A Novel In Vitro Model to Study Staphylococcal Biofilm Formation on Intraocular Lenses under Hydrodynamic Conditions

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Purpose. To develop a novel in vitro model to study the formation of *Staphylococcus epidermidis* biofilm on intraocular lenses (IOLs) from the primary-attachment phase to the biofilm-accumulation phase. The model was designed to replicate intraocular conditions especially by taking into account intraocular hydrodynamics.

Methods. The model consisted of Tygon tubing connected to a vial containing acrylic hydrophobic IOLs. Three septa, placed along the tubing, allowed, respectively, the artificial aqueous humor's arrival and its elimination and the bacterial suspension's inoculation. A first pump allowed the aqueous humor's movement along the circuit, whereas a second one regulated the flow at which the nutritive environment was regenerated. The whole circuit was placed in a 34°C water bath. Every 2 to 4 hours, lenses were taken from this environment. Bound bacteria were removed by scraping of optical faces and counted. All data are presented as the mean, SD, and coefficient of variation (CV). Comparisons among experiments were performed by one-way analysis of variance (ANOVA).

Results. Calculated CVs were close to 30, showing that biofilm formation was homogeneous. Differences between experiments were nonsignificant for each removal time. The model provided the full kinetics of *S. epidermidis* biofilm growth on acrylic hydrophobic IOLs, with a stationary phase reached after 28 hours of incubation.

Conclusions. Biofilm development is modulated by many variables, including environmental factors. The findings in the present study of bacterial colonization of IOLs under intraocular physiological conditions allow understanding and more accurate targeting of biomedical device-related infections such as endophthalmitis. (*Invest Ophthalmol Vis Sci.* 2006;47:3410–3416) DOI:10.1167/iovs.05-1070

Postoperative endophthalmitis remains one of the worst complications after intraocular surgery. Despite improved methods of prophylaxis, surgical techniques, and treatments, endophthalmitis still represents a therapeutic emergency that often leads to definitive functional loss or even anatomic eye loss.[1,2]

The exact mechanisms by which the most frequently involved bacterium *Staphylococcus epidermidis* causes endophthalmitis after cataract surgery have not been completely elucidated.[3,4] Nevertheless, the binding of bacteria to intraocular lenses (IOLs) during implantation is well known to be the primary step of the pathogenesis of endophthalmitis and of pseudophakic chronic intraocular inflammations.[5–10] Subsequent colonization and production of extracellular polysaccharide glycocalyx (slime) on the IOLs surfaces generally lead to the formation of a multilayered confluent biofilm. This biofilm may protect the embedded bacteria against the host's defense mechanisms, such as antibodies and phagocytes, and against antibiotic therapy.[11] Thus, reducing the adherence of bacteria to intraocular implants and inhibiting biofilm production could help to decrease the incidence of endophthalmitis.

Bacterial adhesion to IOLs has been the subject of numerous investigations.[5–7,10,12–19] All in vitro studies performed were stationary experiments, which consisted only of studying the early phase of biofilm formation (primary attachment of bacteria to the substrate) by placing lenses in various bacterial suspensions and removing them after several hours of incubation. Many discrepancies were found between these studies because of variations of experimental conditions such as bacterial strains, incubation times or temperature, and type of medium used.[5,12] Moreover, the lack of similarity between experimental conditions and intraocular physiological conditions made it difficult to extrapolate these in vitro results to the clinical situation.[20]

The purpose of the present work was to develop an in vitro model that allows the study of different microorganisms that attach to various IOLs. In particular, our model was designed to study *S. epidermidis* biofilm formation, including both the primary attachment and the biofilm accumulation phases. To the best of our knowledge, the entire process of biofilm development on IOLs surfaces has not yet been the subject of an experimental investigation. As the influence of the surrounding medium is essential for biofilm formation, this experiment was also designed to replicate the in vivo conditions as closely as possible, especially by taking into account intraocular hydrodynamic conditions and by using artificial aqueous humor.

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Materials and Methods

Intraocular Lenses

Ninety-six sterile IOLs (SA30AT Acrysof hydrophobic acrylic IOLs, provided by Alcon Laboratories, Paris, France), were used throughout the study. All lenses had identical optical diameters (5.5 mm).

Bacterial Strain

The microbiology department of Edouard Herriot Hospital (Staphylococci National reference Centre, Lyon, France) provided a clinical isolate of *S. epidermidis* (N890074), already reported by our team.[5,12] This strain was isolated from infected cerebrospinal fluid after a complication of a ventriculoperitoneal shunt in a child with hydrocephalus. N890074 was identified by the following characteristics: colony and microscopic morphology, lack of coagulase activity on rabbit plasma (bioMerieux, Marcy l'Etoile, France), absence of production of a clumping factor (Staphyslide; bioMerieux), and results of a staph gallery (ID32; bioMerieux). This isolate is able to produce a great amount of slime.[6] By using polymerase chain reaction amplification, we checked that this strain carried the intercellular adhesion (*ica*) locus, which is known to encode production of *S. epidermidis* antigens that mediate adhesion to biomaterials and between the bacterial cells.[5] For the assays, bacterial concentration was spectrophotometrically adjusted to a high inoculum of 10^9 colony-forming units per milliliter (CFU/mL) in a sterile physiologic saline solution (BSS; Alcon Laboratories). Twenty milliliters of the bacterial solution was inoculated by injection into 190 mL of the in vitro flow conditions model described in a later section, to obtain a local bacterial concentration of 10^8 CFU/mL.

Medium Composition

The artificial aqueous humor was constituted of sterile physiological saline solution to which glucose (1 g/L), yeast extract (0.5 g/L), and casein peptone (1 g/L) were added.

Perfusion Model

The model was constituted of a sterile Tygon tubing, 75 cm in length with an inner diameter of 6.4 mm (Masterflex; Fisher Bioblock Scientific, Illirch, France), connected to a 165 mL vial, which would contain the IOLs. The model's volume was estimated to be 190 mL. Three septa, placed along the Tygon tubing, allowed respectively the arrival of the artificial aqueous humor and its elimination into a waste container and the inoculation of the bacterial suspension. The first pump (four-headed peristaltic pump, Masterflex L/S; Bioblock Scientific) allowed the movement (2 mL/min) of the artificial aqueous humor along the circuit, whereas the second one (Dosi-Flow 1-SY; Leventon, Barcelona, Spain) regulated the flow (95 mL/h) at which the nutritive environment was regenerated (Fig. 1).

Sterile IOLs were linked by their haptics with 8-0 sterile silk sutures (Alcon Laboratories) to one of the three to four supporting metallic structures fixed to the head of the recipient vial. Each metallic structure could support four implants. The metallic structure was designed to allow the placement of all IOLs in a vertical position at the same height into the vial's contaminated artificial aqueous humor. The whole system was placed in a 34°C thermostat-controlled bath (Polystat; Fisher Bioblock Scientific) under a laminar flow hood (PSM Type II; Jouan, Inc., Saint-Herblain, France).

Quantification of Adhesion

Every 2 to 4 hours, the IOLs were removed from the test vial. They were rinsed three times in sterile water to eliminate nonadhering bacteria and then transferred into 3 mL of 0.9% saline solution. Bound bacteria were removed by gentle scraping of both optic surfaces. Bacterial aggregates were subsequently dissociated through the needle of a syringe and vortexed for 3 minutes. The resultant suspension was then diluted and spread over a nutritive agar plate (Trypticase-Soja, BioMerieux, Marcy l'Etoile, France). Colonies were counted after 24-hour incubation at 37°C. The number of bacteria was expressed as colony-forming units per unit area of the optic's IOL (CFU/mm^2). After scraping, all IOL optics were stained with crystal violet 0.25% (Sigma-Aldrich) and examined under light microscope to check that all adhering bacteria had been removed.

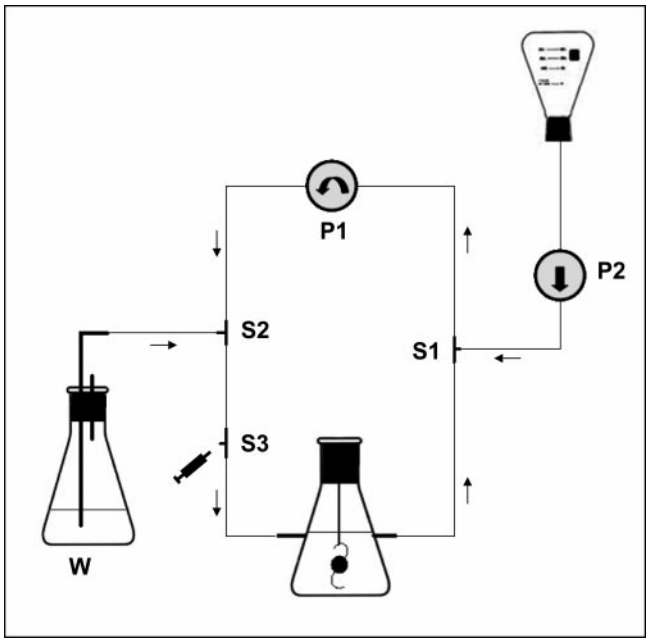

Figure 1. Schematic representation of our biofilm device. Artificial aqueous humor is pumped (P2) from a reservoir into Tygon tubing connected to a vial containing the intraocular lenses. Another pump (P1) allows the movement of the artificial aqueous humor along the circuit. Excess medium is removed to waste (W). Three septa allow the arrival of the pseudo aqueous humor (S1), its elimination into the waste container (S2), and the inoculation of the bacterial suspension (S3).

To estimate biofilm development on our IOLs, we favored biofilm removal from the substratum by mechanical forces before examination and measurement. Scrapping is preferred as it allows, better than sonication, the removal of adherent bacteria without affecting their viability.[21–23] Indeed, recovery efficiency of sonication methods used to quantify microorganisms attached to surfaces has recently been questioned.[24] It appears that ultrasonic treatments depend on ultrasonic apparatus, which are often used to kill bacteria to produce endotoxins. Therefore, it could interfere with an accurate assessment of the biomass. For biofilm measurement we have shown previously that several techniques, such as a viable plate count procedure, bioluminescence, and scanning electron microscopy, may be used.[12] We chose to rely on the most commonly used procedure, which is the viable plate count, in which the resuspended and dispersed biofilm cells are plated onto a solid microbiologic medium, incubated, and counted.[25] This method is known to be efficient in removing and counting biofilms from their supports.[22,25]

Statistical Analysis

The means, standard deviations, and coefficients of variation (CVs) were calculated. The differences between biofilm formation on IOLs linked to the same metallic structure were considered nonsignificant if the corresponding coefficient of variation was close to 30. Comparison among experiments was performed by one-way analysis of variance (ANOVA) conducted on computer (SPSS for Windows, ver. 12.0; SPSS

TABLE 1 Comparison of Biofilm Formation on Each of the Four IOLs Fixed to the Same Metallic Structure and Removed from the Test Vial at the Same Time

	4 h		6 h		8 h		12 h		14 h		16 h		20 h	
Experiment Number	1	5	1	5	1	5	1	5	2	6	2	6	2	6
Mean number of bound bacteria (CFU/mm^2)	7140	12697	24271	25962	34602	27772	75347	55703	115119	134165	361693	322283	242686	304462
Coefficient of Variation	26.07	26.27	42.5	30.38	19.36	10.81	8.29	14.29	9.96	6.48	20.40	11.70	39.43	13.89

Biofilm was considered homogenous, as calculated coefficients of variation were close to 30. Therefore, biofilm formation did not depend on lenses' localization in the vial.

Inc., Chicago, IL). The differences were considered nonsignificant at $P > 0.05$.

RESULTS

The developed model provided the full kinetics of *S. epidermidis* growth on hydrophobic acrylic IOLs (Fig. 2).

The biofilm growth could be described in four phases:

- A latent phase during the first 12 hours of incubation, which corresponds to the beginning of the biofilm process, with a slow accumulation of attached biomass.
- A dynamic or accelerated-growth phase, from hour 12 to hour 24 of incubation, with fast development in the biofilm and a clear accumulation of attached biomass.
- A linear growth phase, corresponding to accumulation of biofilm on the support at constant rate. At the end of this phase, at hour 28 of incubation, the IOL surface was entirely colonized.
- A stabilization phase, which was reached after 28 hours of incubation and is characterized by constant and maximum counts of bound bacteria.

Biofilm on each of the four IOLs fixed to the same metallic structure was considered homogenous, as calculated coefficients of variation were close to 30 (Table 1). Therefore, biofilm formation did not depend on lenses' localizing into the vial. Differences between independent rounds of experiments were considered nonsignificant ($P > 0.05$ for each removal time; Table 2).

Analysis of all scraped and stained IOLs under light microscope confirmed that no bacteria had remained adherent to the surfaces.

DISCUSSION

Bacterial endophthalmitis remains the most feared complication after cataract surgery with IOL implantation. Indeed, an appreciable percentage of endophthalmitis cases remains responsible for a definitive functional loss (50% of the patients recover visual acuity lower than 20/400), or even for anatomic eye loss.[1]

Coagulase-negative staphylococci, particularly *S. epidermidis*, are recognized as one of the most important etiological agents of endophthalmitis after cataract surgery.[26] All published studies showed that *S. epidermidis* is the most common organism contaminating the anterior chamber after uneventful cataract surgery.[27–30] *S. epidermidis* is also the most common microbe found in acute endophthalmitis (50%–70% of cases).[4,31,32] It seems likely that these microorganisms, because of their relative ability to adhere to surfaces, opportunistically infect IOLs during the implantation process or just after. This bacterial binding phase to IOLs surfaces is the first step in IOL colonization. It is eventually followed by bacterial production of a polysaccharide glycocalyx (referred to as slime), in

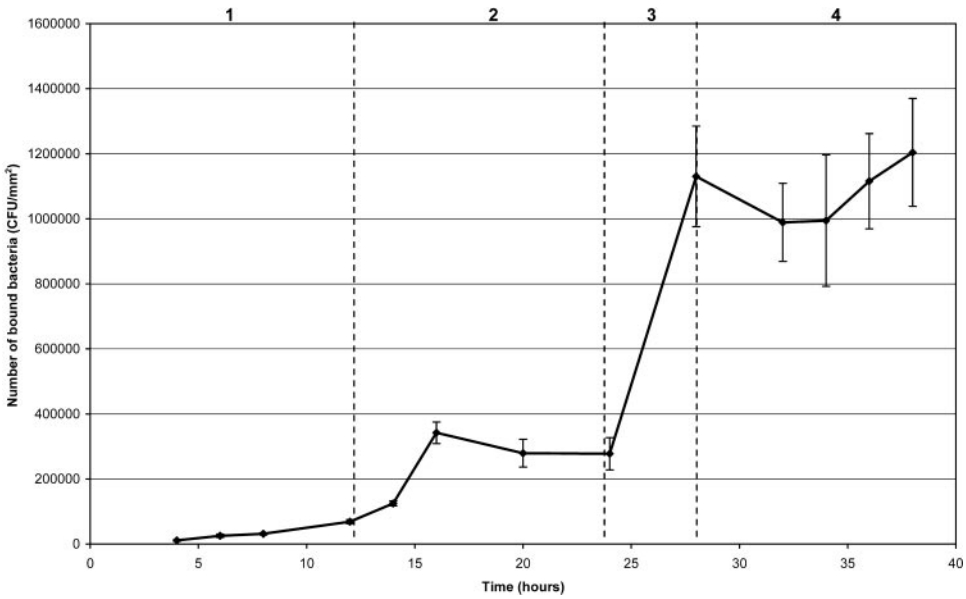

FIGURE 2. *S. epidermidis* kinetics of biofilm formation on hydrophobic acrylic intraocular lenses shown in the four phases of growth: (1) latent; (2) accelerated growth; (3) linear growth; and (4) stabilization.

24 h		28 h		32 h		34 h		36 h		38 h	
3	7	3	7	3	7	4	8	4	8	4	8
278789	278194	$1.14*10^6$	$1.17*10^6$	910502	$1.06*10^6$	990716	998264	$1.00*10^6$	$1.23*10^6$	$1.09*10^6$	$1.31*10^6$
34.25	32.82	31.96	12.20	27.86	14.46	42.72	32.62	28.60	16.56	32.07	16.20

which bacteria are embedded and which finally leads to the formation of a multilayered confluent biofilm.[10,33,34]

Reports of several in vitro experiments studying staphylococcal adhesion to IOLs have been published so far,[5–7,10,13–19] but they are difficult to compare with each other as experimental conditions such as *S. epidermidis* strains, incubation times, medium composition, and methods of adhesion quantification are highly variable. Moreover, these results may not be applicable in vivo as the chosen conditions differ radically from intraocular physiological ones. Indeed each step of staphylococcal biofilm formation, from initial bacterial binding to biofilm maturation, as well as slime production, is modulated by a large number of variables, including environmental factors such as medium composition, temperature, osmolarity, and hydrodynamic forces, to name a few.[35–49] Among *S. epidermidis* strains, these environmental factors have been shown to influence bacterial adhesion and slime production by enhancing the *ica* locus transcription, which particularly encodes for the production of two polysaccharides (PS/A and PIA) that mediate adherence to biomaterials and between the bacterial cells.[35,37,39,50–54] Therefore, a staphylococcal strain classified as biofilm-negative in vivo, can show a biofilm-forming phenotype under the right conditions in vitro. In contrast, a clinical staphylococcal strain isolated in association with a biomedical device infection can unfortunately form no biofilm in vitro in nonphysiological conditions.[55] If any strain can be persuaded to adhere and to form a biofilm in artificial conditions, the relevance of in vitro studies that failed to take physiological environmental factors into consideration as well as the results they provided could be seriously brought into questions.

To overcome these drawbacks, we developed a novel in vitro system designed to replicate as closely as possible the intraocular physiological conditions so that the in vitro behavior of *S. epidermidis* may be applicable in clinical practice.

Under routine planktonic growth conditions, bacteria taken from endophthalmitis samples grow quickly under laboratory conditions. This contrasts with the clinical presentation of endophthalmitis that often occurs late after surgery: 55% present more than 1 week after surgery.[56] Indeed, in intraocular physiological conditions, bacteria rarely interact with such rich growth conditions. The human aqueous humor is actually a poor medium that supports relatively slow and light growth compared with complete chemically defined media.[57,58] To our knowledge, only a single experiment on bacterial adhesion to IOLs tried to create a growth medium close to in vivo composition.[59] The IOLs were incubated with cerebrospinal fluid chosen because of protein content similar to that of aqueous humor.[60,61] However, such growth media based on biological fluids are difficult to obtain in high quantity (190 mL every 2 hours for our device). That is why we decided to rely on balanced salt solutions, which are used as extraocular and intraocular irrigating solutions. Casein peptone and yeast extract were added in minimal quantities to supply essential growth factors such as nitrogen, carbon, minerals, and vitamins.

All in vitro studies performed on bacterial adhesion on IOLs were stationary experiments that consisted of incubating lenses into bacterial solutions of various concentrations. To our knowledge, only one experiment tried to take into considerations the existence of intraocular flows, even if the applied flow rate was a hundred times superior to the intraocular rate.[59] In the human eye, the aqueous humor is secreted into the posterior chamber by the ciliary processes. It then circulates through the pupil into the anterior chamber where it is drained by the trabecular meshwork and the uveoscleral pathways. The average rate of its formation is approximately 2.75 μL/min which means that almost 0.9% of the aqueous humor volume is renewed every minute.[62] Therefore, as it is in our bioreactor, the complete aqueous humor and of course the availability of nutrients in the immediate environment, is entirely renewed every 2 hours.[57] In addition, independent of the forced flow that conducts the aqueous humor from the posterior chamber to the anterior chamber, the aqueous humor natural convection flow has to be considered. Indeed, the human cornea is cooled by the surrounding air and by evaporation of the tear film to a temperature less than 37°C (30°C–34°C).[63–65] The warm blood circulating through the iris is sufficient to maintain its surface at a temperature approaching core body temperature (36–37°C).[63,65] The temperature difference between the cornea and the iris creates a temperature gradient in the eye that causes thermal convection currents in the anterior chamber, with a cooler and denser fluid near the cornea to move downward while the warmer fluid near the iris rises. Our bioreactor has been placed in a 34°C thermostat-controlled bath so as to mimic the average aqueous humor temperature.

Table 2. One-way ANOVA Comparing Biofilm Formation on IOLs from Independent Rounds of Experiments

	4 h	6 h	8 h	12 h	14 h	16 h	20 h	24 h	28 h	32 h	34 h	36 h	38 h
P	0.353	0.674	0.134	0.533	0.062	0.107	0.282	0.200	0.722	0.395	0.981	0.299	0.393

Differences between independent rounds of experiments were considered nonsignificant ($P > 0.05$ for each removal time).

The velocity of the convection flow in the anterior chamber has been estimated to be ~1 mm every 1 to 2 seconds.[65] This flow rate was applied to our bioreactor's first pump in charge of aqueous humor circulation among the circuit. More recently, Heys and Barocas[63] found that the velocities generated by natural convection were on the same order of magnitude as those caused by forced flow from the posterior chamber into the anterior chamber. They estimated these flows to be a Reynolds number of ~1 which means that in the eye, laminar flow conditions prevail.

We believe that hydrodynamic shearing is essential to consider, as they influence biofilm development in many ways. Indeed the structural biofilm architecture is modulated by hydrodynamic flows: in laminar or low shear flow, the biofilm microcolonies often assemble into amorphous aggregates, roughly hemispherical or cylindrical in shape; whereas in turbulent or high flow, the circular symmetry tends to diminish, and filamentous streamers are formed instead.[66,67] It also appears that biofilms can adjust the mechanical properties of their slime in response to hydrodynamic shear. Biofilms grown in high-shear conditions are remarkably strong and resistant to mechanical breakage, as they have a stronger slime matrix and subsequently more strongly adherent cells than do biofilms formed in laminar-shear environments, which have a low tensile strength and break easily.[66,68,69] The transport of the energy source to the center of the biofilm is also an important factor to consider. In general, nutrients are dissolved in the liquid flow and must diffuse first through the mass transfer boundary layer and then through the biofilm matrix to reach the bacterial cells.[70] The thickness of the boundary is inversely correlated to the flow pattern over the biofilm surface, meaning that in low-shear conditions, the thickness of the boundary layer increases and results in a decreased rate of nutrient diffusion into the biofilms.[66,71] Physical forces such as hydrodynamics are also known to cause biofilm detachment via either erosion of single cells or sloughing of large aggregates of biomass.[69,72] This detachment process is also important to consider, as it plays a fundamental role in dissemination, contamination, and, ultimately, long-term survival of bacteria.

Although many papers about biofilm in ophthalmology have been published, no fundamental studies have been performed to date on bacterial growth kinetics. Indeed, all these articles studied bacterial adhesion rather than bacterial colonization on IOLs.[5–7,10,13–19] Most of the studies involved an incubation time between 0.5 and 2 hours,[7,10,14,16,19,73] but it usually takes a few hours, even days under unfavorable environmental conditions such as in our study, for the biofilm to form completely.[14] The study of the whole biofilm formation up to the stabilization phase, and not its first binding phase only, appears to be essential if one wants to gain better understanding and control of IOL-related infections. Furthermore, to develop new therapeutic or prevention strategies against endophthalmitis, it is necessary to elucidate mechanisms in biofilm maturation. For several reasons beyond the scope of this article, bacteria embedded in a biofilm are better able to resist attacks by host defenses or antibiotics.[74] Biofilm bacteria can survive the use of antiseptics and/or antibiotics at concentrations hundreds or even thousands of times higher than the concentrations that kill planktonic cells of the same species.[75–77] For that reason, antibiotics often fail in vivo to eradicate the biofilm organisms that may persist as a nidus of infection.[75] At that point, the only way to end the infection may be to remove the infected device. It is now obvious that any method for the study of or determination of the efficacy of a treatment against such organisms that grow in sessile communities should use biofilms and not planktonic cells to do so.[22,76,78] By providing the full kinetics of biofilm growth on IOLs, our novel bioreactor represents a first step that would certainly be more accurate in targeting infections.

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