Chapter 6
Anti-antimicrobial Approaches to Device-Based Infections

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6.1 Introduction

Biofilms are surface-associated communities of microbial cells that are embedded in a microbe-generated extracellular matrix (**EM**) of polysaccharides, proteins, and DNA. The overall financial impact of biofilm-based infections is estimated to be in the tens of billions of dollars per year [69]. Medical-device-based infections are further complicated in that most, if not all, causative bacterial species are now resistant to multiple antibiotics [43].

Unfortunately, *biofilm infections* are typically treated using the systemic application of antibiotics; compounds are chosen based on their ability to kill or inhibit the growth of *freely suspended microorganisms*. A major concern with this approach is the frequent development of resistance to antibiotics [49]. In fact studies have shown that sublethal doses of antibiotics can actually *exacerbate* biofilm formation [12]. As stated above, biofilm communities tend to be significantly less responsive to antibiotics and antimicrobial stressors than planktonic organisms of the same species [76, 103]. Our work has documented a further complication that the spread of antibiotic resistance genes borne on plasmid DNA, within and between species, is greatly exacerbated within biofilm communities [18, 19, 79]. As a consequence to this increase in resistance, researchers have turned to a number of alternatives to antibiotics, including bacteriophage [104] and bacteriophage lytic enzymes [45], probiotics [59, 87], and human antimicrobial peptides (defensins, cathelicidins, and histatins) [38]. The success of these alternatives awaits much development and optimization.

Unfortunately, most of these alternatives are still based upon some mechanism of killing or terminating the target bacteria, an approach some feel preordains the

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development of resistance in bacteria. It has been recently proposed to develop substances that specifically inhibit bacterial virulence [2]. Such "anti-pathogenic" drugs, in contrast to antibacterial drugs, do not kill bacteria or stop their growth and are assumed not to lead to the development of resistant strains. A very elegant approach comprises the inhibition of regulatory systems that govern the expression of a series of bacterial virulence factors, such as biofilm formation.

Consequently, we review here some recent alternative anti-biofilm approaches that do not necessarily kill bacteria but rather negate biofilm formation. There are numerous prospects of *anti-antibiotic* therapies that are emerging from research; not all of these will be addressed in this chapter. Therapies that will not be discussed here and the reader is urged to explore on their own are: (*a*) biofilm matrix disruption via DNA extraction; (*b*) quorum sensing interference; (*c*) immunotherapies, i.e., vaccines that target bacterial adhesins; and (*d*) biomaterials that heal with such fidelity that they prevent infection similar to the natural healing process.

Here we focus on three novel anti-biofilm strategies: (*1*) disruption of bacterial iron metabolism, (*2*) enhancing phagocytosis, and (*3*) preventing amyloid fibril production within the biofilm extracellular matrix.

6.2 Iron Metabolism Disruption

6.2.1 The Competition for Iron

Iron is critical for bacterial growth and the function of key metabolic enzymes [7, 15, 16, 114], and sequestration of iron is an early evolutionary strategy of host defense [44, 64]. Recent work has also shown that even when sufficient levels are available for bacterial growth, iron limitation blocks development, perhaps by a signaling mechanism [98, 97]. Iron limitation has also been shown to act at several stages of biofilm formation [20]; low iron inhibits bacterial attachment, microcolony formation, and mature biofilm development. Thus, strategies that disrupt iron metabolism present a therapeutic potential against infections caused by biofilm and planktonic bacteria.

One approach to combating infection is to exploit those stresses already imposed on organisms by the in vivo environment or host defenses. Fe metabolism is a major vulnerability for infecting bacteria for two reasons. First, in almost all pathogens, Fe is essential for growth and the functioning of key enzymes, such as those involved in DNA synthesis, electron transport, and oxidative stress defense [23]. Second, free Fe levels are extremely low in vivo (approximately 10^{-18} M) due to multiple host mechanisms that sequester Fe [23]. The importance of Fe limitation in blocking acute infection has been established for numerous bacterial species. In these studies, increasing the amount of available Fe markedly increased acute infections [23, 53]. For example, a single injection of Fe decreased the lethal dose of a *P. aeruginosa* strain (in a murine acute infection model) from $\geq 10^4$ organisms to $\leq 10^1$ [46]. Work

by a number of laboratories has linked Fe metabolism to the pathogenesis of chronic infections, in that high Fe levels (i.e., more Fe than is required for growth) promote biofilm development. High levels of Fe are required for the formation of cell clusters early in biofilm development and for the maturation of biofilms into three-dimensional structures [13, 92, 97]. Fe sequestration may also be protective during infection, as bacteria sampled from infection sites show gene expression profiles indicative of Fe starvation [21, 99, 117]. That host defenses severely limit available Fe and the critical role of Fe in infection suggest that invading organisms may be susceptible to interventions that further disrupt Fe acquisition or metabolism. Exploiting this Fe vulnerability has proven difficult. Fe chelation therapy has been tried, but most microorganisms can also use Fe when it is bound to chelators [115]. Targeting bacterial Fe uptake mechanisms is also problematic because most pathogens have many redundant uptake systems, e.g., *P. aeruginosa* has more than 30 genes encoding different Fe receptors [33]. This redundancy reduces the likelihood that any single therapy could block all Fe uptake systems.

6.2.2 Iron Replacement

Gallium and zinc (group IIIA transition metals) have many atomic features similar to Fe3+, including a nearly identical ionic radius, such that biological systems are unable to distinguish Ga from Fe3+. Unlike Fe3+, Ga does not undergo reduction/oxidation cycling that is critical for Fe to function in many enzymes. Thus, replacing Fe with Ga in such enzymes renders them nonfunctional [29, 84]. Ga can bind to the many siderophores of *Pseudomonas* sp. [11] and can be taken up by other bacteria including *S. aureus*, *S. epidermidis*, *E. coli*, *E. faecalis*, and *S. typhimurium* [15, 41, 40, 61]. Zinc and manganese may also interfere with iron metabolism in bacteria because of similar physicochemical properties and shared uptake pathways. Ga enters cells via the same mechanism used to acquire Fe [30, 31, 85]. Kaneko et al. [65] show that concentrations >1 μM $Ga(NO_3)_3$ inhibited *P. aeruginosa* suspended growth in 1/100 strength TSB medium (Fig. 6.1a). Since the authors were only interested in the specific anti-biofilm effects of Ga, the effects of Ga on biofilm formation were investigated at a low concentration of $Ga(NO_3)_3$ (1 μM), a level that did not impair the growth of suspended *P. aeruginosa* (Fig. 6.1a). In a clinical application, both suspended growth inhibition and anti-biofilm efficacy would be desirable. At concentrations of $Ga(NO_3)_3$ that were sub-inhibitory to suspended cultures, *P. aeruginosa* weakly attached to a glass surface, but biofilm formation was completely negated (Fig. 6.1b) at 0.5 μM.

To determine if Ga would actually kill and eliminate existing *P. aeruginosa* biofilms, Kaneko et al. [65] report growing biofilms for 3 days (with no Ga present) and then switching to medium containing Ga at various concentrations for 48 h. Bacterial viability was assayed using a live–dead stain. Most antimicrobial agents show markedly less activity against biofilms than against planktonic organisms (~1000–10,000-fold less activity, depending on conditions) [103]. In the Kaneko et al. [65] study,

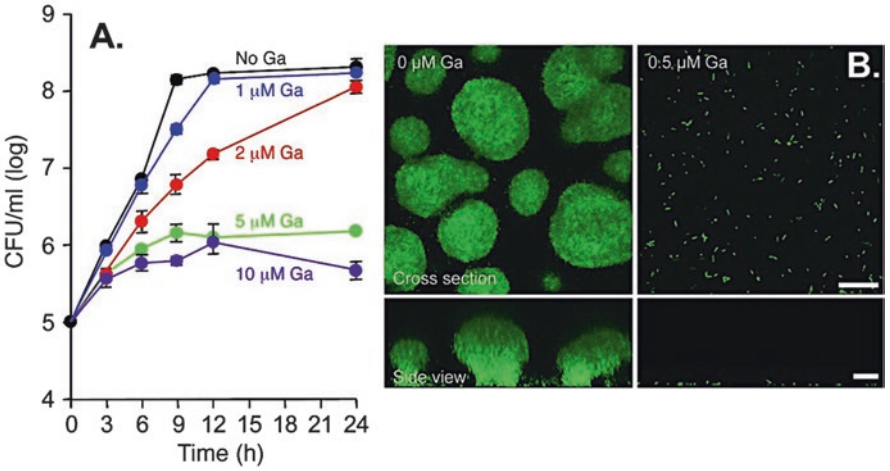

Fig. 6.1 (**a**) Effect of gallium on *P. aeruginosa* growth. $Ga(NO_3)_3$ inhibits *P. aeruginosa* growth in a concentration-dependent manner. Experiments were performed in biofilm medium at 37 °C, and data are the mean of four experiments; error bars indicate SEM. (**b**) Ga prevents *P. aeruginosa* biofilm formation. Confocal microscopic images of GFP-expressing *P. aeruginosa* in flow cells perfused with medium without (*left*) and with (*right*) Ga, 5 days after inoculation. Experiments were performed at 25 °C using 0.5 μg/ml $Ga(NO_3)_3$; this concentration did not inhibited suspended growth (see frame A). Top images, top-down views (*x*-*y* plane); bottom images, side views (*x*-*z* plane); scale bars, 50 μm (Adapted from Kaneko et al. [65])

bacteria within mature biofilms were killed to varying degrees by concentrations of Ga similar to those that killed planktonic cells: 10, 100, and 1000 μM.

Subsequently, there have been a few reports of incorporating gallium into the formulation of certain biomedical implants. In a series of four papers from the Valappil group [95, 108–107], gallium in the form of Ga_2O_3 was incorporated into phosphate-based glasses by a conventional melt quenching method. Intended for the treatment of periodontal disease, the efficacy of the various gallium-loaded glasses was assessed using the bacterial species *Porphyromonas gingivalis*, *Streptococcus gordonii*, *Streptococcus mutans*, and *Pseudomonas aeruginosa* cultivated both in suspension and in biofilms. While certain formulations reduced the planktonic concentration of cells significantly, gallium-loaded glass formulations had only minor-to-no effects on biofilm populations.

6.2.3 Enhanced Cellular Uptake Using Siderophore–Gallium Complexes

In response to sequestered iron in mammalian hosts, successful pathogens can acquire iron within the host via four strategies that target specific iron sources: (1) iron acquisition by degrading heme and heme-containing proteins; (2) iron acquisition by degrading transferrin, lactoferrin, and ferritin; (3) ferric iron acquisition by

siderophores; and (4) uptake of ferrous iron. Many bacteria and fungi (and perhaps mammals) produce *siderophores* (low molecular weight, high-affinity iron chelators) to acquire and transport iron, as detailed elsewhere [6, 55, 82, 116]. Further, many microorganisms have evolved the transport mechanisms to use heterologous siderophores produced by other microbes (xenosiderophores) [116], which is true for the opportunistic pathogen *P. aeruginosa* that produces two different siderophores, pyoverdine and pyochelin [34], but can utilize a variety of heterologous siderophores from other bacteria and fungi, including ferrioxamine B, ferrichrome, and enterobactin [35, 88]. Many pathogenic microorganisms produce siderophores that are directly implicated in their virulence [48, 82]. In these cases, siderophores of bacterial and fungal pathogens can directly remove iron from host proteins such as transferrin to support proliferation in invertebrates [71]. A schematic of Gram-negative and Gram-positive siderophore uptake mechanisms of ferric ion is shown in Fig. 6.2.

While Kaneko et al. [65] document efficacy of $Ga(NO_3)_3$ as both an anti-biofilm and antimicrobial, dosages were still relatively high. Banin et al. [14] report a novel approach for the delivery of gallium, in their case to *P. aeruginosa*, where they used a strong siderophore, desferrioxamine (**DFO**), to bind gallium ion. DFO is a bacte-

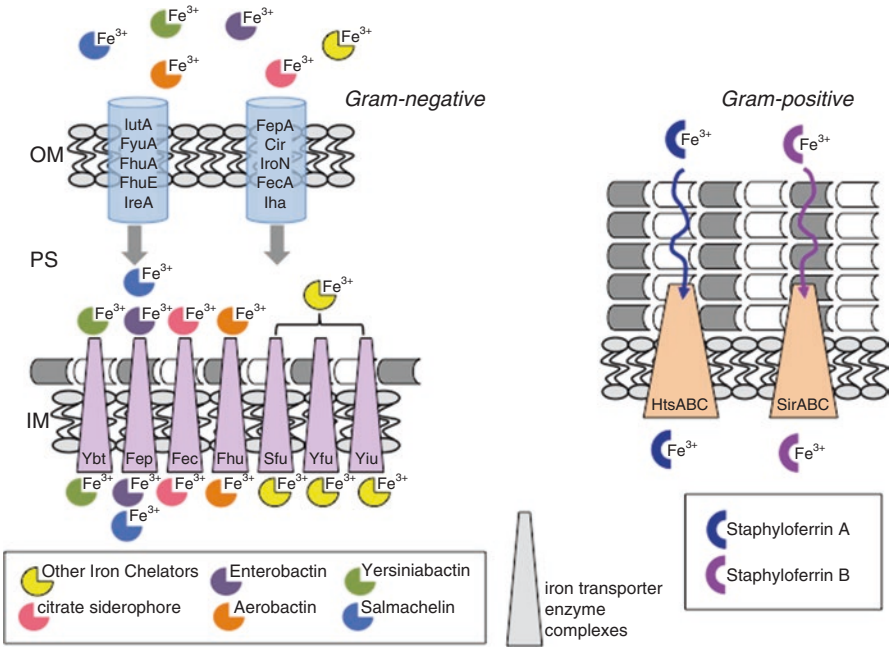

Fig. 6.2 Schemes for ferric iron uptake via siderophores in Gram-negative and Gram-positive bacteria. For Gram-negative (*left panel*), outer membrane receptors (*in blue*) import chelated iron to the periplasmic space where several inner membrane enzymatic transporter complexes (*in pink*) bring chelated iron into the cytoplasm to be uncoupled. In Gram-positive bacteria (*right panel*) the ABC transporters HstABC and SirABC import iron chelated by the siderophores staphyloferrin A and staphyloferrin B, respectively

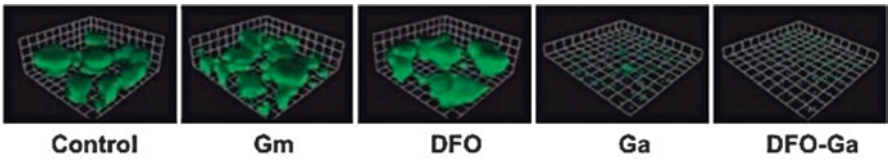

Fig. 6.3 Biofilm formation in flow cells with sub-inhibitory concentrations of DFO (0.001 mM), Ga (0.001 mM), DFO-Ga (0.001 mM), or gentamicin (Gm) (0.1 μg/ml). Shown are 3D reconstructed confocal microscope images of 6-day biofilms grown in the presence of the indicated agent (a side of each square on the grids is 23 μm). *P. aeruginosa* cells are expressing GFP (Reproduced from Banin et al. [14])

rial siderophore produced by the Actinobacteria *Streptomyces pilosus* and is used clinically to treat iron poisoning. DFO was selected as a siderophore carrier of Ga because *P. aeruginosa* possesses two uptake systems for DFO-Fe. Thus, DFO-Ga could deliver gallium to *P. aeruginosa* cells in preference to uncomplexed Fe via either of the two DFO uptake systems. Banin et al. [14] quantify the efficacy of both Zn-DFO and Ga-DFO against *P. aeruginosa* PAO1 growing under normal planktonic conditions and growing as biofilms. A flow cell system was used to examine the influence of sub-inhibitory concentrations of the DFO complexes (5 μM) on biofilm formation. As controls, DFO plus $GaCl_3$ or $ZnCl_3$ was applied separately. *P. aeruginosa* forms mature, thick, structured biofilms when cultivated in flow cells. Although addition of $ZnCl_3$ or DFO (5 μM) does not influence biofilm architecture, Zn-DFO (5 μM) complex does impair biofilm formation, while the use of free gallium (5 μM) or Ga-DFO (5 μM) complex completely blocks biofilm formation (Fig. 6.3). The ability of the DFO complexes to eradicate existing, mature PA (PAO1) biofilms was also examined. Survival of biofilms was measured as viable cell counts. Results show the antibiotic gentamicin (10 μM), about 2x the MIC for planktonic cultures, facilitated a 2 $\log_{10}$ decrease in cell counts, whereas Ga-DFO (1 μM) and Zn-DFO (1 μM) caused a 3–4 $\log_{10}$ decrease in cell counts. When the complexes were combined with antibiotic treatment, the DFO-Zn and gentamicin together were very effective in killing biofilm cells, reducing the viable count by almost 6 $\log_{10}$.

Ma et al. [80] describe the development of two novel anti-biofilm agents, gallium(Ga) and zinc (Zn), complexed with protoporphyrin IX (PP) or mesoprotoporphyrin IX (MP) that are both highly effective in negating suspended bacterial growth and biofilm formation. These chelated gallium or zinc complexes act as iron siderophore analogues, supplanting the natural iron uptake of most bacteria. Ma et al. describe development of a poly(ether urethane) (PEU) film that released either Ga or Zn complexes for a sustained time period; such loaded polymer systems could be developed into entirely new implants (catheters, shunts, tissue engineering scaffolds) or used as outer coatings applied to existing devices, prior to implantation. A segmented biomedical-grade poly(ether urethane) PEU (FDA accepted as BioSpan®) was used as the base polymer because of its excellent mechanical properties. PEU is an FDA-approved blood-contacting material and is

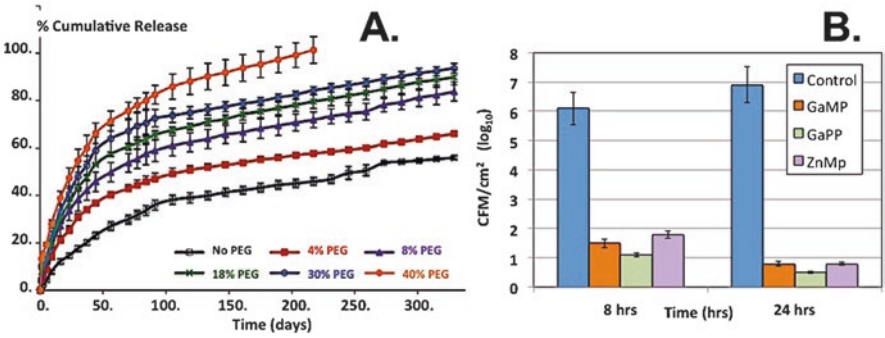

Fig. 6.4 (**a**) Cumulative percent release profiles of gallium/mesoporphyrin (MP) from porous pHEMA scaffolds as function of pore former, PEG, weight percentage. Each specimen contains 0.55% of Ga-MP and PEG (*MW* = 1450) in amounts varying from 2% to 40% (w/w). Temperature = 37 °C *pH* = 7.2. Data is taken from two separate experiments, each with $n = 3$. Error bars are standard deviations (SDs). (**b**) Adhesion of *Staphylococcus epidermidisSE* RP62A on porous pHEMA scaffolds releasing gallium MP complexes. = control pHEMA, no release; = Ga-MP; = Ga-PP; and = ZnMP (Adapted from Ma et al. [80])

commonly used in devices such as heart valves and spinal implants. Poly(ethylene glycol), PEG, was chosen as a pore-forming agent because it dissolves upon hydration, creating pores in the PEU through which drugs can escape. PEG was determined to be a superior pore-forming agent after extensive comparison with bovine serum albumin (BSA) (work previously shown by Kwok et al. [74]). An optimum formulation containing 8% PEG (*MW* = 1450) in the PEU polymer effectively sustained the release of Zn or Ga complexes for at least 3 months (Fig. 6.4a). All drug-loaded PEU films exhibited in vitro ≥ 90% reduction of Gram-positive (*Staphylococcus epidermidis*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria in both suspended and in biofilm culture versus the negative control PEU films that released nothing (Fig. 6.4b). Cytotoxicity and endotoxin evaluation demonstrated no adverse responses to the Ga or Zn complex releasing PEU films. Finally in vivo studies were carried out where PEU films releasing Ga-PP or PEU films releasing nothing were implanted subcutaneously in the dorsal area of mice; 24 h after implantation, mice were injected with 10^6 cell/mL bacterial suspension of either *S. aureus* or *P. aeruginosa* at the site of implantation. Mice implanted with empty PEU films required euthanasia 16 h after bacterial challenge due to obvious signs of local infection; mice that received Ga-PP releasing PEU films survived 3-weekly applied bacterial challenges with no apparent signs of infection.

Since this initial development of Ga siderophore complexes, there have been publications further developing their utility. Abdalla et al. [1] report the results of a study to quantify the growth-inhibitory activity of different Ga compounds against an ATCC strain and clinical isolates of *Mycobacterium abscessus*. Ga-protoporphyrin completely and significantly inhibited both an ATCC strain and clinical isolates of *M. abscessus* at much lower concentrations than $Ga(NO_3)_3$. When *M. abscessus* growth inside the human macrophage THP-1 cell line was assessed, Ga-protoporphyrin

was >20 times more active than $Ga(NO_3)_3$. Arivett et al. [9] similarly examined the antibiotic activity of gallium protoporphyrin IX (Ga-PP) against a collection of multidrug-resistant *A. baumannii* strains. Susceptibility testing demonstrated that Ga-PP inhibited the growth of all tested strains when cultured in cation-adjusted Mueller–Hinton broth, with a MIC of 20 μg/ml. This concentration significantly reduced bacterial viability, while 40 μg/ml killed all cells of the *A. baumannii* ATCC 19606(T) strain and a multidrug-resistant clinical isolate after 24 h incubation. Recovery of ATCC 19606(T) and ACICU strains from infected A549 human alveolar epithelial monolayers was also decreased when the medium was supplemented with Ga-PP, particularly at a 40 μg/ml concentration. Similarly, the co-injection of bacteria with Ga-PP increased the survival of *Galleria mellonella* larvae infected with ATCC 19606(T) or the clinical isolate. Ga-PP was cytotoxic only when monolayers of larvae were exposed to concentrations 16-fold and 1250-fold higher than those showing antibacterial activity, respectively. Chang et al. [28] report on the activity of gallium meso- and protoporphyrin IX against biofilms of multidrug-resistant *Acinetobacter baumannii* isolates. $Ga(NO_3)_3$ was moderately effective at reducing planktonic bacteria (64–128 μM) with little activity against biofilms (≥512 μM). In contrast, Ga-MPIX and Ga-PPIX were highly active against planktonic bacteria (0.25–8 μM). Cytotoxic effects in human fibroblasts were observed following exposure to concentrations exceeding 128 μM of Ga-MP and Ga-PP. Finally, Richter et al. [91] recently report similar results of gallium protoporphyrin in combination with deferiprone (an iron chelate). Deferiprone (20 μM) and Ga-PP (200 μg/mL) monotherapy for 2 h showed 35% and 74% biofilm removal, respectively, whereas simultaneous Def/Ga-PP administration showed 55% biofilm removal. In contrast, the consecutive treatment (2 h deferiprone followed by 2 h Ga-PP) achieved 95% biofilm removal. Cytotoxicity studies indicated no cell hazard in all treatments.

6.3 Enhancing Phagocytosis

6.3.1 Avoiding the Innate Immune Response

The immune system has evolved to protect the host from infection in two ways: *innate* and *adaptive* immunity. *Innate immunity* is the ability to produce a response within minutes or hours after infection through the recognition of molecules expressed by pathogens (e.g., microbial cell wall components, bacterial nucleic acids, formylated peptides, and viral double-stranded RNA). Such molecules are recognized by specialized receptors on cells of the innate immune system, which include dendritic cells, macrophages, neutrophils, natural killer cells, and gamma–delta T cells. Binding of microbial components to innate immune cell receptors (TOL-like receptors, NOD-like receptors, RIG-like receptors, C-type lectin receptors, and N-formyl met-leu-phe receptors) triggers signaling cascades within the cells that induce phagocytosis and the production of antimicrobial products.

Phagocytes also produce growth factors that can regulate adaptive immune responses. While innate immune responses are immediate (and may induce cross-protective immunity), they are typically short-lived.

The primary defense against infection is the innate immunity provided by neutrophils, macrophages, and dendritic cells particularly by activating the complement system. The role of complement activation is to control infections by eliminating microorganisms by opsonization and then clearance from the bloodstream [112]. Components of the complement system also interact with B and T cells to coordinate the adaptive immune response by regulating antigen presentation, promoting the formation of specific antibodies, and maintaining immunological memory [24, 68]. Complement evasion by many Gram-positive bacteria involves incorrect binding of complement recognition factors by bacterial cell wall proteins (proteins A, G, M) [94]. Gram-negative bacteria have also evolved secretory proteins that can degrade complement factors or their binding components or that prove anti-chemotactic or toxic to immune cells. For example, *S. aureus* has the ability to thwart neutrophils and macrophages by (*a*) inhibiting chemotaxis (blocking formylated peptide recognition, blocking C5a binding, secreting leukotoxins, and blocking LFA1–ICAM1-mediated extravasation), (*b*) negating opsonization (via protein G binding IgG antibody molecules by way of their Fc segments, plasmin degradation of bound IgG and C3b, and blocking C3b binding), and (*c*) thwarting phagocytosis (cell wall modifications to resist low endosomal pH, enzymatic degradation of endosomes) [47]. *Staphylococcus epidermidis*, another Gram-positive bacteria and the main species isolated in the majority of nosocomial infections, avoids the immune system through adhesion and biofilm formation [105, 109]. Otto and co-workers [77] have documented that Gram-positive bacteria actually sense antimicrobial peptides released by neutrophils and macrophage and can coordinate a directed defensive response. They discovered an antimicrobial peptide sensor system that controls major specific resistance mechanisms; the sensor contains a classical two-component signal transducer and an unusual third protein, all of which are indispensable for signal transduction and antimicrobial peptide resistance.

Some pathogens obviate the immune response by specifically targeting C-type lectin receptors, particularly dendritic cell-specific ICAM-3-grabbing non-integrins and mannose receptors, which benefits the pathogen by downregulating intracellular signaling and inhibiting maturation and cytokine secretion [72]. For example, entry of the Gram-negative oral pathogen, *Porphyromonas gingivalis*, into monocyte-derived dendritic cells in vitro leads to suboptimal DC maturation. This process requires *P. gingivalis* to express the major fimbriae, FimA [42, 62]. *P. gingivalis* also expresses unique immunosuppressive lipopolysaccharides (LPS) [32, 63, 90] and proteolytic gingipains [89]. The lipopolysaccharide of *P. gingivalis*, relative to those of *E. coli*, stimulates dendritic cells to secrete IL-10, but not IL-12 in vitro [63] and in vivo [90]. These two factors (suboptimal DC maturation and truncated cytokine expression) lead to the induction of a Th2 effector response, which suggests that *P. gingivalis* may target dendritic cell C-type lectin receptors (e.g., dendritic cell-specific ICAM-3-grabbing non-integrins) for entry and for blunting dendritic cell maturation.

6.3.2 *Artificial Opsonins*

Opsonization is the process where microorganisms and inanimate colloids (e.g., liposomes, particulates) are coated with host-produced proteins and lipids (immunoglobulins, complement factors), thus facilitating the binding of the opsonized bacteria or particle to specific receptor molecules present on phagocytes (i.e., neutrophils, macrophage, dendritic cells). IgG antibodies bind to their antigens on the surface of bacteria through coupling of the variable binding sites in the Fab region of the antibody, leaving the Fc region exposed. Phagocytes possess Fc gamma receptors and therefore can bind to the Fc-coated bacteria or particles and then internalize them. Complement fragment, C3b, also specifically binds to surface proteins or polysaccharides on microorganisms thus mediating binding to C3b receptors on the phagocytes. As described above, bacteria have evolved numerous ways to avoid opsonization by IgG and complement and thus avoid phagocytic elimination.

One possible alternative anti-biofilm biomaterial defense is one where the biomaterial would release factors that enhance neutrophil or macrophage phagocytosis of bacteria. There are a number of reports of synthetically derived "opsonins" enhancing bacterial phagocytic clearance.

The Taylor group, in a series of elegant papers, reports the use of several different bispecific fusion proteins that enhanced phagocytosis by macrophage of various pathogens, including *E. coli* [73], *P. aeruginosa* [78], and *S. aureus* [54]. In all cases, their artificial opsonins consisted of (*1*) a molecule that recognizes a surface marker on the pathogen that was chemically coupled with (*2*) a Mab that is specific to the complement receptor 1 (CR1) present on primate erythrocytes. In in vitro and in vivo studies, this series of works from the Taylor group demonstrated that their opsonins promoted binding of the target pathogen first to circulating erythrocytes, which then enhances macrophage phagocytosis of the bacteria. This phagocytosis did not apparently harm the erythrocyte, as verified in both in vitro and in vivo experiments [73].

Kobayashi et al. [70] report improved in vivo and in vitro phagocytosis of a periodontal pathogen, *Porphyromonas gingivalis*, using an artificial opsonin composed of two monoclonal antibody fragments: one against (*a*) the hemagglutinin domain of *P. gingivalis* (anti-r130k-HMGD antibody) and (*b*) the polymorphonuclear leukocyte (PMN) FcαRI (CD89) receptor (FcR). The Kobayashi work selectively targeted Fc receptors that were dominant on PMNs collected from gingival crevicular fluid of chronic periodontitis patients versus Fc receptors dominant on peripheral blood PMNs. Data shows that PMNs exhibited a higher capacity to phagocytose and kill *P. gingivalis* when treated with an opsonin that targeted *P. gingivalis* r130k-HMGD to leukocyte Fc RI as compared to opsonizing the bacteria with only the anti-r130k-HMGD antibody.

Encapsulated bacteria such as virulent strains of *Bacillus anthracis* impair phagocytosis with their capsules unless opsonized by antibodies. Poly-gamma-D-glutamic acid (gamma-PDGA) is the major component of the *B. anthracis* capsule. Bruno et al. [22] used poly-alpha-D-glutamic acid (alpha-PDGA)-coated magnetic beads as surrogates to simulate vegetative *B. anthracis* cells and avoid the hazards

of working with virulent bacteria. They report developing DNA aptamers against the alpha-linked PDGA-MBs. Four of the most frequent candidate aptamer sequences in the pool were coupled at their 5′ ends to Fc fragments of murine IgG to act as artificial opsonins. The effects of candidate aptamer-Fc conjugate addition on macrophage attachment and internalization of alpha-PDGA-coated beads were quantified using P388D1 and RAW 264.7 murine macrophage lines. P388D1 cells were not able to internalize the alpha-PDGA-coated beads, but attachment of the alpha-PDGA-coated beads was enhanced by the conjugates to varying degrees. Ingestion of alpha-PDGA-coated beads by RAW 264.7 cells in the presence of several different candidate aptamer-Fc conjugates demonstrated a statistically significant ($p < 0.01$) increase in phagocytic index, up to threefold in the first 30 min of exposure.

The major disadvantage of these original artificial opsonins described above was their use of bacterial species- or strain-specific Mabs or DNA aptamers; hence they lacked broad bacterial species recognition. The other limitation to these early constructs was that they used a phagocyte recognition moiety (e.g., Mab) that simply bound to the phagocytes, but did not necessarily activate phagocytosis.

Katzenmeyer and Bryers [67] describe a first-generation artificial opsonin that exhibits broad recognition of most Gram-positive bacteria and a phagocytic cell-targeting molecule that stimulates phagocytosis. Here, the antibiotic vancomycin, which binds to peptides terminated in D–Ala–D–Ala that are present in all Gram-positive bacterial peptidoglycan cell wall, was used as a bacterial recognition ligand. To negate the antibiotic membrane penetrating action of vancomycin, multiple copies of vancomycin were coupled to a poly-L-lysine (pLL) branched polymer (MW_N = 47,900) along with copies of the Fc portion of IgG. Vancomycin, when coupled to the large pLL, only acted as a Gram-positive bacteria recognition molecule and not an antibiotic. The vancomycin-based opsonins also exhibited higher affinity for MRSA and VRSA strains versus wild-type *S. aureus*. Pretreatment of Gram-positive strains with this multivalent opsonin *triples* (3X) in vitro phagocytosis compared to untreated controls (Fig. 6.5). These artificial opsonins were lyophilized, crushed into a fine powder, and then incorporated into a hydroxyethyl methacrylate (HEMA) monomer used to form polyHEMA films. In vivo data shows that upon hydration, films releasing the artificial opsonins were able to provide their intended protection to an initial bacterial challenge (10^7 cells/mL *SE*), *but as expected* (opsonin release was designed to terminate after 3 days), polyHEMA films depleted of opsonins failed against a second bacterial challenge at day 3.

6.3.3 *Biomaterial Surface Decorations that Influence Phagocytosis*

Macrophages (**MØ**) are the predominant cell type present at the tissue/biomaterial interface [66], and they are known to play a pivotal role in steering the outcome of implanted biomaterials. Following device implantation, macrophages infiltrate the

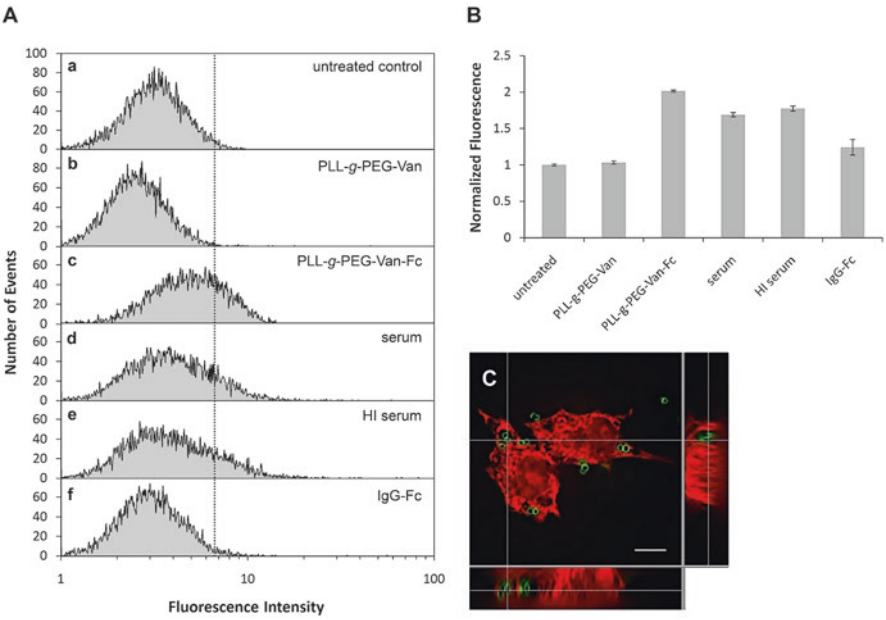

Fig. 6.5 Phagocytosis of *S. epidermidis* by HL-60 human neutrophil cell line. (**a**) Flow cytometry histograms showing the cell-associated fluorescence of the HL-60 population (~10,000 events). *Dotted line* indicates the threshold for identification of fluorescence-positive cells for an untreated negative control. Fluorescence of extracellular FITC-labeled *S. epidermidis* was quenched with trypan blue. (**b**) *S. epidermidis* opsonization with PLL-*g*-PEG-Van-Fc shifts HL-60 population to higher fluorescence intensity indicating a 3x greater extent of phagocytosis compared to opsonization with PLL-*g*-PEG-Van. Opsonization with human IgG-Fc alone does not significantly enhance phagocytosis. (**c**) CLSM image of live FITC-labeled *S. epidermidis* (*green*) ingested by a neutrophil (*red*). *Upper left* image is in the horizontal *x*-*y* plane. *Upper right* (*y*-*z* plane) and lower (*x*-*z* plane) images confirm that the selected bacterium is contained within the phagocyte. Scale bar = 5 μm (Adapted from Katzenmeyer and Bryers [67])

site of injury and mount an inflammatory response to clear wound debris and any pathogens introduced during the implantation procedure; later, they mediate the foreign body reaction, dictating the integration of the implant into the surrounding tissue [5]. The early response of MØ to a bacterial challenge is critical not only for the prevention of catastrophic infections but also to the proper healing and integration of the implant: persistent bacterial presence, even if asymptomatic, can cause prolonged inflammation at the implant site, leading to biomaterial degradation and/or damage to host tissue [110]. Unfortunately, the presence of implanted foreign bodies has been shown to increase host susceptibility to infections [8, 58]. Consequently, macrophage clearance of bacteria at the implant surface during the early post-implantation period is of key importance to the prevention of infections.

Activated MØ are most commonly categorized into two broad subtypes: classically activated (M1) and alternatively activated (M2) MØ. While M2 MØ are considered to play an important role in angiogenesis, neovascularization, and tissue

repair, M1 MØ are primarily responsible for microbial killing [83]. M1 MØ are characterized by markedly enhanced intracellular pathogen killing as well as the production of reactive oxygen and nitrogen intermediates and pro-inflammatory cytokines such as tissue necrosis factor (TNF)-α, interleukin (IL)-12, IL-1, and IL-6 [83]. In the murine system, M1 MØ are easily distinguished by their production of nitric oxide (NO) [56, 81]. M1 activation requires two distinct signals, interferon (IFN)-γ and a microbial stimulus such as bacterial lipopolysaccharide (LPS), both of which trigger the endogenous production of tissue necrosis factor (TNF)-α [36, 51, 83]. Interestingly, recent work has revealed that implant sites are characterized by a predominantly immunosuppressive microenvironment [57]. Areas of implants were shown to express high levels of anti-inflammatory cytokines IL-4 and IL-10, while lacking expression of TNF-α. Notably, IFN-γ, the primary signal necessary for M1 activation, was not detected near the implant site at any time point [57]. Considering the predominant role of M1 MØ in pathogen clearance, it has been suggested that such an immunosuppressive microenvironment may result in the susceptibility of the implant surface to bacterial colonization.

However, the importance of M1 activation on macrophage microbicidal function has never been fully elucidated in the context of implant-associated infections. In fact, controversies exist in the literature regarding the effect of M1 activation on macrophage phagocytic and microbicidal capacity. For example, Speert and Thorson [100] reported that IFN-c treatment diminished both the phagocytosis and killing of *Pseudomonas aeruginosa* by human monocytes, whereas Gratchev et al. [52] found that IFN-c/LPS treatment decreased the overall phagocytic capacity of human monocytes, but increased their killing of *Escherichia coli*.

There are a number of excellent papers that address modulating M1–M2 polarization using biomaterials (e.g., [4] review; [101, 102]), but almost all of these focus on manipulating MØ phenotype for the purpose of promoting tissue regeneration and healing; very few consider the goal of eliminating bacteria at the implant interface by enhancing MØ innate response.

The first such study was an excellent series of three papers that quantified the interaction between MØ and bacteria at the surfaces of materials that were designed to attract and modulate MØ behavior. Well before the concept of MØ polarization, Wagner et al. [113] describe the development of a series of biomaterials designed to promote MØ adhesion and subsequent activation. This paper presents the details behind the design and synthesis of the base PEG-g-PA copolymer, describes the surface modifications by peptides and Mab fragments using various PEO tethers, and provides results of surface analysis for the various materials. The efficacy of these various materials to control (*a*) random protein fouling and (*b*) bacterial cell adhesion and biofilm formation was also determined. A companion paper [110] evaluated the response of monocyte/MØ only (adhesion, cytokine expression, and oxidative burst) to PEG-g-PA copolymers that were modified with either (*a*) adhesion-promoting peptides (YRGDS, YRGES, and YEILDV) or (*b*) fragments of monoclonal antibodies specific to macrophage integrin receptors (anti-VLA4, anti-β1, anti-β2, and anti-CD64). Peptides bound to PEG-g-PA may facilitate macrophage adhesion, but they may also activate the cells, thus leading to inflammation.

Consequently, fragments of monoclonal antibodies (Mabs), known not to be involved in inflammatory stimulus, were also tethered to the based substratum. A third article [111] quantified MØ response to these decorated material interfaces, now in the presence of bacteria (*Staphylococcus epidermidis* and *Pseudomonas aeruginosa*). Materials modified with adhesion peptides marginally enhanced (2x) MØ attachment versus controls, but these materials tended to activate MØ to rapidly overexpress pro-inflammatory cytokines such that upon bacterial challenge they were less effective at phagocytosis. Conversely, PEG-g-PA materials modified by fragments of monoclonal antibodies significantly enhanced (7x) MØ adhesion, but initial "per cell" activation levels were markedly reduced compared to peptide-modified materials; thus these materials promoted significant phagocytosis upon bacterial challenge. MØ adhering to antibody fragment modified surfaces also exhibited sustained enhanced phagocytic response and higher bacterial killing efficiencies when compared with peptide-modified materials.

Park and Bryers [86] report a study on the effect of M1 activation on surface-adherent MØ interactions with bacteria relevant in implant-associated infections. To model the interaction of implant-adherent, M1-activated MØ with bacteria, an in vitro biomaterial platform was developed to evoke surface-adherent macrophage M1 activation, i.e., M1-activating ligands IFN-c and LPS were co-immobilized onto a model surface. Such a system was designed to evoke the specific activation of adherent macrophages—and macrophages only—by the surface-immobilized ligands, throughout the duration of the macrophage–bacteria interaction. Glass was silanized with a silane-PEG-biotin base layer, followed by an intermediate layer of streptavidin, to which biotin-conjugated ligands IFN-γ and LPS were coupled. These M1-activating ligands IFN-γ and LPS were immobilized to the substratum, each individually or in combination. Adherent macrophage response to the ligand-functionalized surfaces was evaluated using primary mouse bone marrow-derived MØ (BMDM). Finally, the phagocytic and microbicidal capacity of surface-adherent BMDM was assessed using the bacterium, *S. epidermidis*.

PEG–IFN-c/LPS-coated substrata enhanced the production of both IL-12(p40) and NO, indicating M1 activation of the adherent MØ. PEG:LPS, on the other hand, elicited an intermediate increase in the production of IL-12(p40) but did not markedly affect NO production, indicating a MØ phenotype distinct from M1 activation. Overall, ligand-presenting surfaces elicited lower levels of MØ activation compared to treatment with soluble ligands, which was attributed to differences in the total amounts of IFN-γ and LPS presented to the cells. The phagocytic capacity of IFN-γ-primed, innately activated, and M1-activated adherent macrophages was evaluated using live *S. epidermidis* cells. Although the differences were not statistically significant, a consistent trend toward lower phagocytosis (relative to untreated macrophages) was observed in M1-activated MØ at all time points (Fig. 6.6a). M1 activation has been shown to downregulate the expression of non-opsonic receptors such as mannose and scavenger receptors; Park and Bryers [86] suggest that non-opsonic pathways are important in the phagocytosis of *S. epidermidis*, since M1 activation appears to decrease their internalization. Bacterial killing assays were

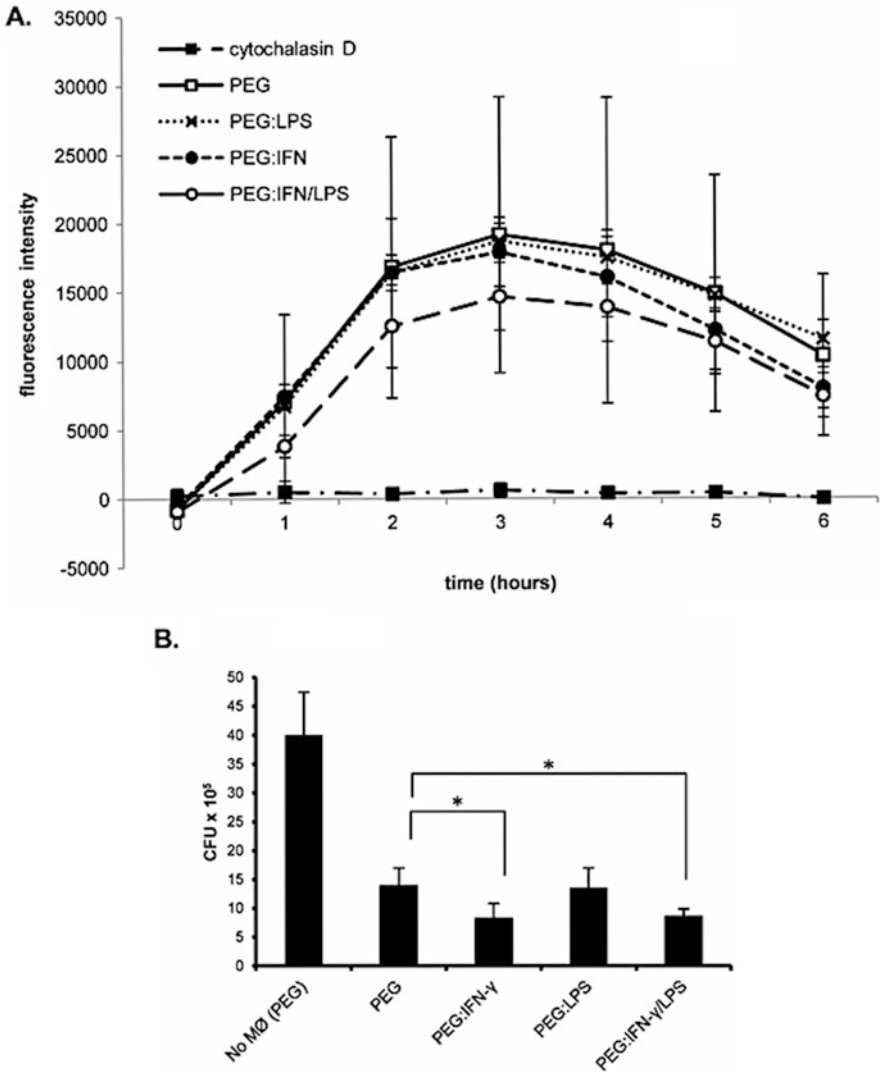

Fig. 6.6 (**a**) BMDM phagocytosis of *S. epidermidis* RP62A. BMDM were pre-cultured on the ligand-presenting surface for 24 h or with cytochalasin D (10 lM) for 1 h. pHrodoTM-labeled *S. epidermidis* RP62A cells were added to the macrophages at bacteria/macrophage ratio of 20:1, in 5% mouse serum, and incubated at 37 °C, 5% CO_2. The fluorescence of the wells (e.g., 550 nm/em 595 nm) was measured every hour for 6 h. All values are expressed as the mean value 6 SD ($N = 3$ for all treatments). One-way ANOVA was performed to test significance of differences among mean values; no significant differences were found (at $p < 0.05$). (**b**) BMDM killing of *S. epidermidis* RP62A. BMDM were cultured on the ligand-presenting surface for 24 h and then challenged with *S. epidermidis* RP62A at a bacteria/macrophage ratio of 1:1 in the presence of 5% mouse serum. After 4 h co-incubation at 37 °C, 5% CO2 BMDM were lysed, and surviving bacteria were quantitated via standard plate count method. Data is expressed as the mean colony-forming unit count (CFU × 10^5) per well ±SD. One-way ANOVA with post hoc testing was performed to test significance of differences among mean values. All macrophage-containing wells had significantly fewer surviving bacteria than bacteria-only wells ($p < 0.05$); *asterisks* (*) denote values significantly different relative to PEG ($p < 0.05$) (Reproduced from Park and Bryers [86])

also performed using a low bacteria: MØ ratio (1:1) (Fig. 6.6b). Compared to untreated macrophages, IFN-γ-primed and M1-activated MØ were shown to have increased ability to kill *S. epidermidis* cells.

6.4 Disrupting Biofilm Extracellular Matrix Amyloid Formation

Biofilms are surface-associated communities of microbial cells that are embedded in a microbe-generated extracellular matrix (**EM**) consisting of polysaccharides, proteins, and nucleic acids. Proteins in the EM can take on many structures, but the most commonly found is *amyloid*. While β-sheet amyloid fibers are the hallmark of human neurodegenerative diseases, increasing research suggests numerous Gram-positive and Gram-negative bacteria, including many multidrug-resistant (MDR) strains, e.g., *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*, resort to amyloid fibril formation to instigate bacterial aggregation, biofilm formation, adherence, and colonization of mucosal surfaces as an underlying mechanism of virulence [39]. As such, these functional amyloids, actually the soluble oligomeric intermediate precursors en route to fibril production, represent a novel target to prevent or disrupt biofilm formation.

6.4.1 Amyloid Fibril Formation Within Biofilm EM

Amyloids are remarkably stable protein polymers that form β-sheet-rich fibrils with a diameter of 5–10 nm. The *amyloid fold* is unique in that a range of proteins with totally different primary sequences can change structure, aggregate, and ultimately form the same fibrillar structures. The first step in amyloid fibril formation is aggregation of monomers into oligomeric intermediates, or "seeds" that share a common intermediate structure known as an α-sheet. Once seeds form, they nucleate fibril elongation, with the final amyloid structure being essentially a stack of β-sheet-rich monomers, aligned so that each β-strand is perpendicular to the fibril axis.

Because amyloid fibrils were identified in connection with various diseases, it was assumed that the fibrils themselves were toxic [25]. However, accumulating data suggest that mature amyloid fibrils are relatively inert, non-cytotoxic, and even protective. Instead, amyloid-related toxicity is caused by small soluble oligomers formed as an intermediate step in fibril polymerization. These structurally dynamic oligomeric intermediates can perforate lipid membranes [75]; hence disrupting existing fibrils is not advised. Thus, one novel approach to prevent biofilm formation would be by sequestering the α-sheet intermediates prior to their shift to the inert β-sheet fibril.

Microbes have harnessed the inherent toxicity of amyloid oligomers to kill surrounding cells or negate phagocytic cell chemotaxis. For example, the small hydrophobic microcin E492 (MccE492) is a soluble protein produced by *Klebsiella pneumonia*, and it exerts toxicity by forming pores in lipid membranes [39]. Fibril formation of MccE492 completely ablates toxicity to susceptible target cells. Changes in environmental pH also affect the aggregation of the listeriolysin O (LLO) protein of *Listeria monocytogenes*. LLO forms pores in the phagolysosome, allowing *L. monocytogenes* to escape into the cytoplasm during infection. Under alkaline pH, LLO can readily aggregate into fibril structures that bind the amyloid dyes thioflavin T (*ThT*) and Congo red. As with MccE492, LLO does not demonstrate pore-forming capabilities when in the fibril form. Indeed, since the small oligomers are considered the toxic species in amyloid formation, one obvious mechanism bacteria have for avoiding self-toxicity has been the rapid passage through the oligomeric stage to fibril formation.

Microbial amyloids are important in mediating mechanical invasion of abiotic and biotic substrata. In animal hosts, evidence indicates that these amyloid structures also contribute to tissue colonization by activating host proteases that are involved in hemostasis, inflammation, and remodeling of the host extracellular matrix (*ECM*) [39]. Activation of proteases by amyloids is also implicated in modulating blood coagulation. *Enterobacteriaceae* assemble adhesive amyloid fibrils termed *curli* at the bacterial cell surface to mediate cell–cell and cell surface interactions that promote bacterial adhesion to mammalian and plant cells as well as inert surfaces such as glass, stainless steel, and polymers. Curli also serve as an adhesive and structural component of the biofilm ECM [17, 27]. Amyloid adhesins and amyloid-integrated biofilms, in particular, are prevalent among diverse phyla (e.g., *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*) that thrive in drinking water reservoirs and other environmental habitats. In addition to curli, bacteria can assemble hundreds of extracellular adhesive fibrils known as *pili*, which contribute to bacterial pathogenesis in the human host. Type I pili, crucial to uropathogenic *E. coli* infections, contain the *FimH* adhesin at their tip, which mediates binding to mannosylated receptors present on the luminal surfaces of mammalian bladder epithelial cells—an event that is critical in the pathogenesis of urinary tract infections.

Recently amyloidogenic extracellular fibrils composed of small peptides called *phenol soluble modulins* (*PSMs*) were identified as components in Gram-positive *Staphylococcus aureus* biofilms [96]. While *Staphylococcus aureus* is a commensal organism in the nasal pharynx, the species can also cause a variety of illnesses (minor skin infections, bacteremia, and sepsis), many of which involve biofilm formation within host cells. That PSMs form amyloid fibrils is particularly novel because soluble PSMs have a variety of reported functions. PSMs, isolated either from *Staphylococcus aureus* or *S. epidermidis*, reportedly recruit, activate, and lyse human neutrophils and can kill competing bacteria. Soluble PSMs also effectively act as a biofilm dissociation factor, but upon amyloid fibril formation, PSMs lose that ability. However, PSM fibrils are required for *Staphylococcus aureus* biofilms to tolerate (*a*) various dispersion agents (e.g., dispersin B, DNAse I, protease K) and (*b*) elevated mechanical

stress, again demonstrating functional roles in both the monomeric and fibrous states. The recently described *B. subtilis* amyloid TasA protein may also perform roles as a toxin and as a biofilm stability factor, as prior to its described amyloid properties, TasA was reported to display antimicrobial activity [93].

It was previously assumed that mycobacteria did not produce pili. However, Alteri et al. [3] recently reported that *Mycobacterium tuberculosis* produces fine (2–3 nm wide), aggregative, flexible *pili* that are recognized by IgG antibodies contained in sera obtained from patients with active TB, indicating that *Mycobacterium tuberculosis* produce pili or pili-associated antigen during human infection. Purified *Mycobacterium tuberculosis* pili are composed of low molecular weight protein subunits encoded by the *Mycobacterium tuberculosis* H37Rv ORF, designated *Rv3312A*. *Mycobacterium tuberculosis* pili can bind to the extracellular matrix protein laminin in vitro, suggesting that *Mycobacterium tuberculosis* pili possess adhesive properties. Isogenic pili mutants lose the ability to produce pili in vitro and demonstrate decreased laminin binding. *Mycobacterium tuberculosis* pili share morphological, biochemical (bind ThT and Congo red), and functional properties attributed to other bacterial pili, especially with curli.

6.4.2 Biofilm Prevention/Disruption with Amyloid Inhibitors

Romero et al. [93] screened a subset of bioactive molecules archived at the BIOMOL–ICCB Known Bioactives collection at the ICCB Longwood Screening Facility (Harvard Medical School, Boston, MA), and they found that two molecules, AA-861 (a benzoquinone derivative) and parthenolide (a sesquiterpene lactone), inhibited the formation of *B. subtilis* biofilms by preventing the assembly of TasA oligomers into functional amyloid-like fibrils. Both small molecules were also able to disrupt, to varying degrees of effectiveness, preformed 12-h-old *B. subtilis* biofilms, albeit at relatively high concentrations (100–200 μM), although this disruption may inadvertently disperse the toxic oligomers if carried out in vivo. Both small molecules were also able to inhibit biofilm formation of *B. cereus* and *E. coli* at high concentrations, but they had no effect on *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms. Cegelski et al. [27] recently reported on two different ring-fused 2-pyridones with pilicide activity that inhibited *EC* curli polymerization. This inhibition of both curli and type I pili was correlated with reduced virulence and a decreased ability of *E. coli* to colonize host tissues and form biofilms. They did not test these two compounds on preformed biofilms. The downside of these four amyloid inhibitors is that their inhibitory effects are not broad spectrum. While they inhibit the biofilm formation of the species to which they were screened, the molecules have no, or the opposite, effect on other species. AA-861 and parthenolide inhibited *B. subtilis* biofilm formation but stimulated amyloid production in other species; a similar reversal of effects was reported for the two ring-fused 2-pyridones when applied to species other than *E. coli* [27]. Finally, all four compounds alone (no bacteria present) exhibited significant mammalian cell cytotoxicity at the concentrations used.

6.4.2.1 Designer Amyloid Inhibitors

What is really required is an amyloid fibril inhibitor that is designed based upon the ability to bind to the soluble oligomer of any protein via recognition of a common nonstandard secondary structure shared by all oligomers. Structural similarities between soluble oligomers from a range of unrelated proteins have been demonstrated by generation of an antibody that recognizes a common backbone conformation [50]. However, the amorphous nature of the toxic oligomer prevents its high-resolution structural characterization. Atomistic molecular dynamics (MD) simulations, however, provide a means to obtain information about these states. Based on previous simulations, Daggett and co-workers [10, 37] proposed that toxic intermediates from different amyloid proteins adopt a common, nonstandard secondary structure called the *α-sheet* and proposed that the oligomer-specific antibody above [50] binds to this structure. The Daggett group has now designed, synthesized, and experimentally characterized a series of small peptides that adopt stable, monomeric α-sheet structure complementary to the α-sheet structure observed in amyloid proteins [60]. These α-sheet peptides are recognized by the A11 oligomer-specific antibody introduced above [50], and they inhibit aggregation in three different human amyloid systems: transthyretin, which is implicated in systemic amyloid disease and a major contributor to heart disease; the amyloid β-peptide, which is linked to Alzheimer's disease; and amylin (or IAPP), which is linked to type 2 diabetes. In effect, these anti-α-sheet peptide inhibitors are themselves α-sheets.

Our research group has hypothesized that the α-sheet structure in the toxic soluble oligomers associated with human diseases also forms during bacterial amyloid fibril production during biofilm formation. Thus, these designed α-sheet inhibitors should recognize and bind the bacterial soluble oligomers, regardless of the species and original protein structure since they assume a common structural intermediate form prior to fibrillization.

The effects of various anti-α-sheet peptide inhibitors on amyloid fibril formation and overall biofilm formation for a series of amyloid-forming bacterial species were quantified as a function of inhibitor concentration. After a 1.5 h inoculation of wells in a 24-well tissue culture plate with suspended cells, cell suspensions were aspirated and any remaining planktonic bacteria removed by gentle PBS rinses. Then, appropriate sterile fresh medium is added to each well, along with varying concentrations of the various amyloid inhibitors to be screened. Control wells did not receive an inhibitor. The shift in ThT (10 μM per well) fluorescence intensity upon binding to any amyloid fibrils (a classic fibril formation assay) within the biofilm matrix was measured directly from the microwell plates. Transmission EM and AFM images were collected on intact biofilm.

We have some preliminary data supporting our hypothesis that anti-α-sheet compounds inhibit aggregation and amyloid formation in bacterial systems and that our common α-sheet oligomer intermediate hypothesis is valid. We have tested a number of α-sheet inhibitors against a series of amyloid-forming bacterial species. We used the ThT binding assay described above to determine the ability of α-sheet designs to inhibit amyloid formation in a series of different bacterial species biofilms;

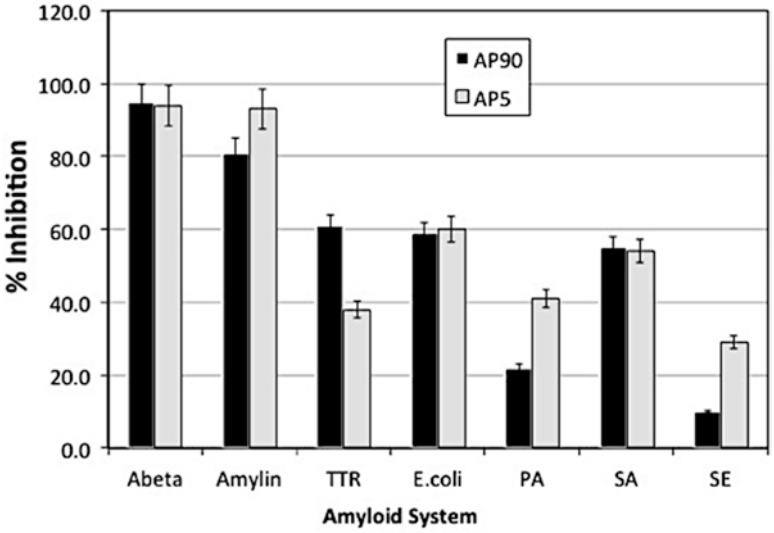

Fig. 6.7 Inhibition of amyloid formation in seven unrelated systems by two different α-sheet designs. Aβ and amylin aggregation were probed with ThT and TTR with Congo red, and the bacteria were assessed by crystal violet staining. Details, including SDs, have been presented for Aβ, amylin, and TTR; the bacterial results are very preliminary and merely suggest there is some effect upon adding α-sheet designs. Bacterial systems: *E. coli*, *P. aeruginosa* (*PA*), *S. aureus* (*SA*), and *S. epidermidis* (*SE*)

several designs did significantly reduce the amount of amyloid formed (Fig. 6.7), which also resulted in less biofilm formed in the case of *S. aureus* secreting PSMs (Fig. 6.8). It is important to emphasize that our best performing compound, A90, shows activity against both *S. aureus* and *P. aeruginosa*, bacterial species that express two totally different amyloid precursor proteins. This result supports our contention that the α-sheet structure is a critical intermediate in amyloid formation independent of the targeted proteins, sequences, structures, or even organisms.

6.5 Concluding Remarks

Existing anti-infective biomaterials can "deliver" anti-infective agents in one of two basic ways: agents are either directly tethered to the surface of the biomaterial or agents entrapped within the base biomaterial are released upon hydration into the adjacent surroundings. Unfortunately, no matter how lethal the anti-infective agent or how novel the "release" technology, anti-infective biomaterials based on toxic drug release *can never* provide active protection over the lifetime of indwelling implants. For example, heart valves can become infected by *Enterococcus faecalis* 20 years after implantation.

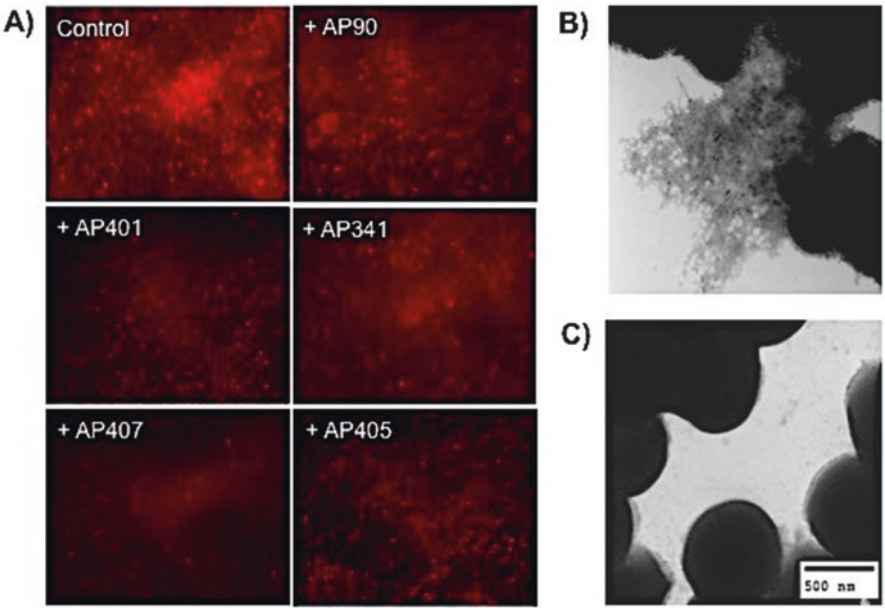

Fig. 6.8 *S. aureus* biofilm structures become less robust when grown in the presence of designed anti-α-sheet peptide inhibitors. (**a**) *S. aureus MN8* + mCherry biofilms were grown on glass substrates for 24 h and then cells were washed and fixed. Addition of peptide inhibitors (80 μM) reduced adhesion to the slide in some cases (e.g., design AP407), causing cells to detach during the wash step. Images are representative of duplicate wells. (**b**) In *S. aureus* SH1000 WT strain biofilms grown in regular LB medium, PSM amyloid fibrils are visible (TEM images) as deposits in spaces between cells. (**c**) Upon addition of the peptide design AP90 (80 μM), no extracellular fibril deposits were observed (Adapted from Bleem et al. in press)

What is required is anti-antibiotic or a non-killing anti-biofilm approach that seeks to negate bacteria colonization and reduce inflammation at an implantation site. Such approaches include (*a*) biofilm matrix disruption via DNA extraction; (*b*) quorum sensing interference; (*c*) immunotherapies, i.e., vaccines that target bacterial adhesins; (*d*) biomaterials that heal with such fidelity that they prevent infection similar to the natural healing process; (*e*) disruption of bacterial iron metabolism; (*f*) enhancing phagocytosis; and (*g*) preventing amyloid fibril production within the biofilm extracellular matrix; the latter three are detailed here.

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