Biofilms: An Underappreciated Mechanism of Treatment Failure and Recurrence in Vaginal Infections

Christina A. Muzny and Jane R. Schwebke

Division of Infectious Diseases, University of Alabama at Birmingham

(See the Editorial Commentary by Sobel on pages 607–8.)

Biofilms are microbial communities of surface-attached cells embedded in a self-produced extracellular matrix. They are of major medical significance because they decrease susceptibility to antimicrobial agents and enhance the spread of antimicrobial resistance. Biofilm-associated bacterial and fungal microorganisms have increasingly been recognized to play a role in multiple infectious diseases, particularly in their persistence and recurrence. More recently, biofilms have also been implicated in vaginal infections, notably bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC), particularly in the setting of treatment failure and recurrence. The purpose of this review is to discuss the impact of biofilms on the management and treatment of BV and recurrent VVC and highlight the need for additional research and development of novel therapeutics targeting pathogenic vaginal biofilms.

Keywords. biofilms; bacterial vaginosis; recurrent vulvovaginal candidiasis; treatment failure.

BIOFILM DEVELOPMENT

Biofilms have been described since Antonie van Leeuwenhoek examined the "little living animalcules, very prettily a-moving" in the plaque of his teeth in 1683, but the concept of biofilm growth was not officially described until 1978 [1]. With the advent of sophisticated microscopic techniques, biofilms are now characterized as highly organized sessile microbial communities of bacteria, fungi, or both. Attachment of these microorganisms to an interface is considered an initiating event in the biofilm-process, triggering the self-production of an encasing extracellular matrix in addition to an altered phenotype with respect to growth rate and gene transcription [2]. Biofilms are of major medical significance because they decrease susceptibility to antimicrobial agents and enhance the spread of antimicrobial resistance [3]. Additionally, they provide a safe haven for other opportunistic pathogens to thrive and be a source of infection [4].

Biofilm formation occurs when planktonic (or free-floating) microorganisms encounter a surface and adhere in a reversible fashion while they "explore" the location to ascertain whether it offers nutrients or other advantages. The ability to adhere is a fast process [5]. If the "decision" favors permanent settlement, adherent cells up-regulate genes involved in matrix production and biofilm formation begins [6]. The extracellular matrix provides the physical architecture for microbial interactions, facilitating feedback (sensing and signaling) among the cells [7]. Quorum sensing (QS), or cell-to-cell signaling, is the controlled expression of specific genes in response to extracellular signal molecules or "autoinducers" produced by the microorganisms. QS allows for a unified response that benefits the microbial population as a whole; indeed, QS communication circuits have been found to play a role in the coordinated

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Correspondence: Christina A. Muzny, MD, Assistant Professor of Medicine, Division of Infectious Diseases, University of Alabama at Birmingham, ZRB 242, 1530 3rd Ave S, Birmingham, AL 35294 (cmuzny@uab.edu).

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Table 1. Examples of Human Infections That May Involve Biofilms

Native valve endocarditis
Prosthetic valve endocarditis
Chronic otitis media
Chronic sinusitis
Chronic bacterial prostatitis
Cystic fibrosis pneumonia
Periodontitis
Central venous catheter associated infections
Urinary catheter associated infections
Contact lens related eye infections (ie, keratitis)
Intrauterine device related infections
Gonococcal cervicitis
Bacterial vaginosis
Recurrent vulvovaginal candidiasis

regulation of a diverse array of physiologic activities, including biofilm development and detachment [6, 8]. A mature biofilm community is composed of tower- and mushroom-shaped microcolonies containing sessile cells enclosed by matrix material [2]. Open water channels are interspersed between the microcolonies for nutrient circulation. Biofilm communities have properties similar to a viscous fluid [6]. Basic community structure is universal with only minor variations noted [2].

Biofilm-associated bacterial and fungal microorganisms have increasingly been recognized to play a role in multiple infectious diseases, particularly in their persistence and recurrence (Table 1) [9]. Biofilms have also been found to colonize a wide variety of medical devices, putting patients at risk for device-related infections [2, 5]. More recently, biofilms have been implicated in vaginal infections. Biofilms formed by *Gardnerella vaginalis* and *Candida* spp., key pathogens in bacterial vaginosis (BV) and recurrent vulvovaginal candidiasis (VVC), respectively, represent potentially important virulence attributes and mechanisms of resistance often encountered clinically. The purpose of this review is to discuss the impact of biofilms on the management and treatment of BV and recurrent VVC and highlight the need for additional research and development of novel therapeutics targeting pathogenic vaginal biofilms.

BIOFILMS: PATHOGNOMONIC IN VAGINITIS

It is well accepted that BV results from a loss of the normal lactobacillus-predominant vaginal flora and a synergistic relationship between a large number of microorganisms including *Gardnerella vaginalis* and other anaerobes (BV-associated bacteria, BVAB) [10]; however, the trigger that initiates these alterations is controversial. The epidemiology of BV suggests that it is acquired via sexual transmission, but it is unknown whether BV results from acquisition of *G. vaginalis* as the "founder" organism, leading to the complex changes in vaginal flora [11], or whether BV is transmitted as a polymicrobial consortium [12]. Nonetheless, recent data have shown that BV is associated with the development of an adherent polymicrobial biofilm containing abundant *G. vaginalis* and smaller numbers of BVAB, including *Atopobium vaginae*, on vaginal epithelial cells that is apparent by fluorescent in situ hybridization of vaginal biopsy specimens from women with BV [13]. Subsequent desquamation of these cells coated with bacterial biofilm results in the formation of "clue cells" visualized on saline microscopy of vaginal secretions. Indeed, *G. vaginalis* has been found to have a greater virulence potential (more adherent, more cytotoxic, and has the greatest ability to form biofilm) relative to other BVAB [14–17], supporting the hypothesis that *G. vaginalis* biofilm formation may be an initiating event in the pathogenesis of BV. In an in vitro model for *G. vaginalis* biofilm formation, susceptibilities of biofilms (cohesive *G. vaginalis*) vs planktonic (dispersed *G. vaginalis*) cultures of this organism to H_2O_2 and lactic acid (substances normally produced by lactobacilli in the healthy vagina that reduce the vaginal pH to <4.5 and prevent colonization by pathogenic anaerobes) were compared [18]. *Gardnerella vaginalis* biofilms tolerated 5-fold and 4–8-fold higher concentrations of H_2O_2 and lactic acid, respectively, than planktonic cultures, whereas proteolytic dissolution of the biofilms increased susceptibility of *G. vaginalis* to H_2O_2 and lactic acid. This suggests that biofilm formation contributes to *G. vaginalis* survival and that biofilm disruption resensitizes otherwise resistant biofilm-associated pathogens. This is consistent with biofilm resistance as a phenotypic phenomenon and has important implications for management.

Building on the discovery of the BV biofilm, Swidsinski et al refined the picture of BV transmissibility in a study of women with symptomatic BV and their partners as well as married pregnant women and their partners [19]. In this study, women with symptomatic BV consistently presented with cohesive *G. vaginalis* (indicative of the presence of *G. vaginalis* in a biofilm mode of growth) as did their partners. Among the married pregnant women and their partners, cohesive *G. vaginalis* was also consistently found among the partners of women with cohesive *G. vaginalis*. This concordance was not observed for dispersed (planktonic) *G. vaginalis*. The authors concluded that the biofilm mode of growth represents the infectious or transmissible mode of *G. vaginalis* and BV, whereas the presence of dispersed *G. vaginalis* seems to be of less clinical significance.

Treatment of BV is recommended for symptomatic women [20]; however, despite an initial response, BV recurs or persists in a significant proportion of women [21–23]. This is likely due to persistence of the biofilm, now documented by vaginal biopsy, following FDA-approved therapies such as metronidazole [24, 25]. Alternative treatment approaches targeting the

underlying biofilm are thus needed. In addition, opponents of the sexual transmission theory of BV pathogenesis cite older studies in which treatment of the male sexual partner did not prevent recurrence of BV in the female. The methodological flaws of these studies are nicely described in a recent systematic review by Mehta [26]. Taking these methodological flaws into account, a new phase III clinical trial is currently underway to determine if the treatment of male sexual partners of women with recurrent BV significantly decreases the recurrence rate of BV in the female [Schwebke, unpublished data].

Like BV, VVC is also common, affecting up to 75% of women during their lifetime; 5%–8% subsequently develop recurrent VVC (RVVC), defined as 4 or more episodes per year in the absence of predisposing factors [27]. In addition to causing symptomatic disease, *Candida* spp. can also colonize the vagina in approximately 15%–20% of asymptomatic women [27, 28]. *Candida albicans* is the most common *Candida* spp. associated with VVC although *C. glabrata*, *C. tropicalis*, and rarely other *Candida* spp. are also implicated [28].

It has been hypothesized that virulence factors other than antimicrobial resistance contribute to the pathogenesis of RVVC, including germ tube formation (associated with adherence) and biofilm production (consequence of adherence) [29]. *Candida* spp., particularly *C. albicans*, are well known for forming biofilms on the acrylics of dentures, on implantable devices in the bloodstream, on urinary catheters, and on mucosal surfaces including the oral cavity [30, 31]; it is thus possible that *Candida* biofilm formation may also occur on mucosal tissues of the female genital tract, perhaps during times of increased fungal burden [32]. Although no vaginal biopsy studies parallel to those in the BV literature have been performed in women with VVC or RVVC, in vivo and *ex vivo* murine vaginitis models examining *C. albicans* biofilm formation on vaginal epithelial cells have confirmed this suspicion [32]. In this study, wild-type *C. albicans* strains formed biofilms on the vaginal mucosa as indicated by the high fungal burden and microscopic analysis demonstrating typical biofilm architecture and presence of extracellular matrix co-localized with the presence of fungi. In contrast, mutant strains defective in regulation of morphogenesis (ie, hyphal formation) and biofilm production exhibited weak to no biofilm formation. The ability of 5 types of *Candida* spp. (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. guilliermondii*) isolated from patients with VVC to form biofilms has also been investigated in vitro [29]. In this study, *C. albicans* and *C. parapsilosis* demonstrated the strongest ability to adhere and form biofilms. *Candida albicans* biofilms have also been grown on reconstituted human vaginal epithelial cells, which further supports the virulence attributes of this organism in the lower genital tract [33]. *Candida glabrata*, the second most prevalent pathogenic fungal species in humans after *C. albicans* [34], is also able to form biofilms. A recent in vitro/in vivo study in rats noted that *C. glabrata* is a unique microorganism, which, despite its lack of transition to the hyphal form, formed thick biofilms inside foreign bodies in vivo [35]. Considering the results of these studies, it will be interesting to investigate whether the presence of a biofilm determines whether *Candida* spp. behave as pathogens or colonizers on the vaginal mucosa, allowing a switch from commensalism to a pathogenic state.

Compared with conventional azole antifungal medications, one of the more effective therapies for RVVC is intravaginal boric acid [36], which also has demonstrated clinical utility for recurrent BV [37]. Although its mechanism of action is unknown, boric acid effectively inhibits *Candida* [38] and *G. vaginalis* biofilm formation in vitro (personal communication, Elinor Pulcini, PhD, Center for Biofilm Engineering, Bozeman, Montana), which may account for its efficacy in vivo. Although the role of *G. vaginalis* biofilms in BV has been characterized [13, 14, 24, 25], further research is needed to clarify the role of *Candida* biofilms in the pathogenesis of VVC, particularly RVVC. Novel agents with the ability to interrupt *Candida* [5] and *G. vaginalis* biofilms [11] offer a promising approach to the management of common vaginal infections as primary and/or adjunctive treatments but require additional study.

MECHANISMS OF BIOFILM RESISTANCE

Bacterial biofilms are highly resistant to antimicrobial agents and host defenses by multiple mechanisms that are inherently multicellular (ie, they act together to provide the biofilm with multiple levels of defense against antimicrobial agents) [39, 40]. This resistance typically only applies to microorganisms embedded within the biofilm matrix (ie, *G. vaginalis*) as planktonic organisms are usually more susceptible to killing [18, 39]. Known mechanisms of biofilm resistance include (1) slow or incomplete penetration of antibiotics and host immune cells through the matrix, (2) physiological changes in the biofilm microenvironment due to slow growth and starvation responses, (3) phenotypic change in biofilm cells, similar to spore formation, (4) QS between biofilm microorganisms, (5) expression of efflux pumps, which remove solutes (such as antimicrobials) out of the cells, and (6) "persister cells," small fractions of microorganisms that are able to survive antibiotic concentrations well above minimal inhibitory concentrations [31, 40].

In vitro studies have shown that some antimicrobial agents can readily permeate the biofilm as there is no generic barrier to the diffusion of solutes through the extracellular matrix [41]. However, if an antimicrobial agent is deactivated in the upper layers of the biofilm by catalytic enzyme(s), penetration can be delayed [42]. For example, ampicillin can penetrate a biofilm formed by a β-lactamase-negative *Klebsiella pneumoniae* but not a biofilm formed by a β-lactamase-positive wild-type strain of the same organism where deactivation of ampicillin

occurs in the biofilm surface layers more rapidly than it is able to diffuse [43]. Regarding physiologic changes in the biofilm microenvironment, oxygen may be completely consumed by microorganisms in the surface layers, leading to anaerobic niches in deeper layers that may render pathogenic organisms more resistant to antimicrobials that are less effective under hypoxic conditions [44]. Similarly, accumulation of acidic waste products produced by biofilm microorganisms may lead to pH changes which can directly antagonize the action of an antibiotic [45]. Depletion of substrates can also cause microorganisms as part of a biofilm community to enter a dormant state, protecting them from killing by antimicrobials such as β-lactams, which require actively dividing cells to be effective [39]. It is also hypothesized that a small subpopulation of biofilm cells (ie, "persister cells") can evolve to a unique, highly protected state, similar to spores, which are more likely to survive in the ongoing presence of an antibiotic [39]. Additionally, expression of efflux pumps in biofilm resistance to antimicrobials has also been shown to occur [40]. Efflux pump activity may also play a role in biofilm development as it has been shown that efflux systems are implicated in QS regulation, allowing the intrusion or extrusion of molecules by biofilm cells [40].

Like bacterial biofilms, *Candida* biofilms also demonstrate reduced susceptibility to antifungal agents such as azoles and are less sensitive to killing by the host immune system [31]. Resistance mechanisms in *Candida* biofilms include differential regulation of drug targets (ie, changing the drug target structure so the antifungal is incapable of binding the target), up-regulation of drug efflux pumps, "persister cells" living in a dormant state with inactive targets, and presence of matrix components that prevent antifungals from reaching their targets [31, 46, 47].

RETHINKING TREATMENTS: TARGETING PATHOGENIC VAGINAL BIOFILMS

With the growing realization that biofilms play an important role in the pathogenesis of common vaginal infections such as BV and RVVC and in the setting of low cure rates using current FDA-approved therapies [24, 25], novel therapeutic agents targeting biofilms are needed. This is especially true for BV, owing to the associated serious secondary health complications such as preterm birth and acquisition and transmission of sexually transmitted infections (STIs), including human immunodeficiency virus [48, 49]. Simplified in vitro biofilm models (such as microtiter-plate assays used to grow biofilms and flow cell models that closely mimic natural shear conditions that occur in biofilm development) have been utilized to address basic questions about biofilm formation, physiology, and architecture [9]. Indeed, disruption of the biofilm matrix has been shown to re-sensitize otherwise resistant organisms, as in the case of *G. vaginalis*, consistent with the phenotypic nature of biofilm resistance [18]. However, there are currently very few treatment options targeting vaginal biofilms in any stage of development.

With respect to BV, research on the ability of novel therapeutic agents to disrupt the biofilm has been hampered by the lack of a validated in vitro model of this infection [50]. Despite this challenge, a recent study using a murine vaginal colonization model for *G. vaginalis* demonstrated that *G. vaginalis* biofilms contain extracellular DNA and that enzymatic disruption of this DNA with DNase inhibited the biofilm, suggesting its use as a potential adjunct to existing BV therapies [51]. Hooven et al have also recently evaluated a synthetic retrocyclin (an antimicrobial peptide) in vitro against *G. vaginalis* biofilms, with mixed results [52]. As mentioned previously, intravaginal boric acid has clinical utility in the setting of both recurrent BV and VVC, perhaps by influencing the biofilm [38] and enhancing the antibacterial effect of conventional antimicrobial therapy [37]. In addition, Swidsinski et al recently evaluated the use of octenidine among women with recurrent BV, a local antiseptic previously shown to be highly effective in several biofilm-associated infections including oral infections, orthopedic steel implant biofilms, and biofilms involved in wound infections [53]. The initial cure rate following a 7-day course was high at 87.5%; however, relapse at 6 months due to bacterial resistance was significant at 66.6%. Repeated treatment with a 28-day course led to a cure rate of 75% but was not sustained and was associated with emergence of complete resistance in a considerable proportion of patients. The authors concluded that although initial treatment with this agent was highly effective, the efficacy of repeated and prolonged treatment dropped quickly.

Quorum sensing inhibitors (QSIs) have recently been identified as antibiofilm agents for a number of bacterial species including *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Bacillus cereus* [54–56]. QS inhibition can be achieved by inhibiting signal synthesis or direct degradation of the signal, inhibition of binding of the signal molecule to the receptor, and/or inhibition of the signal transduction cascade. It is currently unknown whether these nonantibiotic compounds could be useful in biofilm-related infections in humans, including pathogenic vaginal biofilms (perhaps as an adjunct to traditional antibiotic therapy). Future research should explore this possibility. It has also been noted that D-amino acids, produced by many bacteria including *P. aeruginosa* and *Staphylococcus aureus*, trigger biofilm disassembly by causing the release of amyloid fibers that link the biofilm cells together [57]. This typically occurs when biofilms have aged, nutrients have diminished, and wastes have accumulated. It would also be useful to determine if these agents could be used as a strategy for biofilm disassembly in the treatment of common vaginal infections such as BV. Complicating research and development in this area is the current lack of validated vaginal infection biofilm models, which limits the ability to evaluate potential

antibiofilm agents in a standardized way. Once this issue has been resolved, clinical trials should be conducted using combination antimicrobial therapy for vaginal infections (such as BV) and biofilm inhibiting agents.

Despite current advances in the understanding of pathogenic vaginal biofilms, particularly with regards to BV, additional research and development in the area of vaginal biofilm infections is needed with the goal of developing validated biofilm models for both BV and RVVC, expanding current treatment options, improving outcomes, and stemming secondary public health risks, which remain significant.

Notes

Disclaimer. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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