Biofilm-Related Periprosthetic Joint Infections

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The Use of Planktonic Versus Biofilm Bacteria in Animal Models

Currently, the majority of animal studies that are used to model biofilm-related infections involve the use of an initial inoculum of planktonic bacterial cells from batch cultures [1–24]. The expectation has been that planktonic cells would attach to the surface of a biomaterial, medical device, or surrounding tissue and subsequently form a biofilm. Although valuable, data that has been derived from these experiments may not provide clinicians and biomaterials scientists additional clinical insight into how bacteria that reside in well-established, mature biofilms impact device-related and other human infections when they initially contaminate an implant site.

Following several decades of important observations from investigators that bacteria preferentially adhere to solid surfaces and to one another [25, 26], in 1978 Costerton et al. formally hypothesized that bacteria in nature reside primarily in the biofilm phenotype [27]. Strong support for this hypothesis continues to be shown in the literature that involves collecting, analyzing, imaging, and characterizing bacterial biofilms found in nature, human tissues, and clinically retrieved devices [28–34]. Additionally, since the initial hypothesis of Costerton et al., estimates have suggested that 99.9 % of bacteria in natural ecosystems reside in the biofilm phenotype [35]. Intriguingly, The Centers for Disease Control has estimated that biofilms cause 65 % of infections in the developed world [36]. A public announcement from The National Institutes of Health has stated, "Biofilms are clinically important, accounting for over 80 percent of microbial infections in the body" (see announcement PA-07-288).

Based on these observations and information, it is important to consider that when bacteria come in contact with wound sites, biomaterials, or portals of entry in humans, i.e., inoculate patients, there is strong evidence to suggest that the majority of these bacteria are inherently residing in well-established, mature biofilms. A specific example of this scenario is that of a patient who suffers from a Type IIIB open fracture, which is reduced with a fracture fixation device.

A Type IIIB severe fracture has been defined by Gustilo et al. [37] as having "Extensive soft-tissue injury loss with periosteal stripping and

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bone exposure" that "is usually associated with massive contamination." Rates of infection that accompany open fractures may reach as high as 50 [38–40] and 60 % in at least one reported instance [41]. The potential for open fractures to be massively contaminated is highlighted by the work of Bakken [42] and Torsvik et al. [43] who have shown that even 1 g of soil may contain between 10^7 and 10^{10} bacteria, the majority of which are estimated to reside in the biofilm phenotype [35]. These data indicate that biofilm-dwelling bacteria have the potential to initially contaminate open wound sites.

Limitations of Using Planktonic Cells as Initial Inocula

At least three proposed rationales can be given for why the use of planktonic cells has potentially limited investigators' abilities to detect clinically relevant outcomes of device biofilm-related infections. (1) Planktonic cells are more readily cleared by the immune system than cells residing in a biofilm [44–46]. Thus, when planktonic cells are used in in vivo models, it may be that a portion are eradicated before they can form biofilms. This may contribute to the low reproducibility for the induction of osteomyelitis, which has been suggested by Gaudin et al. [47] as a common problem with animal models of osteomyelitis. (2) It is well documented that planktonic bacterial cells are more susceptible to antibiotics than those residing in a biofilm [48, 49]. Therefore, if antibiotics are administered immediately following inoculation, they may affect planktonic cells more effectively than they would if bacteria in well-established biofilms were used as initial inocula. (3) When planktonic cells are added to an in vivo system, the possibility exists that they may be dispersed rapidly away from the site of initial inoculation, which would dilute the concentration of bacteria per given area—potentially making it easier for the body to handle the bacterial load and prevent attachment to a medical device.

In addition to these limitations that may accompany the use of planktonic cells as initial inocula, investigators have depended heavily on minimum inhibitory concentrations (MICs) to determine the dose of antimicrobial that should be delivered, either from a device coating or intravenously, to prevent and/or treat biofilm-related infections. The limitation of the MIC value in this specific instance is that it is based on data derived from planktonic cells from batch culture. Specifically, a MIC is defined by the Clinical and Laboratory Standards Institute (CLSI) as the dose of antimicrobial that is needed to result in a three log reduction ($10^5 \rightarrow 10^2$) of planktonic bacteria over a 24 h period (see CLSI standard M26-A). Antimicrobial efficacy tests as standardized by the Environmental Protection Agency (e.g., SOP Number: MB-09-04 and SOP Number: MB-06-05) are also based on planktonic bacterial responses. At least one standard of the American Society for Testing and Materials (ASTM E645-07) was found to recommend that microbicides be tested against biofilms. Citing these planktonic cell-based standards, Ceri et al. suggest that additional standards must be developed to treat and/or prevent recurring and untreatable infections that are the result of biofilm contamination and/or subsequent biofilm formation on medical devices [50].

The 10^5 Rule May Not Apply to Biofilm

Studies have shown that to prevent infection, bacterial loads must be kept below 10^5 cells/g of tissue [51–55]. This is a rule of thumb used by various clinicians as an indicator of infection [54]. However, this number is strain-dependent and is based on planktonic bacterial cell counts. Citing Bowler [56], Edwards and Harding have stated, "The clinical relevance of the theory that bacterial counts of over 10^5 represent clinical infection has been questioned" [52]. The work of Bernthal et al. [57] may provide support for this statement. They showed that low-grade infection developed in a mouse model of joint arthroplasty when 5×10^2, 5×10^3, or 5×10^4 planktonic bacteria

were used as initial inocula. Antoci et al. [58] found that infection developed in a rat model of periprosthetic infection (PPI) wherein 1×10^3 bacteria were used as initial inocula. It may be that even smaller numbers of cells are required to cause infection if they reside in the biofilm phenotype. Indeed, the ability of low number, mature biofilms to resist antimicrobial treatment and immune system components may enhance our understanding of how bacteria cause infection when initial inocula are on the order of tens, thousands, or tens of thousands of cells as opposed to the hundreds of thousands or hundreds of millions in planktonic form that are commonly used for in vivo studies.

Wolcott et al. [59] have recently undertaken a study wherein they showed that in the early stages of development, biofilms were more sensitive to antimicrobials when compared to biofilms that had matured for more than 24 or 48 h. Their data further suggested that even if similar numbers of cells were present, the maturity, and not so much the number of cells within the biofilm, had a significant influence on its ability to resist antimicrobial perturbations. Their work was designed to model a specific clinical application and effectively addressed those scenarios. Importantly, however, this work followed the predominant pattern of biofilm research wherein enormous numbers of cells accumulated over time within the biofilm growth system. Yet, it may not always be accurate to analyze biofilms as they undergo an increase in their number of cells. Though dynamic, biofilms in real life systems may not display the same growth rates as those generated under optimal conditions in the laboratory. Rather, in natural systems biofilms may increase in cellular number over a longer period of time, mature to a level of equilibrium, and, when challenged by modifications in their environment, respond appropriately.

The hypothesis is that these equilibrated, matured, slow growing biofilms are what primarily contaminate wound sites, surgical sites, parenteral routes, and medical devices within humans. Thus, to model contamination of a wound site with matured, equilibrated biofilms, similar to how they are found in nature, studies may benefit from growing biofilms to threshold levels, allowing them to mature, and then exposing them to wound sites, antibiotics, or other antimicrobial agents in in vitro and/or in vivo systems.

Limitations of Using Biofilms as Initial Inocula

While animal studies may benefit from utilizing biofilms as initial inocula, there are limitations to consider in doing so. First, current technologies for growing biofilms in a laboratory setting, i.e., in vitro, are largely unable to translate to in vivo applications. For example, if biofilms are grown on the surface of a polymeric slide within a Drip Flow Biofilm Reactor, it would be impractical to implant the biofilm-ridden slide in an animal. After a careful literature review, it appears that there is currently only one study in the literature wherein a biofilm reactor has been developed for the specific intent of growing biofilms on the surface of a polymeric membrane such that the biofilms could be used as initial inocula in an animal model (discussed in more detail below) [60, 61].

Second, the use of biofilms as initial inocula is application-dependent. If an infection is well known to be caused by planktonic bacterial cells, it would be inappropriate to use biofilms as initial inocula to model such an infection.

Third, repeatability has the potential to be a complicating aspect of using biofilms as initial inocula (this is also an important aspect of using planktonic bacteria as initial inocula). If biofilms are grown on the surface of a material and, for example, are scraped off, the scraping technique of one person may differ from another. This may further result in variable numbers of bacteria being used as initial inocula. If scraping of biofilms is to be performed, care would need to be taken to standardize the scraping procedure as has been done by Goeres et al. [62]. Similarly, if biofilms are grown on the surface of a material and not scraped off, the procedure for growing biofilms should be standardized and the repeatability confirmed as has been shown by Williams et al. [61].

Number of Bacteria in a Biofilm That May Be Used as Initial Inocula

It does not appear that all biofilms carry the same infectious potential and it is proposed that most have minimal pathogenicity. If the opposite were true, it is likely that many more people would suffer from infections including gingivitis, periodontitis, sinusitis, conjunctivitis, cellulitis, gastroenteritis, vaginitis, and/or colitis. Each human being is colonized with billions of bacteria, the majority of which appear to reside in well-established biofilms [63]. As such, infection may be considered an anomaly that extends beyond the normal host/bacterial relationship. Infection may also occur as humans are exposed to well-known pathogens that reside in biofilms from soil samples, on grocery carts, in food, within the human microbiome, on office desks, in shower heads, women's purses, grocery bags, and a plethora of other locations all over the world.

The number of bacteria that should be used as initial inocula in animal models of infection is application-dependent. Conditions may be considerably different in an animal that is intended to model a patient of total joint replacement or some other elective surgery. Elective surgeries are performed under scrupulously aseptic conditions, yet despite these efforts, rates of infection still range from 1 to 4 % and at times higher [64–71]. If an animal model were used to replicate an elective surgery scenario for biomaterial development, it may be more appropriate to use a low number biofilm as the initial inoculum than what might be used for a massively contaminated open fracture model. Additional consideration would also need to be given for the inclusion of organisms associated with human skin.

When biofilms are grown in the laboratory, it is common to see them reach incredibly high numbers—on the order of 10^7 or 10^{10} cells per given area. Biofilms that contain high numbers of cells can also be found in nature [25, 27, 29, 42, 43]. Similarly, bacterial cells that have been directly observed on and in the human body have been shown to reside in the biofilm phenotype [63, 72]. Biopsy punches of human skin have been estimated to contain ~10^6 cells/cm^2 and it is well documented that the hardy biofilm former, *Staphylococcus epidermidis*, comprises a large portion of these resident commensal bacteria [63, 73, 74]. In the large intestine, several hundred grams of bacteria can be found with numbers reaching an astounding 10^{11} or 10^{12} cells/g of tissue comprising hundreds of species [63, 75, 76]. Notably, 60 % of fecal solids have been shown to be comprising bacteria [77].

Although biofilms are ubiquitous and they tend to dwell in communities that can have very high numbers of cells, it may nevertheless be incorrect to assume that wound sites or surgical sites only become infected when they are contaminated with high number biofilms. To the contrary, a biofilm, or a portion of biofilm that has broken off, that contaminates a wound site may consist of as few as 10^2 or 10^4 cells, if not fewer.

Consider the paradigm of a patient who undergoes elective surgery, such as total joint replacement. After the patient's skin is prepped, 10^6 cells/cm^2 of normal flora may be reduced in number to less than 10^3 cells/cm^2 (a 99.9 % reduction, which is the most common claim of antiseptics). Note that the majority of these have been shown to reside in the biofilm phenotype. Importantly, groups have shown that even following antiseptic treatment, viable cells continue to reside several layers deep in skin [51, 78]. In an unpublished observation, the late Bill Costerton observed matrix-enclosed bacterial biofilms between stratified squamous cells in the distal 5–7 layers of human prepped skin (Fig. 7.1) [79]. While an incision is made during surgery, these viable, biofilm-dwelling bacteria may be transported from the deeper layers of skin through a patient's integument (Fig. 7.2). As such, they may have direct access to subdermal tissues, as well as to the surfaces of transcutaneous or other implanted biomaterials. As there is no data in the literature that involves small number biofilms contaminating wound and/or surgical sites, surgeons and investigators are left to wonder what effect these might have on the development of infection in these scenarios.

There are myriad other paradigms that could be considered with similar scenarios of low

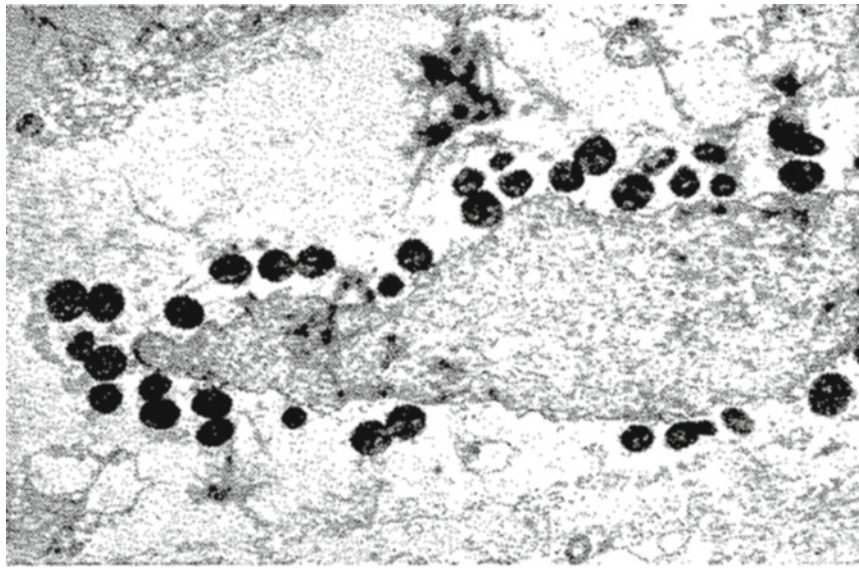

Fig. 7.1 Transmission electron microscope image of an extensive biofilm of Gram-positive bacteria on a skin cell deep (±70 μm) in a moist area between Bill Costerton's toes. Do not attempt this at home. Original image can be found on page 101 of "The Biofilm Primer," by Dr. Bill Costerton [81]. Image used with permission

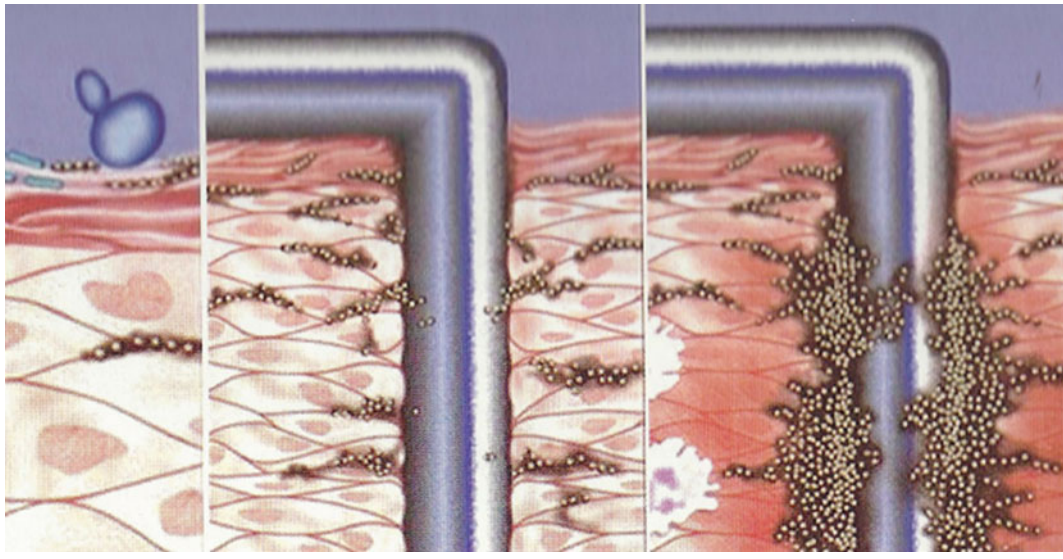

Fig. 7.2 Conceptual drawing of microbial colonization of human skin. In the *left panel* cells of *Staphylococcus epidermidis* (*black*) are seen to inhabit the deeper layers of skin, while cells of this species and of Gram-negative bacteria and fungi (*blue*) all occupy the distal layers of this squamous epithelium. The *central panel* shows that, when the skin has been prepared for surgery and a staple has been inserted, the surface of the skin is uncolonized, but living biofilms of *S. epidermidis* occupy the deeper layers in the vicinity of this foreign body. The *right panel* shows the development of an extensive *S. epidermidis* biofilm on the surfaces of the staple and the initiation of a mild inflammatory response involving the mobilization of leukocytes. Original image can be found on page 102 of "The Biofilm Primer," by Dr. Bill Costerton [81]. Image used with permission

numbers of cells within a biofilm contaminating wound and/or surgical sites. What remains is the fact that hypothesis-driven research needs to be undertaken to determine the impact that low number biofilms have on human health as they attach to and form on the surface of biomaterial devices. Furthermore, there does not appear to be a comparative study in the literature to determine the effect that fewer versus higher numbers of cells in a biofilm, which derive from the same bacterial strain(s), have on the formation of biofilms on biomaterials. For now, the understanding of critical doses required to cause infection is based solely on concentrations of planktonic bacteria.

Possible Methods of Growing Biofilm for Use as Initial Inocula

Connell et al. [80] have recently developed a remarkable method of growing biofilms in small numbers using micron-sized "lobster traps." Although countless possibilities exist for in vitro experimentation with these traps, they are currently limited in that they are adhered to a solid surface. However, modifications to the substrate could make it possible for them to be used as initial inocula in an in vivo model.

As was mentioned previously, a membrane biofilm reactor system has been developed with the specific intent of growing biofilms that could be used as initial inocula in an animal model of infection [61, 81]. Within this reactor, biofilms of methicillin-resistant *Staphylococcus aureus* (MRSA) were shown to develop into three-dimensional pillar-like structures on the surface of the membranes (Fig. 7.3). When used as initial inocula in an animal model of a simulated Type IIIB open fracture, these biofilms resulted in chronic infections that resembled biofilm-related infections that are seen clinically [60].

Importantly, despite the promising results of this work, there is one crucial factor to take into consideration. In the above study, biofilms were grown for a 48 h period, rinsed to remove loosely adherent or nonadherent cells, and transferred in a broth solution prior to using them as initial inocula. These steps were undertaken in an attempt to reduce the possibility of having planktonic cells present. However, the potential still existed that a portion of cells present could have been in the planktonic phenotype. As such, the question may arise; was it the biofilm bacteria or the planktonic bacteria that caused infection? Two responses can be given.

First, it is likely impossible with current technologies to separate all planktonic bacteria from those that reside in the biofilm phenotype such that an inoculum with biofilm bacteria alone is absolutely definitive. Yet, it is also unlikely that such a distinct separation exists between planktonic and biofilm bacteria in natural ecosystems. This may suggest that using an inoculum that has a mixture of the two, with those in the biofilm phenotype being more heavily selected, is clinically relevant.

Second, an additional animal model is currently being used to test the ability of the MRSA strain discussed above to cause infection when inoculated in the planktonic phenotype from batch culture. When the onset of infection was compared between these two animal models, there was a drastic difference in the rapidity and severity of infection that set in with the planktonic bacteria. In that instance, none of the animals survived past 11 days. In contrast, those that were treated with biofilms as initial inocula displayed signs of infection that were much less severe and which progressed at a much slower pace. More specifically, those animals displayed limited signs of pain or distress even out to 12 weeks, but each of them developed a significant osteomyelitic infection.

This contrast in the speed and severity of infection may provide clinical evidence that using biofilms as initial inocula is more correlative to biofilm-related infections that are present in patients. In patients, biofilm-related infections appear to be latent infections that develop slowly over time and which may persist for extensive periods [33]. So although these current animal models provide a promising step in the direction of using biofilms as initial inocula, there are many factors to take into account: a host's health, the pathogenicity of an organism, the ability for an organism to develop into a biofilm, the degree

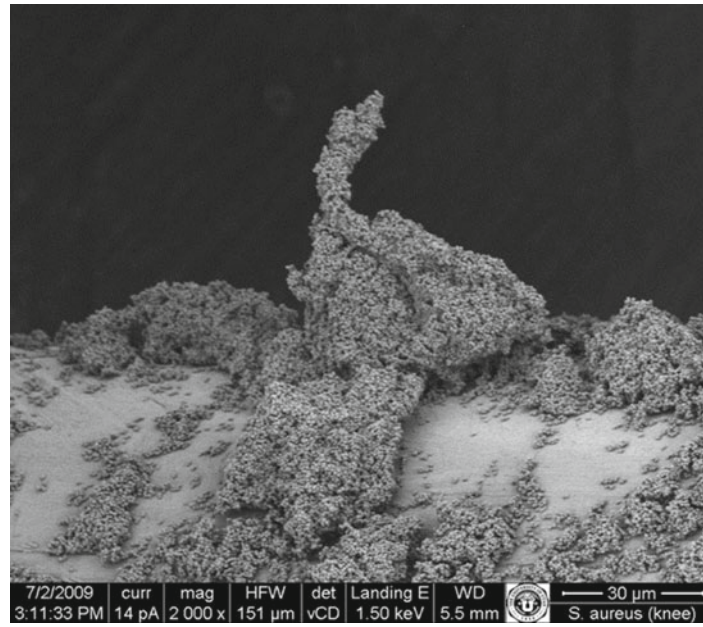

Fig. 7.3 Scanning electron microscope image of a methicillin-resistant *Staphylococcus aureus* (MRSA) biofilm that was grown on the surface of a PEEK membrane within a membrane biofilm reactor. Image used with permission [63]

of contamination, the ratio of cells in the planktonic phenotype to those in the biofilm phenotype, etc. Thus, this issue of planktonic versus biofilm infection is still a limitation and will require additional future testing to overcome the challenges of separating the bacterial phenotypes before more definitive statements can be made.

At this time, with the variety of biofilm reactor devices that are currently available, such as the CDC biofilm reactor, the modified CDC biofilm reactor, the Drip Flow Biofilm Reactor, and "lobster traps," the outlook is promising for a transition in biofilm investigation to occur from the in vitro paradigm to the in vivo setting.

Animal Models That Have Involved Biofilms as Initial Inocula

After a careful literature review, there appear to be two studies wherein well-established, mature biofilms have been used as initial inocula in animal models of infection. The first was published in 2010 by Zhao et al. [82]. To model chronic wounds in diabetic mice, Zhao et al. grew biofilms of *Pseudomonas aeruginosa* on the surface of polycarbonate membrane filters. Biofilms grew on the surface of filters as they were placed on agar that contained a lawn of *P. aeruginosa*. Each membrane was subsequently placed on a wound that had been created on the dorsal skin of a mouse. During the monitoring period, no mice showed signs of systemic infection, yet delayed wound healing was present in those that were treated with biofilm.

The second study wherein biofilms were used as initial inocula was mentioned previously and was performed by Williams et al. [60]. In this study, biofilms of MRSA were grown on the surface of PEEK membranes and placed in apposition to the proximal medial aspect of sheep tibiae. Each membrane was covered with a simulated fracture fixation plate in order to model the clinical scenario of a patient who has bacteria compressed between a fracture fixation and the surface of bone (Fig. 7.4). Infection developed in 100 % of animals exposed to biofilm and, as was mentioned, the infection cycle was similar to biofilm-related infections that are seen clinically.

Importantly, both of these models were developed with very high inocula of bacteria in biofilms. Thus, it remains to be determined if low number biofilms have a similar effect on the development of infection. Nevertheless, both of

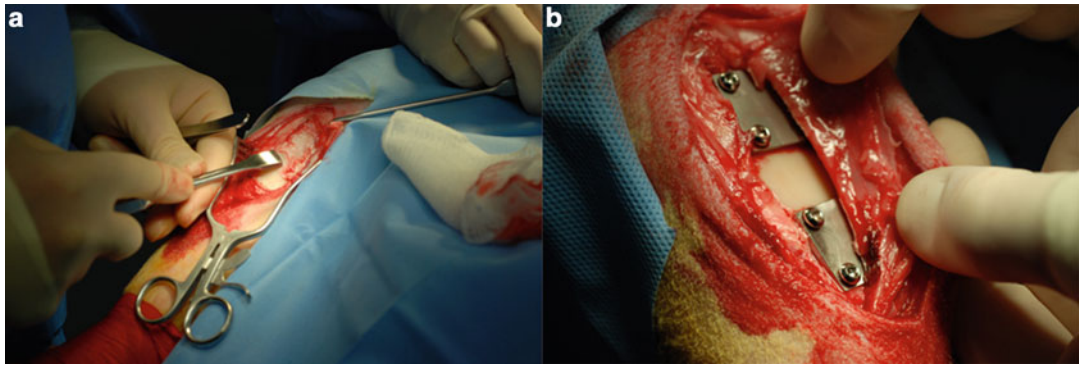

Fig. 7.4 Photographs taken during the surgical placement of PEEK membranes and stainless steel plates in the proximal medial aspect of a sheep tibia as published by Williams et al. [60]. (**a**) The periosteum of each sheep was removed in order to model a Type IIIB open fracture. (**b**) Two stainless steel plates, each of which had a PEEK membrane underneath it that was placed in direct apposition to the bone, were secured to the proximal medial aspect of the tibia

these studies provide an indication that using biofilms as initial inocula has the potential to result in infections that are chronic in nature. Furthermore, these models provide a platform for additional animal work to be performed with biofilms as initial inocula.

Future of Biofilm Studies

The impact of biofilm-dwelling bacteria on human health is becoming ever more apparent. Chronic wounds are now considered to be the result of acute infection that begins with biofilm contamination as opposed to a non-healing wound that is later contaminated and suffers from biofilm formation/infection [83–86]. Heart disease is now indicated to be compounded by biofilm-dwelling bacteria from oral plaque that enter the vasculature [87, 88]. Overall human health is believed to be significantly influenced by an intricate balance of biofilm-dwelling bacteria in gut flora [75]. In short, the impact of biofilms on human wellbeing and disease cannot be overestimated.

Looking to the future of biofilm and biomaterials research, additional approaches for in vitro analyses and design modifications to in vivo models that encompass the use of preformed, well-established, sessile communities of mature biofilms that model those found in nature, in patients, and within the environment can be envisioned. As studies are undertaken to analyze the impact of low number biofilms on infection outcomes, results may indicate that less than 10^5 cells/g of tissue, or per area, will be required to cause infection.

If the efficacy of antimicrobials is tested against high and low number biofilms, those on the order of 10^7–10^9 and 10^2–10^4 cells, respectively, we may uncover deeper insights into the concentrations of antimicrobial in, for example, antimicrobial eluting biomaterials, that are needed to prevent and eradicate biofilm-related infections from developing. We can only wonder at this time how many antimicrobials and antimicrobial eluting biomaterials have been prevented from progressing to clinical, home, industrial, and/or environmental use based on the fact that MIC values, which are primarily the result of planktonic cellular response, have been used to determine the amount that was needed to eradicate bacteria residing in well-established biofilms.

The opposite may be true as well. There is no indication that antibiotics that have been put into clinical use have shown efficacy against low and/or high number biofilms on implants. Although this trend may change as an understanding of the role of biofilm increases, this paradigm has potentially been a contributing factor to the development of antibiotic resistance. More specifically, in various systems, bacteria residing in

biofilms may have been exposed to lower concentrations than are needed to prevent their growth and eradicate them within in vitro and in vivo systems. However, a cavalier approach of simply increasing dosages of antimicrobials alone or used in eluting biomaterials could potentially lead to toxic effects in vivo and cause additional problems. Thus, future work will be needed to elucidate the efficacy and toxicity of antimicrobials used alone or in eluting biomaterials against biofilms in clinical studies.

There is evidence to suggest that bacteria dwelling in the biofilm phenotype have the potential to initially contaminate open wound sites and/or surgical sites of patients. These biofilms may attach to subdermal tissues or the surfaces of implanted devices resulting in chronic, biofilm-related infection. In addition, the impact that low number biofilms have on human infection as well as using well-established, mature biofilms as initial inocula for in vitro and in vivo models may help further the optimization of antimicrobial treatments, such as those used in coatings on biomaterials. In doing so, an understanding of the impact that biofilms from natural systems have as initial contaminants of wounds may also be increased. Most importantly, a shift in the use of biofilms for inoculation methods and analytical techniques may help biomaterial researchers take a step forward, and thus obtain the advantage in the battle against biofilm implant-related infections.

Relevance of Biofilms to the Field of Periprosthetic Infections

There are at least three methods by which bacteria may contaminate, colonize, and form biofilms on the surface of a total joint replacement device and ultimately cause biofilm-related PPI. The first is the possibility for bacteria from a surgeon, other healthcare worker, or the operating room itself to contaminate a surgical site during surgery. The second is for bacteria from the patient's own body to contaminate the surgical site/implant surface. As mentioned, it is hypothesized that biofilm-dwelling bacteria from the deeper layers of a patient's skin, which may not be killed by a surgical scrub, can migrate toward or inoculate the surface of an implant during surgery. The third possibility is for bacteria to spread hematogenously from one area of a patient's body to the surface of an implanted device. Though not yet well documented, this third method may be one cause of late onset PPI. Yet, late onset infections may also be the result of low number biofilms that take days, months, or perhaps even years to colonize an implant surface, reach an infectious dose, and cause PPI.

As our understanding grows of the role that biofilms play in multiple environments including PPI, clinicians and scientists will have the ability to better prevent and treat biofilm implant-related infections. In light of the many problems that accompany biofilm-related infections, such as antibiotic resistance, hospital-acquired infections, patient morbidity, and rising healthcare costs, there is significant motivation to address these issues. Using biofilms as initial inocula in clinically relevant and application-dependent animal models may provide the innovative and unique strategies that are necessary to prevent PPI.

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