that has dried for 70 h. The samples were cultured with the secondary antibody solution at a concentration of 2 μg ml^{-1} for 2 h.

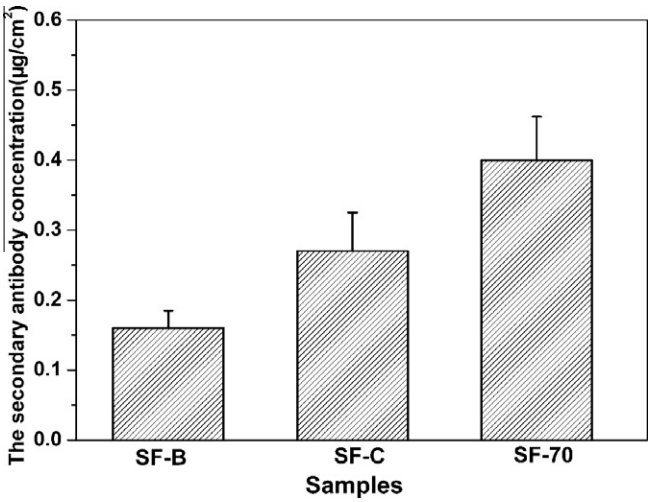

Fig. 3. The concentration of the secondary antibody that was bound on the surface of silk fibroin films containing the primary antibody: SF-B, antibody directly blended with silk fibroin solution; SF-C, antibody immobilized by EDC crosslinking; and SF-70, antibody cast on the half-solid silk fibroin that has dried for 70 h. The samples were incubated with the secondary antibody solution at a concentration of 2 μg ml^{-1} for 2 h, $n = 5$, bars represent standard deviation.

but is more easily leached from the substrate. In the present study, it was found that water-insoluble silk fibroin films with silk I structure had weak hydrophobic/hydrophilic interactions with the antibody, resulting in low loadings when the silk fibroin films were directly cultured with the secondary antibody solution (Fig. 2a). The antibody should be mainly mechanically trapped in insoluble silk I structure in the drying process. It was then confirmed in our recent study. Enzymes could be entrapped in silk fibroin films with silk I structure and only release following the degradation of the films [28]. The detailed immobilization mechanism will be further studied in our future research.

3.3. Antibody distribution

Confocal scanning microscopy further confirmed the enrichment of antibody near the surface. As shown in Fig. 4, when directly blended with silk fibroin solution, the antibody was mainly distributed inside the film. In contrast, when silk fibroin first changed to a semisolid state and then antibody solution was added on the surface of the silk, most of the antibody was distributed near the surface within 20 μm of depth in SF-70 films. In the slow drying process, the silk fibroin changed from solution to a semisolid state in which silk fibroin content was >50 wt.%, retaining a random structure. Silk fibroin quickly transformed to the silk I structure within 2 h, resulting in the formation of water insoluble films. In the semisolid state, the antibody solution could quickly spread on the surface (Fig. 2c), while the infiltration to the inside of the film was limited due to the solidification of the silk fibroin. Once water insoluble silk fibroin was formed due to silk I structures, the antibody was immobilized near the surface.

3.4. Antibody stability

The immobilized antibody on the surface of the silk fibroin films (SF-70) was tested for its stability in dry conditions for up to 28 days (Fig. 5). A slight reduction in percent binding to the secondary antibody was noted at 2 weeks, then stability was maintained over the next 2 weeks at 4 °C. At 25 °C reduced antibody binding was found and about 40% of activity was maintained over the 4 weeks under the conditions of study.

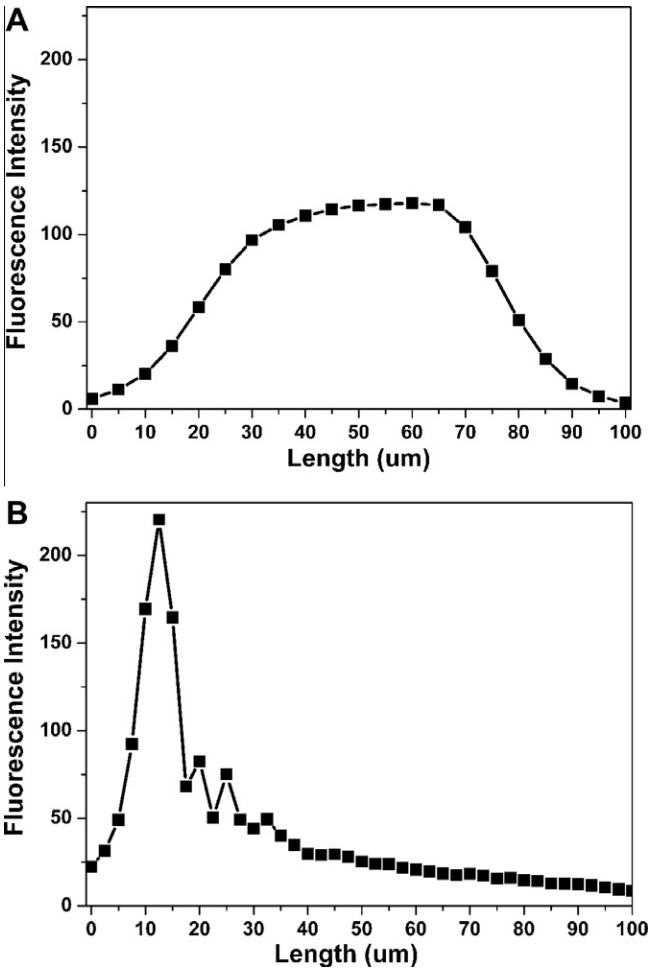

Fig. 4. Confocal scanning microscopy of the antibody distribution in silk films: (a) SF-B, antibody directly blended with silk fibroin solution; and (b) SF-70, antibody cast on the half-solid silk fibroin that has dried for 70 h.

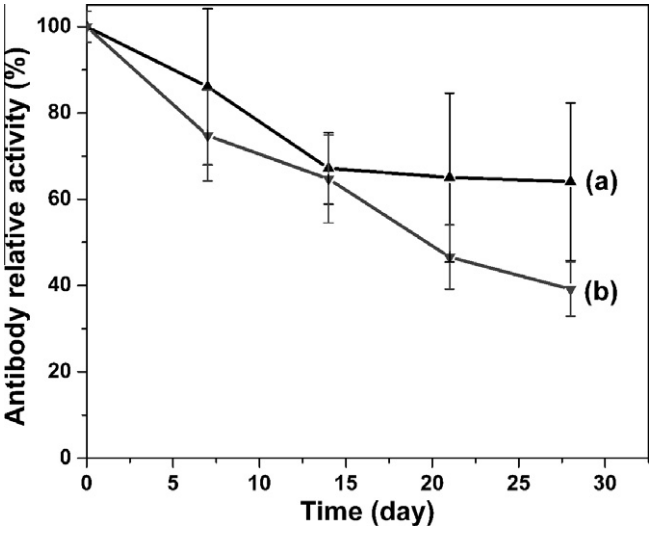

Fig. 5. The stability of antibody enriched on the surface of silk fibroin films when stored at different temperatures in dry conditions at (a) 4 °C and (b) 25 °C, $n = 5$, bars represent standard deviation.

4. Conclusions

A new procedure for immobilization of antibody on silk fibroin substrates was developed. The antibody was immobilized via the

conformational transitions of the silk protein, avoiding the use of chemical crosslinking reactions. By controlling the curing conditions, the antibody could be enriched and fixed near the surface of the silk films. Compared with chemical crosslinking, the conformationally driven immobilization efficiency was increased significantly. Based on recent studies of silk fibroin for biosensors, the present study offers new options in surface functionalization of these types of devices.

Acknowledgements

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figure 2 is difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.actbio.2011.03.001.

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