Sensors and Materials, Vol. 23, No. 4 (2011) 195–205
MYU Tokyo

S & M 0838

Application of Insoluble Fibroin Film as Conditioning Film for Biofilm Formation

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(Received September 30, 2010; accepted November 29, 2010)

Key words: fibroin, contact angle, attachment, biofilm

The aim of this study is to investigate microbial attachment to insoluble fibroin film. The insoluble fibroin film used had tensile strength and contact angle values of 28.79 MPa and 70°, respectively. The attachment of *Escherichia coli*, *Bacillus subtilis*, *Enterobactor cloacae*, *Sphingomonas yanoikuyae*, and *Pseudomonas stutzeri* to insoluble fibroin film occurred rapidly and was maintained for 72 h. The role of the hydrophobic/hydrophilic interactions between microbial attachment and the substratum was investigated using the contact angle. Alginate film (10% or 50% $CaCl_2$ treatment), latex, and urethane had contact angle values of 39°, 15°, 74°, and 116°, respectively. The number of attached *E. coli* cells to insoluble fibroin film was higher than that to urethane. Microbial attachment to the substratum is affected by cell surface characteristics such as hydrophobicity/hydrophilicity. Attachments for the lower-contact-angle microbes were higher than those for the higher-contact-angle microbes. Although the insoluble fibroin film has a relatively higher contact angle value, it has an ability of immobilizing a variety of microbes. These results suggest that the high microbial attachment to insoluble fibroin film is caused not only by the hydrophobicity but also by the characteristics of fibroin.

1. Introduction

Silk has been used commercially for biomedical sutures for decades, and in textile production for centuries. Silk is a continuous filament fiber created by the *Bombyx mori* silkworm, and consists of two types of self-assembled protein: fibroin and sericin.[1] These two proteins both contain the same 18 amino acids, including glycine, alanine, and serine, in different amounts. Sericin is a family of glue-like proteins that hold two fibroin fibers together.[2,3] Fibroin is a main product in silk processing and is a valuable natural fiber possessing gloss and a pleasant texture. Besides its use as a textile

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fiber, fibroin has recently been found to easily be reformed into gels,[4,5] sponges,[6] powders,[7] membranes,[8,9] and films.[10–12] Applications in some processed foods have been proposed and a partial silk material is used in several products such as noodles and sponge cakes.[13,14] Lowered blood cholesterol and glucose levels and decreased alcohol absorption were also observed in fibroin-fed rats.[15]

Silk fiber is known to be one of the strongest and toughest materials because of the dominance of well-orientated β-sheet structures of polypeptide chains.[16,17] However, prepared silk fibroin films, in which the random coil conformation is predominant, are brittle, and need to be treated with plasticizers. Plasticizers such as polyethylene glycol, polypropylene glycol, and glycerol could improve the physical properties of silk fibroin film.[18] Previously, we investigated the factors affecting the insolubilization of fibroin film, and demonstrated that glycerol concentration, drying conditions including temperature and humidity, and pH were closely involved in insoluble fibroin film formation.[19] Most recently, Lu *et al.* have demonstrated that the use of glycerol in combination with silk fibroin provides important benefits to the film properties.[20] Since the fibroin film has excellent biocompatibility and bioabsorbability, and a low level of inflammatory potential, it has been studied as a scaffold for tissue engineering,[21–23] and a material for enzyme stabilization.[24,25] Thus, the application of insoluble fibroin film for the formation of biofilms and immobilization of microbes on the surface is expected.

A biofilm is a population of cells growing on a surface surrounded by an extracellular polysaccharide matrix. The study of biofilms has been of significant interest over the last decade.[26] Biofilm development is a complex process and can be regulated by different factors such as cell surface structure, growth medium, and substratum.[27] Clinically, biofilms are important as a source of persistent infections. They are responsible for dental caries and nosocomial infections, as well as a variety of other infections and diseases.[28] Hence, the studies of biofilms have been focused on the removal and prevention of biofilms. On the other hand, biofilms are particularly suitable for use in bioremediation and biofilm reactors.[29,30] Some advantages of the application of biofilms in a reactor are that comparatively high productivities and high cell densities are achieved.[30] In these reactors, reaction rates are usually high as compared with those in the other types of reactor.

The technology that immobilizes microbes at high cell densities has been applied to biosensors. A microbial biosensor is an analytical device that couples microbes with a transducer to enable rapid accurate and sensitive detection of target analytes in fields as diverse as medicine, environmental monitoring, defense, and food processing and safety.[31] Recently, genetically engineered microbes based on fusing of the *lux*, *gfp* or *lacZ* gene reporters to an inducible gene promoter have been widely applied to assay toxicity and bioavailability.[31] Since microbial biosensors require operational stability and long-term usage, so the choice of immobilization method plays an important role in achieving excellent capability. The physical method is one of the simplest methods for microbe immobilization. Typically, a microbe is incubated with an immobilization matrix, such as alumina and glass beads,[32,33] followed by rinsing with a buffer to remove unattached cells. However, this immobilization method alone generally leads to poor long-term stability because of the detachment of microbes. The immobilization of

microbes by entrapment can be achieved by either the retention of the cells in a dialysis or filter membrane or in chemical/biological polymers/gels.[32] A major disadvantage of entrapment immobilization is the additional diffusion resistance offered by the entrapment materials, which will result in lower sensitivity and detection limit.

In this study, we investigated the attachment of microbes to insoluble fibroin films, and demonstrated that the insoluble fibroin film can collect a variety of microbes in a short period of time. These results suggested that the application of insoluble fibroin film for the immobilization of microbes will provide an advantage in the development of microbial biosensors.

2. Materials and Methods

2.1 *Preparation of insoluble fibroin film*

Insoluble fibroin film was prepared in accordance with the methods of Tsutsumi *et al.*[19] In brief, cocoons of *Bombyx mori* were boiled in a 0.5% (w/v) solution of sodium carbonate and then rinsed with deionized water. The extracted silk fibroin was dissolved in a 50% (w/v) solution of calcium chloride. This solution was dialyzed against deionized water for 2 days to remove salt. Glycerol was added at a final concentration of 0.5% to the 6.5 mg/ml solution of fibroin. Insoluble fibroin films were then prepared by casting the solution onto smooth polypropylene plates and drying at 30°C and 70% relative humidity.

2.2 *Preparation of alginate film*

Equal amounts of 1% (w/v) solution of sodium alginate and 0.1% (w/v) solution of calcium chloride were mixed, and the mixture was subsequently dried. The dried film was then immersed in a 10% (w/v) or 50% (w/v) solution of calcium chloride for 1 min. The prepared film was further dried and alginate film was obtained.

2.3 *Tensile strength measurement*

The insoluble fibroin film was cut into rectangles (approximately 150×600 mm^2). The tensile strength testing was carried out at a crosshead speed of 10 mm/min using a tensile testing machine (RTC-1350A, A and D company, Japan).

2.4 *Contact angle measurement*

The contact angles were measured using a contact angle device (DM-301, Kyowa Interface Science, Japan). For surface analysis of the characteristics, deionized water was dropped on the surface, and then video images were taken. Video images were automatically inputted to an attached computer in which the contact angles were measured using an image analysis program.

2.5 *Bacterial strains and culture conditions*

Escherichia coli XL1 Blue (Cosmo Bio., Japan), *Bacillus subtilis* ISW 1214 (Takara, Japan), *Enterobactor cloacae* NBRC 13536 and *Staphylococcus aureus* NBRC 14462

were cultured in nutrient broth (Becton, Dickinson and Company, Sparks, MD USA) and grown at 37°C. *Sphingomonas yanoikuyae* NBRC 101704 and *Pseudomonas stutzeri* NBRC 12510 were grown in marine broth (Becton, Dickinson and Company, Sparks, MD USA) and nutrient broth at 30°C, respectively. *Candida tropicalis* IFO 1440 and *Candida krusei* NBRC 1395 were cultured in sabouraud medium (Kanto Kagaku, Japan) at 20°C. *Lactobacillus plantarum* ATCC 1497 was grown in MRS medium (Kanto Kagaku, Japan) at 37°C.

2.6 *Attachment experiments*

Insoluble fibroin film, alginate film, urethane, and latex were cut into squares (approximately 150×150 mm^2). Three-hundred microliters of the bacteria culture was diluted in 2.0 ml of appropriate medium in a sterile dish, and the films were inoculated in this mixture for 72 h. To analyze the microbial attachment for a short-term period (for 1 min), 2.0 ml of the bacteria culture was incubated with films in a sterile dish. The bacteria were then incubated with a film at an appropriate temperature for 1 min or 72 h, after which the film was rinsed with sterilized water three times to remove nonattached bacteria. Thereafter, the attached bacteria on the surface of the film were extracted by vortexing in 2.0 ml of sterilized water. The numbers of attached cells were determined on appropriate solid media. Results were expressed in colony forming units (CFU)/cm^2.

3. Results and Discussion

3.1 *Tensile strength of insoluble fibroin film*

The thickness and tensile strength of the insoluble fibroin film were 0.07 mm and 28.79 MPa, respectively. Because protein-based films composed by natural biopolymers are brittle, plasticizers such as glycerol, polyethylene glycol, and polypropylene glycol are used to improve the physical properties of the films.[18] The brittleness is mainly due to extensive intermolecular interactions. Plasticizers can reduce the intermolecular interactions, soften the rigidity of the film structure, and increase the mobility of polypeptide chains, resulting in improvement in the flexibility and the extensibility of the films. Ma and Song reported that fibroin film treated with 3% glycerol had a tensile strength of 14.24 MPa.[18] Our results showed that the insoluble fibroin films prepared in this study had a higher tensile strength value than the 3% glycerol treated fibroin films. Moreover, our fibroin films had a higher tensile strength value compared with synthetic polymer films such as commercially used low-density polyethylene (LDPE) having tensile strength values of 9–15 MPa. The insoluble fibroin films used in this study had a tensile strength value 2 times that of LDPE.

3.2 *Contact angle of insoluble fibroin film*

The contact angle is the angle at which a liquid interface meets a solid surface. The insoluble fibroin film, alginate film (10% or 50% $CaCl_2$ treatment), latex, and urethane used in this study had contact angles of 70°, 39°, 15°, 74°, and 116°, respectively. In general, if the liquid is very strongly attracted to the solid surface, the droplet will completely spread out on the solid surface and the contact angle will be close to 0°. On

many highly hydrophilic surfaces, water droplets will exhibit contact angles of 0° to 30°. If the solid surface is hydrophobic, the contact angle will be larger than 90°. Our results indicated that the insoluble fibroin film, alginate film, and latex can be classified as hydrophilic materials, and the urethane can be classified as a hydrophobic material.

3.3 *Microbial attachment to insoluble fibroin film for a long-term period*

The attachment of five strains of microbe, i.e., *E. coli*, *B. subtilis*, *E. cloacae*, *S. yanoikuyae*, and *P. stutzeri*, to insoluble fibroin film was studied (Fig. 1). The *E. coli*, *B. subtilis*, and *E. cloacae* were incubated with insoluble fibroin film for 72 h at 37°C in the nutrient broth. The *S. yanoikuyae* and *P. stutzeri* were incubated with insoluble fibroin film for 72 h at 30°C in the marine broth and nutrient broth, respectively. After rinsing to remove nonattached cells, the numbers of microbes attached per unit surface area were determined by viable counting. The numbers of *B. subtilis* and *E. cloacae* cells attached to insoluble fibroin film after 3 h were 10^6 CFU/cm^2 and 10^4 CFU/cm^2, respectively. The number of cells increased with elapsed time, and reached 10^8 CFU/cm^2 at 72 h. Similarly, the number of *S. yanoikuyae* cells was 10^4 CFU/cm^2 at 3 h, and increased to 10^6 CFU/cm^2 at 72 h. *E. coli* and *P. stutzeri* existed stably on the insoluble fibroin film for 72 h, although the numbers of cells varied. Moreover, the number of *E. cloacae* cells attached to the insoluble fibroin film was maintained at 10^6 CFU/cm^2 for 2 weeks (data not shown). These results indicated that the insoluble fibroin film could effectively collect and retain a variety of microbes on the surface at a high cell density for 72 h.

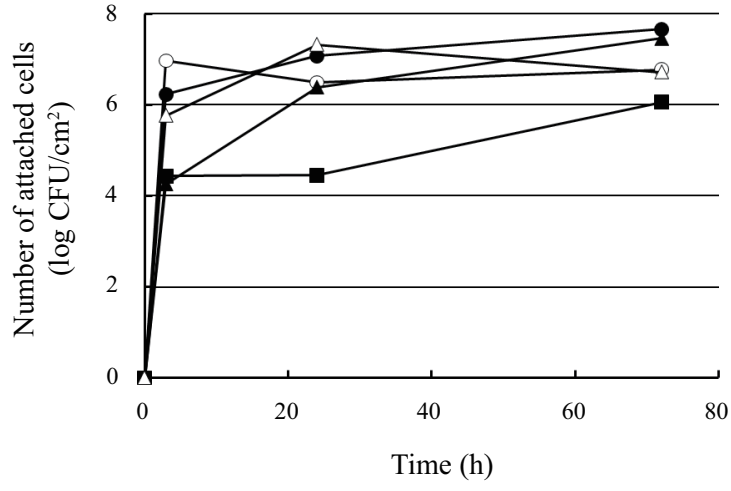

Fig. 1. Kinetics of attachment of several microbes to insoluble fibroin film. The number of cells attached was determined by viable counting. Symbols: △, *E. coli*; ▲, *B. subtilis*; ●, *E. cloacae*; ■, *S. yanoikuyae*; ○, *P. stutzeri*.

3.4 *Microbial attachment to insoluble fibroin film for a short-term period*

Primary attachment constitutes the serendipitous meeting between a substratum and a microbe, which takes place within only seconds. It was thought that the microbial attachment to the insoluble fibroin film occurred in a short period of time. Then, the number of *E. coli* cells attached to insoluble fibroin film for 60 min was determined (Fig. 2). The number of *E. coli* cells attached to insoluble fibroin film was 10^6 CFU/cm^2 at 1 min, and that was maintained for 60 min. In addition to the result of *E. coli*, the numbers of *B. subtilis*, *E. cloacae*, *S. yanoikuyae*, and *P. stutzeri* cells attached to insoluble fibroin film at 1 min were 10^4 CFU/cm^2, 10^6 CFU/cm^2, 10^4 CFU/cm^2, and 10^6 CFU/cm^2, respectively. These results indicated that the insoluble fibroin film is a material that can collect a variety of microbes at high cell density in a short period of time.

3.5 *Relationship between microbial attachment and surface contact angle of substratum*

Characteristics such as surface hydrophobicity, surface charge, and surface chemical composition have been shown to affect the attachment of cells. Nonspecific cell to surface interactions have been explained by a number of physicochemical interactions including van der Waals, electrostatic interactions, and hydrophobic interactions.[34,35] The surface hydrophobicity has been identified as an influential factor for microbial attachment.[36,37] Then, the numbers of *E. coli* cells attached to latex classified as a hydrophilic material, and urethane classified as a hydrophobic material were compared with those of insoluble fibroin film to assess whether the insoluble fibroin film has a superior ability to collect microbes (Fig. 3). The numbers of attached *E. coli* cells at 1 min to latex and urethane were 10^5 CFU/cm^2 and 10^4 CFU/cm^2, respectively. The

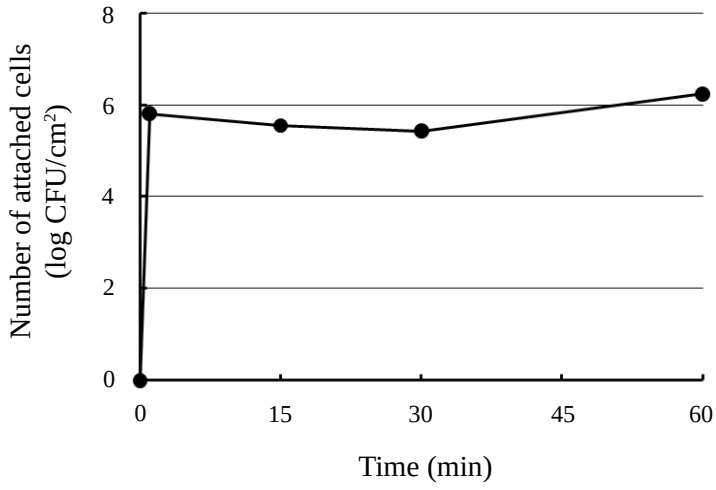

Fig. 2. Kinetics of attachment of *E. coli* to insoluble fibroin film. The number of *E. coli* cells attached was determined by viable counting.

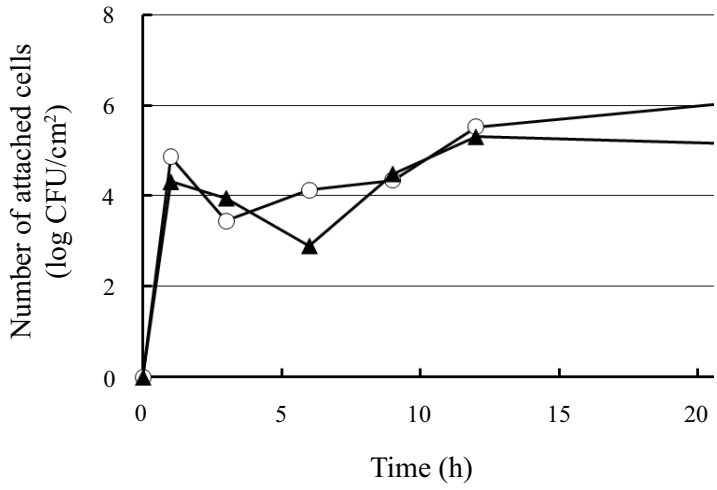

Fig. 3. Kinetics of attachment of *E. coli* to latex and urethane. The number of *E. coli* cells attached was determined by viable counting. Symbols: ○, latex; ▲, urethane.

numbers of *E. coli* cells increased on the surface of latex and urethane, and reached 10^6 CFU/cm^2 and 10^5 CFU/cm^2 at 24 h, respectively. Compared with urethane, latex could collect a large number of microbes. On the other hand, the number of attached cells to the insoluble fibroin film was 10^6 CFU/cm^2 at 1 min, and that was increased and reached 10^7 CFU/cm^2 at 24 h (Figs. 2 and 1). These results suggest that the ability of insoluble fibroin film to collect microbes is higher than those of latex and urethane.

The relationship between the *E. coli* attachment and surface contact angle is represented in Fig. 4. The numbers of *E. coli* cells attached to the alginate films (10% and 50% $CaCl_2$ treatment) have a value approximately equal to that of insoluble fibroin film. On the other hand, the numbers of *E. coli* cells attached to latex and urethane were lower than that of the insoluble fibroin film. The insoluble fibroin film has an ability to collect microbes despite the fact that it has a higher contact angle value than that of alginate films. These results indicated that the high microbial attachment to insoluble fibroin film is not only caused by the force of hydrophobicity but also the characteristics of fibroin.

In the most basic form, bacterial attachment can be divided into two stages: the docking stage and the locking stage.[27] In the docking stage, the attachment is caused by attractive or repulsive forces including electrostatic and hydrophobic interactions, steric hindrance, and van der Waals forces. In the locking stage, loosely bound organisms consolidate the attachment process by producing exopolysaccharides that form a complex with surface materials. Since the microbial attachment to insoluble fibroin film occurred in a short period of time, the primary attachment in the docking stage may be involved in the excellent bioabsorbability of insoluble fibroin film. Detailed information of the

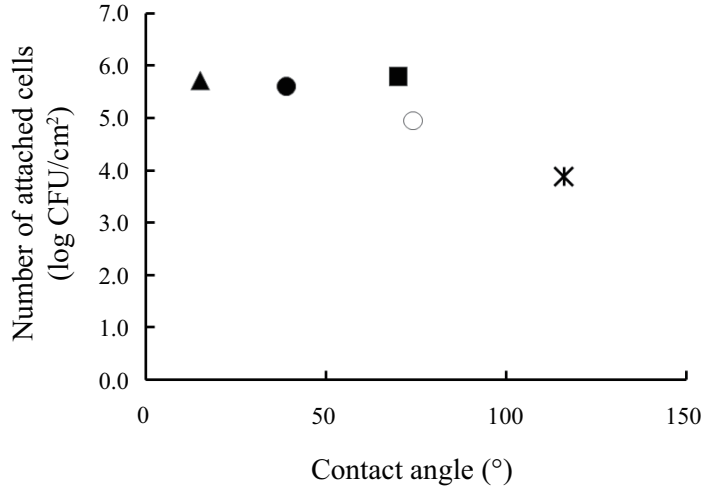

Fig. 4. Relationship between contact angle and attachment to various substrata for *E. coli*. The number of cells attached for a minute was determined by viable counting. Symbols: ▲, alginate film (50% $CaCl_2$ treatment); ●, alginate film (10% $CaCl_2$ treatment); ■, insoluble fibroin film; ○, latex; *, urethane.

relationship between microbial attachment and fibroin is not yet clarified, and further investigation is necessary.

3.6 *Relationship between microbial attachment and surface hydrophobicity of microbes*

We investigated the relationships between microbial attachment to a variety of materials and the cell surface hydrophobicity of four strains of microbe, i.e., *S. aureus*, *L. plantarum*, *C. tropicalis*, and *C. krusei*. Previous reports indicated that *S. aureus*, *L. plantarum*, *C. tropicalis*, and *C. krusei* have contact angle values of 15.6°, 36°, 94.2°, and 118°, respectively.[38–40] *S. aureus* and *L. plantarum* were classified as hydrophilic microbes, and *C. tropicalis* and *C. krusei* were classified as hydrophobic microbes. Figure 5 shows the relationship between microbial attachment and microbial surface hydrophobicity. The numbers of *S. aureus* and *L. plantarum* cells attached to hydorophilic materials such as alginate film and insoluble fibroin film were approximately 10^3-fold higher than those to urethane. The attachments of *C. tropicalis* and *C. krusei* cells were similar to those of hydrophilic microbes in that they are highly attached to the hydrophilic materials, although the numbers of cells attached were lower than those of hydrophilic microbes. Van Loosdrecht *et al.* showed that the microbial attachment to a solid surface such as glass was related to the hydrophobicity of the microbes.[41] Hydrophobicity has also been identified as an important factor in microbial attachment to human epithelial cells,[42] soybean leaves,[43] and air-water interfaces.[44,45] Our results

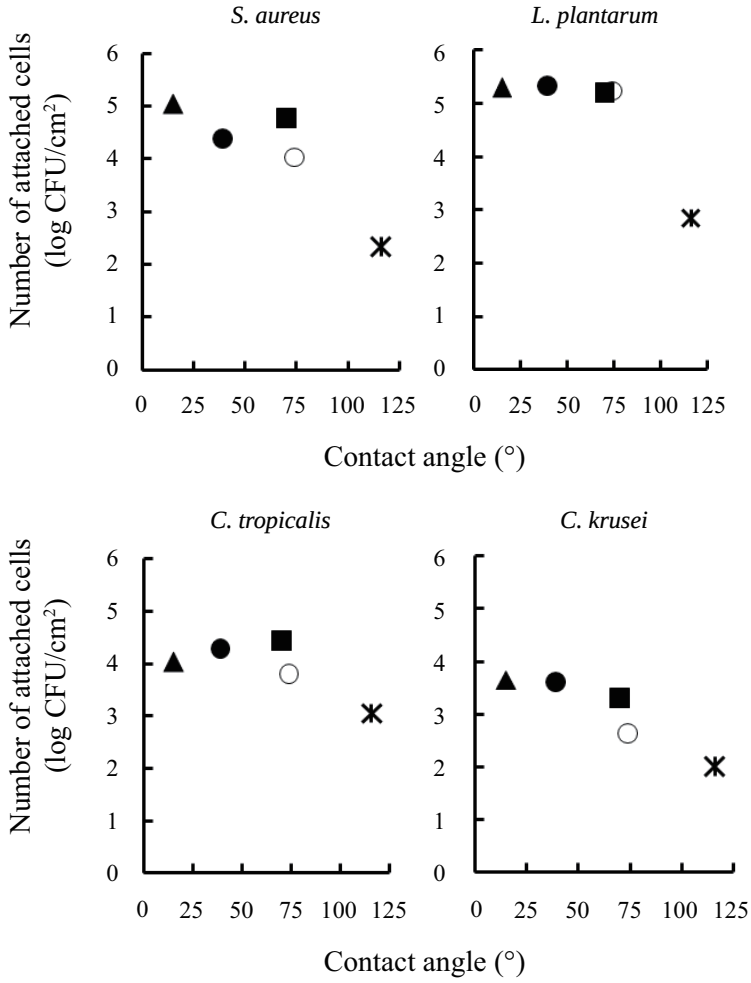

Fig. 5. Relationships between contact angle and attachment to various substrata for *S. aureus*, *L. plantarum*, *C. tropicalis*, and *C. krusei*. The number of cells attached for 1 min was determined by viable counting. Symbols: ▲, alginate film (50% $CaCl_2$ treatment); ●, alginate film (10% $CaCl_2$ treatment); ■, insoluble fibroin film; ○, latex; *, urethane.

indicated that the microbial surface hydrophobicity plays an important role for the attachment to a variety of substrata. The insoluble fibroin film, however, has a high ability of collecting both hydrophilic and hydrophobic microbes at high cell densities, and has a high probability of application for the immobilization of a variety of microbes. Since the fibroin film abundantly contains glycine, alanine, and serine, these amino acids may be involved in the microbial attachment. Although the reason for the ability of the

insoluble fibroin film to collect microbes is unclear, it is thought that the application of the insoluble fibroin film for the immobilization of microbes will lead to the development of a microbial biosensor that has storage, operational, and environmental stability.

Acknowledgment

This work was supported in part by the Regional Innovation Cluster Program of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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