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Amyloid formation: functional friend or fearful foe?

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Abstract

Amyloid formation has been most studied in the context of neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, as well as in amyloidosis. However, it is becoming increasingly clear that amyloid is also present in the healthy setting; for example non-toxic amyloid formation is important for melanin synthesis and in innate immunity. Furthermore, bacteria have mechanisms to produce functional amyloid structures with important roles in bacterial physiology and interaction with host cells. Here we will discuss some novel aspects of fibril-forming proteins in humans and bacteria. First, the amyloid-forming properties of the antimicrobial peptide human defensin 6 (HD6) will be considered. Intriguingly, unlike other antimicrobial peptides, HD6 does not kill bacteria. However, recent data show that HD6 can form amyloid structures at the gut mucosa with strong affinity for bacterial surfaces. These so-called 'nanonets' block bacterial invasion by entangling the bacteria in net-like structures. Next, the role of functional amyloid fibrils in human semen will be discussed. These fibrils were discovered through their property to enhance HIV infection but they may also have other yet unknown functions. Finally, the role of amyloid formation in bacteria will be reviewed. The recent finding that bacteria can make amyloid in a controlled fashion without toxic effects is of particular interest and may have implications for human disease. The role of amyloid in health and disease is beginning to be unraveled, and here we will review some of the most recent findings in this exciting area.

Introduction

Amyloids were originally believed to be merely misfolded proteins that can lead to human disorders, including diabetes, amyloidosis, and neurodegenerative diseases, but now increasing evidence suggests that controlled amyloid formation is involved in normal physiological processes, such as in melanin synthesis and innate immunity signaling [1-5]. Primarily functional amyloids are also produced by microorganisms, mainly as surface fibers with diverse roles in microbe-microbe and microbe–host interactions [6-8].

Here we will discuss some novel aspects of fibril-forming proteins and peptides of both human and bacterial origin. First, we will consider the mysterious function of the human antimicrobial peptide human defensin 6 (HD6), which is expressed by Paneth cells in the small intestine. Unlike other antimicrobial peptides, correctly folded HD6 does not exert direct antimicrobial activity, which has been difficult to explain. However, recently it was shown that HD6 forms amyloid-like structures at mucosal surfaces in the gut with high affinity for bacterial surface proteins [9]. These so-called 'nanonets' block bacterial invasion and systemic infection by virtue of their binding to bacterial surfaces and capacity to 'encapsulate' the bacteria in a net-like structure. Next, we will review another interesting facet of amyloid biology in humans, i.e. the role of amyloid fibrils in human semen in the context of HIV transmission [10]. Münch *et al.* have identified several amyloid-forming protein fragments in human semen that increase HIV infection of cellular targets [10]. This finding opens up novel avenues to potentially block HIV transmission by targeting host-derived factors in human semen. Finally, the role of amyloid in bacteria will be discussed. Given that amyloid-forming properties of proteins are widespread in nature, it is not surprising that bacteria also make their own amyloid proteins. Bacterial amyloids possess key functions in bacterial physiology but also in relation to host–microbe crosstalk, with important implications for bacterial disease in humans. Altogether, the role of amyloid in host–bacteria crosstalk is just beginning to be unraveled. Here we will discuss some of the most recent findings in this exciting area of research.

Amyloid nanonets: an unusual function for the antimicrobial peptide HD6

Antimicrobial peptides are an evolutionarily conserved component of innate immunity found throughout nature [11]. These peptides provide host defense, characteristically by direct microbicidal activity. Often these peptides have a broad spectrum of activity that can include bacteria, fungi, parasites, and viruses. Most antimicrobial peptides are cationic and amphiphilic, which enables them to mediate membrane disruptive action, one of several mechanisms of activity. Some antimicrobial peptides have additional specialized functions such as signaling or immunomodulatory activity [12] and a few have lectin-like activity [13].

Defensins are key components of innate immunity

Defensins, a major family of antimicrobial peptides in mammals, are expressed in phagocytic white cells and at mucosal surfaces. Functioning as endogenous antibiotics, their biological role is to protect against pathogenic microbes, as well as to influence the composition of the colonizing commensal microbiota. Nearly all characterized defensins have microbicidal activity against bacteria, and some are also active against fungi, protozoa,

and viruses. One notable exception is HD6, a defensin that is highly expressed in the small intestine [9, 14]. Recent studies indicate that the protective function of HD6 may be ascribed not to direct microbicidal activity but rather to its propensity to self-assemble to form net-like fibrils that can entangle and agglutinate microbes preventing their invasion of the intestinal mucosa [9].

In their native oxidized form, defensins have three conserved intramolecular disulfide bonds that stabilize a characteristic β-sheet amphiphilic structure. Depending on their size, which ranges from ∼2–5 kDa, and their disulfide connectivity, mammalian defensins are grouped into three subclasses designated α-, β-, and ө-defensins. Humans express six α-defensins, with HD1–4 (sometimes term HNP1–4) expressed in polymorphonuclear leukocytes, and HD5 and HD6 expressed primarily in the small intestine. Although the tertiary structures of human α-defensins are similar, owing to the conserved positioning of the CysI–CysVI, CysII–CysIV, and CysIII–CysV linkages, their primary structures differ significantly, giving rise to distinct biochemical properties.

HD5 impacts the composition of the microbiota

In the small intestine, HD5 and HD6 are abundant in secretory granules of Paneth cells, which are specialized epithelial cells located adjacent to stem cells at the base of the intestinal crypts [15]. *In vitro* studies of HD5 showed that it has potent bactericidal activity against both Gram-positive and Gram-negative bacteria [16]. Like many defensins, HD5 also has antifungal and antiviral activities [16, 17], and can act as a lectin (i.e. bind to glycoproteins) [13]. *In vivo* studies using a transgenic mouse model show that HD5 has profound biological activity. Transgenic expression of HD5 in mouse Paneth cells at physiologically relevant concentrations can protect the mice from challenge with lethal concentrations of the enteric pathogen *Salmonella typhimurium* [18]. Host protection was afforded by the ability of HD5 to decrease the bacterial load in the small intestinal lumen. In addition, expression of HD5 in the transgenic mice significantly altered the colonizing microbiota in the small intestinal lumen [19]. This effect on the microbiota was ascribed to the direct antibacterial activity of HD5.

HD6 protects against bacterial infection, without antimicrobial activity

However, in contrast to HD5 and other defensins, HD6 folded in its native disulfide-linked form lacks both direct antibacterial activity *in vitro* [9, 20, 21] and lectin-like activity [13], thus the function of this abundant defensin in the small intestine remains unclear. Unexpectedly, *in vivo* studies demonstrated that transgenic expression of HD6 in mouse Paneth cells at physiologically relevant concentrations can also protect the mice from challenge with lethal concentrations of *S. typhimurium* [9]. However, the mechanism of protection was different from that of HD5. In the case of HD6, there was no reduction in pathogen load in the lumen, consistent with the negligible activity of HD6 assessed *in vitro*. Rather, there was a significant decrease in bacterial numbers in internal organs, suggesting that HD6 was somehow blocking invasion [9]. Indeed, bacterial counts in HD6 transgenic mice compared to wild-type littermates infected with virulent *S. typhimurium* were reduced to a similar extent as observed for wild-type mice infected with an invasion-deficient mutant of *S. typhimurium* [22]. *In vitro* studies of bacterial invasion of cultured intestinal epithelial

cells showed that HD6 could block invasion not only by *S. typhimurium* [9] but also by *Yersinia enterocolitica* [9] and *Listeria monocytogenes* [23], two other enteric pathogens that use entirely different mechanisms of invasion. Data did not support the likelihood that HD6 exerted its protective effects by inhibiting bacterial expression of invasion pathways or by affecting host cells [9].

HD6 forms nanonets with amyloid characteristics

A critical clue for understanding the mechanism of HD6 protection came from scanning electron microscopy images of interactions between HD6 and *S. typhimurium.* These data revealed the formation of fibrils that emanated from the bacterial surface [9]. These fibrils were approximately 80 nm wide, branched at intervals, and could merge with other fibrils to form net-like structures (nanonets) that often enveloped multiple bacteria. Chairatana and Nolan reported transmission electron images showing that HD6 alone can spontaneously form fibrils and nanonet structures in neutral buffer [23]. Previous crystallographic studies suggested that HD6 has the potential to assemble into an elongated, high-ordered structure [24]. In sharp contrast to the characteristic dimer structures formed by HD1–5 [24, 25], crystal structure analysis demonstrated that HD6 forms an atypical dimer, which is critically stabilized by dimer–dimer association, resulting in a stable tetramer (likely the repeating unit of the elongated HD6 structure). HD6 tetramerization is mediated by extensive reciprocal inter-dimer interactions [24].

HD6 nanonets bind to fimbriae and flagellae on the bacterial surface

Data suggest that proteins on the bacterial surface are the anchor point and nidus for fibril and nanonet formation. As HD6 lacks lectin-like activity [13], it seemed unlikely that bacterial surface carbohydrates were the targets of HD6 binding. Flagellin and type I fimbriae are the two major surface proteins of *S. typhimurium* [26]. Surface plasmon resonance data revealed that HD6 binds to *Salmonella* flagellin, the monomeric subunit of flagella [9]. Furthermore, in contrast to the marked bacterial agglutination and HD6 nanonet formation evident by scanning electron microscopy of wild-type *S. typhimurium*, less HD6-driven agglutination was evident with a *Salmonella* mutant lacking type I fimbriae, Similarly, there was decreased agglutination by HD6 of a flagellin-deficient mutant. Agglutination by HD6 was completely abolished in a *Salmonella* mutant lacking both fimbriae and flagella, and neither nanofibrils nor nanonets were seen with this double mutant [9].

Structural insight: histidine27 is a key amino acid residue for nanonet formation

A number of structure–function experiments increased understanding of the key amino acids of HD6 that enable this peptide to self-assemble and protect against invasion. A previous study of HD1 using alanine-scanning mutagenesis identified the aromatic residue Trp26 as a key functional residue [27]. A comparison of α-defensin sequences in Hominidae (gorillas, chimpanzees, and orangutans) revealed… that most peptides, including HD5, have an aromatic residue at this position [9]. The notable exception is HD6 and its orthologs, which contain histidine at the corresponding position. Therefore, structure–function analysis of HD6 was first used to examine the role of this His residue by mutating the correspondingly placed His27 of HD6 to a tryptophan (H27W-HD6), which rendered the primary structure

more similar to that of other α-defensins at this position. Notably, H27W-HD6 lost its ability to form nanonets and to block invasion in *in vitro* assays with *S. typhimurium*, suggesting that His27 is critical for HD6 activity [9], possibly through an intermolecular salt bridge between His27 and an adjacent C-terminal carboxylate (α-carboxylate of Leu32) in an adjacent monomer in the tetrameric core [9]. Other experiments with an alanine substitution at this position showed residual activity of H27A [23], supporting the notion that interactions besides the proposed salt bridge His27–Leu32 are major structural features driving self-assembly.

A major advance in identifying such features came from the work of Chairatana and Nolan[23], showing that a hydrophobic pocket sits at the interface of the four subunits of the HD6 tetramer. In this pocket, two subunits each contribute Val22, Met23, and Ile25, while the adjacent two monomers each contribute Phe2, Phe29, and Leu32. Sequential mutation of these residues revealed that F2A-HD6 and F29A-HD6 lost the ability to form fibrils and could neither agglutinate bacteria nor block invasion. I22T-HD6 and V25T-HD6 had attenuated propensity for self-assembly and reduced activity in agglutination and invasion assays. These data identify a fundamentally important structural feature of HD6 that contributes to the unique activity of this α-defensin.

The current model for HD6 action is that the peptide initially binds stochastically to proteinaceous bacterial surface molecules. These initially bound HD6 molecules provide a nidus that triggers self-assembly and builds nanonets; this can agglutinate the bacteria and/or provide localized fibrils that can impede the formation of close physical contacts required for microbial invasion of epithelial cells (Fig. 1).

Future perspectives

The ability of HD6 to serve as an efficient anti-infectious agent without having direct antimicrobial effects provides a new target for combating bacterial disease. Traditional antibiotics, and to a certain extent also antimicrobial peptides, exert a selective pressure on bacterial growth, which rapidly selects for resistant mutants. The simple disarmament of bacterial virulence by encapsulation in an HD6-derived nanonet could offer an effective alternative to traditional antibiotic activity. It would be interesting to further study the regulation of the nanonet formation process. How can amyloid formation occur without causing damage to host cells? It could be speculated that powerful mechanisms to avoid potential toxic effects of HD6-derived nanonets are present in the intestine. Understanding these mechanisms may improve efforts to deal with amyloid formation in other tissues, including the brain (in Alzheimer's disease and Parkinson's disease) and internal organs (in systemic amyloidosis). Given that amyloid fibers are eliminated via autophagy, a cellular degradation process linked to Crohn's disease and other inflammatory disorders [28, 29], it could be hypothesized that a dysregulated nanonet degradation could be involved in inflammatory bowel disease. Thus, detailed insight into nanonet degradation could provide novel therapeutic options for inflammatory disorders.

Viral infection-enhancing amyloids in human semen

Amyloids exist in normal semen from young, healthy men who have no evidence of amyloid-related diseases. Here, we discuss how these amyloids were identified, their biophysical properties, and their potential clinical importance, particularly their ability to enhance infection by sexually transmitted viral pathogens such as HIV.

Identification and characterization of amyloid fibrils from human semen

Despite the availability of life-saving antiretroviral therapy, HIV continues to be a major public health burden, and ∼2.3 million new infections still occur each year [30]. Most new infections are acquired through sexual transmission, during which semen is typically the carrier (during male-to-female and male-to-male transmissions) or is usually present to some extent (during female-to-male transmission).

To better understand how semen affects HIV infection, Münch *et al.* generated and screened a complex peptide/protein library derived from human seminal plasma [(SP) the cell free supernatant obtained after centrifugation] for novel inhibitors and enhancers of viral infection. No inhibitors of viral infection were identified through screening of this library for the ability to affect HIV infection in a cell-based assay. Surprisingly, however, two fractions significantly enhanced infection rates [10]. These fractions contained peptides derived from two regions of the abundant seminal protein prostatic acid phosphatase (PAP). Functional and structural analyses demonstrated that these peptides form amyloid fibrils that enhance HIV infection [10, 31]. The initially characterized peptide, from the C-terminal region of PAP, was named semen-derived enhancer of viral infection (SEVI) [10]. The other peptide, from a more N-proximal region of PAP, was named SEVI-2 [31]. These fibrils meet the criteria for classical amyloids, i.e. they (i) exhibit green birefringence under light polarization after staining with the amyloid dye Congo Red, (ii) exhibit shifts in their fluorescence spectrum upon binding to thioflavin T, (iii) show characteristic patterns by X-ray powder diffraction, and (iv) form fibrillar structures visible by both transmission electron and atomic force microscopy. These fibrils have a remarkable ability to increase HIV infection rates: only 1–3 virions are sufficient for productive infection in the presence of fibrils, whereas 1000–10,000 virions are required in the absence of the fibrils [10].

Unexpectedly, it was found that PAP-derived peptides were not the only amyloidogenic fragments present in human semen. In attempts to isolate amyloid fibrils directly from human semen with an antibody that recognizes a sequence-independent amyloid conformation, we serendipitously identified an additional set of semen amyloids [32]. These amyloids are derived from semenogelin 1 and the highly homologous semenogelin 2, which are the main constituents of the semen coagulum, a viscous gel that encases spermatozoa before liquefaction of semen. These amyloids, termed SEM (fragments from semenogelins) fibrils, do not share sequence homology with SEVI or SEVI-2 but have similar structural and biophysical properties. Both classes of semen fibrils are derived from peptides with high isoelectric points, and zeta potential measurements confirmed that they are all highly cationic at neutral pH [32, 33]. This property enables the fibrils to bind strongly to the negatively charged membranes of both HIV virions and cells, which leads to increased viral attachment and fusion to cellular targets (Fig. 2).

Indeed, abrogating the cationic properties of the fibrils through anionic polymers, site-directed mutagenesis, or a molecular tweezer diminishes their ability to enhance infection [32-34]. It is interesting that SEVI and SEM fibrils appear to achieve a cationic surface through different mechanisms: SEVI relies on the C-terminal region to form the core of the amyloid, whereas SEM fibrils appear to use multiple core regions interspersed by cationic exposed regions [35, 36]. The stabilities of SEVI and SEM fibrils are pH dependent, perhaps in part because of their cationic nature. SEVI and SEM fibrils preformed at neutral pH disassemble completely when transferred to medium of pH 2.5 and moderately disassemble at pH 5.5 [35, 36]. These observations have implications for heterosexual HIV transmission to women. Mixing of healthy vaginal fluid (pH ∼5.2) [37] and semen (pH ∼7.7) [38] generates a solution with a pH of 5–6 [39, 40], at which fibrils are still mostly intact and could therefore promote HIV infection. Of interest, bacterial vaginosis, a common disorder in which changes in the vaginal commensal flora render vaginal fluid less acidic, is associated with a 60% increase in risk of HIV transmission to women [41]. Although there are likely many reasons for this increased risk, one possibility is that the mixing of semen with vaginal fluid from a woman with bacterial vaginosis might lead to more neutral conditions, and thus greater stability of the infection-promoting semen fibrils, compared to mixing with normal vaginal fluid; this would favor the stability of the infection-promoting semen fibrils.

Early studies of SEVI and SEM amyloids primarily characterized synthetic versions of the fibrils. Recently, however, using confocal imaging and immunogold electron microscopy, we detected endogenous forms of both types of fibrils in human semen, including in fresh ejaculates that had never been subjected to freeze/thaw cycles [42, 43]. Endogenous seminal amyloids could be detected by transmission electron and atomic force microscopy or with the amyloidotropic dyes Proteostat, thioflavin T, and pFTAA [42]. Similar in size and shape to synthetic fibrils, these endogenous amyloids bind directly to HIV virions and are readily detectable in semen from both uninfected and HIV-infected individuals [42]. These observations confirmed the findings of many previous studies that synthetic semen fibrils are natural enhancers of HIV infection and, importantly, revealed semen as the first human biological fluid to harbor amyloids in the absence of disease.

Effects of semen on HIV infection

As discussed above, amyloid fibrils are readily detected in human semen and can potently enhance HIV infection. How do the fibrils influence the overall effects of semen on HIV infection? Until recently, there had been few studies to examine such effects, likely because this biological fluid is quite toxic to cells *in vitro*, making it difficult to use in cell culture-based assays. These cytotoxic effects complicate the evaluation of semen *in vitro*, therefore it is critical to perform studies under conditions that preserve the viability of target cells [44]. A large number of studies have demonstrated that, under *in vitro* conditions that preserve target cell viability, semen and SP markedly enhance HIV infection [10, 32, 33, 42-50]. For example, when HIV was pretreated with 90% (v/v) semen and diluted 15-fold on addition to reporter cells, infection rates were up to 40-fold higher than in the absence of semen [44]. Importantly, the infection-enhancing effects of semen are most prominent at low viral inocula, which to some extent mimics the *in vivo* situation as only small amounts of

virus are present in semen [10, 44], and these effects are lost as the viral inoculum is increased because the fibrils become saturated with virus. As a result, the HIV-promoting effects cannot be observed in systems that require high viral inocula, such as explant studies [51, 52]. When low viral doses are used, semen enhances infection of cell types relevant for sexual transmission, including macrophages [44] and T cells isolated from cervical and endometrial tissues [33, 44]. Semen also increases HIV *trans*-infection from cervical epithelial cells to T cells by ∼80-fold [10]. These observations suggest that semen, the main vector for HIV transmission, markedly enhances viral infection *in vitro* under biologically relevant, low-dose inoculum conditions.

That fibrils are important contributors to the ability of semen to enhance HIV infection is supported by three observations. First, donor-dependent variability in the extent of semen-mediated enhancement of HIV infection correlates with the levels of amyloidogenic SEVI and SEM peptides, as measured by enzyme-linked immunosorbent assay and quantitative mass spectrometry [32, 43, 44]. Secondly, semen lacking these peptides, as a consequence of a congenital condition termed ejaculatory duct obstruction, cannot enhance HIV infection [32]. Thirdly, extension of the period of semen liquefaction results in progressive loss of the amyloidogenic SEM peptide in a manner that parallels the progressive loss of the infection-enhancing activity of semen. However, because semen retains significant infection-enhancing activity for more than 8 h after emission, it has ample opportunity to enhance viral transmission *in vivo*, which can occur within 1 h after exposure in animal models [53]. Furthermore, the detection of amyloidogenic SEM peptides in seminal vesicles [32] suggests that amyloid seeds are already present even before ejaculation, consistent with the observation that fresh ejaculates contain amyloid fibrils and strongly enhance HIV infection [32]. Therefore, the 'window of opportunity' for semen fibril-mediated enhancement of HIV infection ranges from the time of semen deposition to >8 h later.

Although the data discussed above strongly support a role for fibrils in semen-mediated enhancement of HIV infection, the fibrils are not the only factors involved. For example, fibronectin, an abundant component of semen that on its own does not significantly increase HIV infection rates, synergistically increases the ability of fibrils to enhance HIV infection [49]. Semen also contains factors that inhibit HIV infection, such as high molecular mass complexes that block dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)-mediated transfer of HIV-1 from dendritic cells to CD4 T cells [54]. The overall effects of SP on HIV transmission will most certainly result from the combined effects of a variety of semen factors, in conjunction with other mucosal components such as cervicovaginal fluid.

The effects of semen on HIV transmission *in vivo* are not known and cannot be studied experimentally in humans, because of ethical concerns. To date only three studies have examined the effect of semen/SP in primates. In two early studies, the effect of SP on vaginal infection of rhesus macaques by simian immunodeficiency virus (SIV) was analyzed [55, 56]. In both these early studies, vaginal SIV infection increased in the presence of SP, but only when low viral inocula were used. Recently, in a similar study, we confirmed that SP may facilitate transmission at low viral doses, and found that macaques infected in the presence of SP had 6.9-fold higher peak viral loads [57]. Unfortunately, in all three of these

in vivo studies, animals in the control groups differed greatly in their susceptibility to vaginal SIV infection resulting in insufficient statistical power. Another problem is that SP enhances SIV markedly less than HIV-1 infection [10], which reduces the ability of SIV-based studies to assess the infection-promoting effects of semen on HIV *in vivo.* Thus, the effect of semen on HIV transmission *in vivo* remains unclear. Future non-human primate studies using viruses that are more similar to HIV-1 (e.g. simian human immune deficiency viruses (SHIV) that carry primary transmitted-founder virus envelopes and that can be transmitted mucosally [58]) should be explored to provide more conclusive data regarding whether or not semen increases sexual HIV transmission rates.

Effects of semen and semen fibrils on antiretrovirals

If the potent effects of semen on HIV infection *in vitro* translate to the *in vivo* setting, this could have important implications for HIV prevention. Clinical trials of topical microbicides to limit HIV transmission have proved disappointing, showing at best an overall protection rate of only 39% [59-61]. Drug adherence was probably a main contributor to the low level of efficacy, but other factors were also likely to have a role [62]. An unexplored possibility is that the effects of semen during transmission may have diminished the efficacy of the microbicides. Supporting the notion that semen may limit microbicide efficacy is evidence from *in vitro* studies demonstrating that semen markedly reduces the activity of microbicides that target HIV [50]. In immortalized cell lines, semen increased the IC_{50} of polyanions, neutralizing antibodies, nucleotide reverse transcriptase inhibitors (including tenofovir), non-nucleoside reverse transcriptase inhibitors, and inhibitors of HIV integrase and protease by 8- to 21-fold. The inhibitory effect was likely due to the activity of seminal amyloids, as synthetic fibrils exert a similar effect, and semen lacking the infection-promoting fibrils does not reduce microbicide activity. Similar results were observed after HIV-1 infection of peripheral blood mononuclear cells, in which semen and semen fibrils increased IC_{50} values of microbicides by 7- to 20-fold. The one exception was the microbicide candidate maraviroc (MVC), an inhibitor of the cellular CCR5 receptor, which retained its activity in the presence of SP.

The mechanism that enables semen to impair the activity of some but not all microbicides requires further investigation. It is interesting that, in the aforementioned study, MVC was the only microbicide candidate tested that targets a host cell component rather than viral components. Why might MVC retain its activity in the presence of SP? One possibility relates to the observation that semen fibrils concentrate viruses onto the cell surface [10, 44]. Thus, the level of antiretroviral drugs required would presumably increase with the number of virions concentrated at the cell surface. By contrast, a saturating MVC concentration that occupies all CCR5 molecules would theoretically prevent HIV entry regardless of the number of surface-bound virions. Overall, these data suggest that microbicides targeting host components should remain effective in the presence of SP; however, for optimal activity, drugs targeting HIV may need to incorporate semen fibril antagonists.

Antagonizing the activity of semen fibrils

Counteracting the infection-enhancing activity of semen fibrils may be a promising novel approach for HIV microbicide development. Two properties of semen fibrils are required for

their ability to enhance HIV infection. The first is the ability of the cationic fibrils to bind electrostatically to membranes. Simply blocking the cationic residues of the fibrils with polyanions, however, is not an option for microbicide development, because polyanions tend to increase rather than decrease susceptibility to HIV transmission, possibly by increasing genital inflammation [63]. An alternative is surfen, a non-polyanionic small molecule that prevents the fibrils from binding to HIV and cellular targets [45] and has anti-inflammatory properties [64]. Inter-chelators of fibrils prevent semen and semen fibrils from enhancing HIV infection, also through a non-electrostatic mechanism [48].

The second requirement of semen fibrils for infection-enhancing activity is a fibrillar conformation. Monomeric semenogelin and PAP peptides are inactive, suggesting that conversion of the mature fibrils to peptides would antagonize their infection-promoting activity. In support of this notion, a non-natural L-amino acid inhibitor against the SEVI peptide disrupts fibril formation and blocks viral enhancement activity [65]. Compounds that directly disassemble preformed semen fibrils have not been described, although the polyphenol epigallocatechin-3-gallate targets SEVI for degradation through an undefined molecular mechanism [47].

Very recently, it has been demonstrated that CLR01, a lysine- and arginine-specific molecular tweezer, acts as a dual-function inhibitor of HIV [34]. This small molecular compound inhibits amyloid formation by engaging specific lysine or arginine residues within a variety of disease-associated amyloidogenic proteins including amyloid-β protein [66]. CLR01 not only remodeled preformed semen amyloids but also abrogated semen-mediated enhancement of viral infection by preventing the formation of virion–amyloid complexes. Moreover, CLR01 has a direct antiviral activity as it disrupts the viral membrane and hence destroys the infectivity of HIV and other enveloped viruses such as hepatitis C virus, human cytomegalovirus (CMV), and herpes simplex virus (HSV) type 2. Thus, CLR01 counteracts both semen amyloids that may be important for viral transmission and the pathogen itself. These combined anti-amyloid and antiviral activities make CLR01 a promising topical microbicide candidate for blocking infection by HIV and other sexually transmitted viruses.

Chemical modifications of semen fibril antagonists to increase their potency and half-life in semen would potentially be valuable not only for the development of novel HIV microbicide candidates, but also for the development of preventative agents against other sexually transmitted viruses (such as CMV and HSV), whose infection levels of which are also enhanced by SEVI and SEM fibrils [67, 68].

Are semen fibrils more than just viral enhancers?

Easterhoff *et al.* demonstrated that synthetic SEVI fibrils bind bacteria through electrostatic interactions thereby promoting bacterial aggregation and phagocytosis by macrophages [69]. However, whether endogenous semen amyloids exert similar antimicrobial effects remains unknown. Of note, antagonizing the infection-promoting activity of semen fibrils may interfere with their normal, as yet unknown, activity. The presence of amyloidogenic SEM orthologs in non-human greater apes [43] suggests that these fibrils are evolutionarily conserved. Of interest, in primates more distantly related to humans, other regions of the

semenogelin protein encode cationic, amyloidogenic peptides that have properties similar to those of human SEVI and SEM fibrils [43]. The existence of multiple cationic amyloid peptides in primates suggests that these fibrils may play a role in primate reproductive fitness. Although SP is not absolutely necessary for reproduction, as demonstrated by the success of *in vitro* fertilization, it can increase the efficiency of implantation and promote proper embryonic development [70-73]. Whether seminal amyloids play any part in promoting reproductive success remains to be determined.

Future perspectives

Future investigations to determine whether semen fibrils play a role in promoting conception or embryonic development would be of interest, both to assess the potential consequences of antagonizing their activity for microbicide development and to gain a better understanding of how semen components participate in reproduction. Another interesting question is why amyloid is present in semen in healthy young men and can promote infection of sexually transmitted viruses. From an evolutionary perspective it is possible that amyloid in semen could be advantageous, in terms of either survival or reproductive success. Clearly, much remains to be learned about seminal amyloids.

Bacterial amyloids: important for biofilm formation and host–microbe crosstalk

In contrast to eukaryotic amyloids that are conventionally viewed as being pathogenic, bacterial amyloids are mostly functional [7, 74, 75]. Bacterial amyloids are usually extracellular fibers, i. e. long thin appendages extending from the cell surface, with amyloid structures built up in a highly controlled way through a unique assembly pathway [76, 77]. Amyloid fibers seem to be abundant in the bacterial world and are recognized as major structural and functional extracellular matrix components of environmental and pathogenic biofilms [78, 79]. Biofilm formation is the natural 'lifestyle' of most bacterial organisms, with cells embedded in a self-produced extracellular matrix [80], which aids the assembly of multicellular communities of differentiated bacterial cells comparable to tissue formation in higher organisms [81]. Of note, cells in biofilms show elevated tolerance to stress and antibiotics as well as to immune-mediated attack; this contributes to the persistence of this bacterial state in the environment and in association with the host [82-84].

Curli fimbriae are amyloid structures in bacteria that mediate biofilm formation

Amyloid fibers are widespread in Gram-positive and Gram-negative bacteria [85-88]. One of the most thoroughly investigated types of bacterial amyloid is curli. Curli are commonly produced by *Escherichia coli* and *S. typhimurium*, but expression has also been experimentally demonstrated in additional enterobacterial species, such as *Citrobacter* spp and *Enterobacter* spp, with genetic predisposition shown to be widespread in nature [89, 90]. Curli fimbriae are assembled extracellularly by a specific assembly pathway, the extracellular nucleator/precipitation pathway, distinct from the assembly mode of the chaperone-usher pathway of type 1 fimbriae and type IV pili [86]. Of note, in biofilms, curli fimbriae tightly interact with the exopolysaccharide component cellulose, which produces a protective extracellular matrix termed 'bacterial wood' [91].

Curli fimbriae bind to human proteins and affect inflammation

As amyloids, curli fimbriae have the unique property of interacting with various structurally and chemically distinct substrates of bacterial and human origin. For example, curli bind to human proteins and activate cascades leading to fibrinolysis and coagulation [92]. On the other hand, curli bind to the immune stimulatory bacterial protein flagellin in monomeric form, which, in the curli-bound form, is able to trigger a proinflammatory cytokine response [87]. Unexpectedly, an innate immune component, the antimicrobial peptide LL-37, not only binds to amyloid curli, but also prevents their formation [77]. Co-expression of cellulose decreases the binding of curli to fibronectin [86], and also dramatically alters other curli-mediated phenotypes, such as adhesion, invasion of host cells, and induction of a proinflammatory cytokine response [88–90].

Curli expression has been shown to be important for bacterial virulence and a target of the subsequent immune response [93, 94]. Interestingly, bacterial amyloids have been proposed to initiate amyloidogenic processes in the host via cross-seeding mechanisms, which could have implications for neurodegenerative and other amyloid-based human diseases [95, 96]. In an animal model of sepsis, elevated NO induction and a drop in blood pressure is associated with curli production [93]. Amyloid curli are pathogen-associated molecular pattern (PAMP) molecules recognized by toll-like receptor 2 and the adaptor molecule CD14, in conjunction with the NLRP3 inflammasome, which leads to augmented barrier function and enhanced IL production at the intestinal mucosa [8]. This recent work also provides the molecular basis for early observations that curli production is associated with higher cytokine production *in vitro* [93]. Intriguingly, curli also bind human DNA, which stimulates autoantibodies and triggers systemic lupus erythematosus-like symptoms [6]; this might be one of the mechanisms of autoimmunity caused by the human (gut) microbiome.

Uropathogenic *E. coli* frequently express curli fimbriae at body temperature, with curli fiber-producing bacteria detected in human urine [82]. However, the role of curli during urinary tract infections (UTIs) is complex; curli have been found to possess time-dependent roles in a mouse model of UTI. In the short term, a UTI *E. coli* isolate expressing curli fimbriae at ambient and body temperature exhibited enhanced colonization compared to an isogenic mutant lacking curli. After 2 days, a significantly reduced number of the curli-expressing strain was found in the urine. One explanation for this dual role of curli during UTIs might be that they facilitate adhesion, but at the same time trigger a more vigorous immune response than a curli-deficient mutant. Notably, a higher concentration of MIP-2 (the homolog to human IL-8) was detected after infection with the curli-expressing strain, which supports the notion that curli are proinflammatory during UTI [82]. It was previously shown that specific inhibitors of curli biogenesis in *E. coli* reduced both biofilm formation and virulence in a mouse model of UTI, which suggests that curli may play an important role during infection [97] (Fig. 3).

Future perspectives

Amyloids are truly functional in bacteria, and play key roles in biofilm formation as well as in host–microbe cross-talk. It is remarkable that distinct and often counteracting functionalities are observed for curli in biofilm formation and interaction with the host. The

observed multiple roles of curli in bacteria–host interaction are in line with those of other adhesive and immunogenic surface appendages, such as fimbriae and flagella, where various mechanisms of immune evasion (e.g. tight regulation of expression *in vivo*) contribute to virulence. Consistent with this principle, curli-expressing bacteria are required for initiation of infection, but can also constitute a prominent target for host immunity, thus contributing to a complex scenario [77]. However in *Shigella* spp., a pathogen that thrives in the epithelial cell layer, curli genes are inactivated in conjunction with the rdar biofilm pathway, which includes cellulose genes and a major biofilm regulator [96]. The rdar biofilm pathway is also inactivated or expression is downregulated in related invasive pathogens such as enteroinvasive *E. coli* or *Salmonella typhi*, respectively, whereas, for example, the pathway is upregulated in sepsis isolates [93]. Another thought-provoking proposal is that bacterial amyloids act as cross-seeding molecules, which may initiate Alzheimer's disease or Parkinson's disease [97]. A number of observations support this hypothesis, such as the fact that amyloid fibrils are found in gastrointestinal nerve plexa early in the course of these neurodegenerative diseases [98, 99]. In addition, the olfactory system is affected early in Parkinson's disease and the olfactory nerve is situated close to the nasal mucosa and the normal bacterial flora associated with this anatomical niche. In fact, it could be speculated that bacteria producing amyloid fibrils, that share molecular patterns with tau or Abeta42, could initiate such a fibrillation process [97]. This idea is in line with the cell–cell spread that has been suggested to occur in Alzheimer's disease or Parkinson's disease [98]. However, it should be noted that formal evidence in support of this hypothesis is lacking.

Finally, the protein homeostasis machinery has a central role in the regulation of amyloid formation. It was surprising that CsgA amyloid formation did not readily cause cell damage [76]. Indeed, CsgC was recently identified as an important holding chaperone for CsgA, the key subunit of the curli amyloid. Without CsgC, CsgA formed intracellular amyloid, which killed the bacterial cell. Interestingly, CsgC could also prevent amyloid formation in tau, but not in Abeta, suggesting highly specific mechanisms of regulation of bacterial amyloid formation extending to higher organisms, including humans [76, 100].

Summary

In the first part of this review, we discussed how HD6 uses the amyloid fold to prevent pathogenic bacteria from invading the gut. In the second part we focused on how amyloids derived from seminal proteins may facilitate HIV transmission. Notably, both HD6 and semen amyloid can be described as 'functional amyloids' in the sense that they do not clump together and cause inflammation and subsequent death of host cells, which is in contrast to the effects of disease-associated amyloids on mammalian cells. Finally, we considered how bacteria utilize amyloid structures in biofilm formation and in the crosstalk with human cells.

We reviewed recent evidence of how bacteria can produce amyloid structures without damaging the bacterial cell. Next steps in this exciting area of research include applying our understanding of amyloid activity to identify novel ways of preventing or treating amyloid-associated human diseases, such as Alzheimer's disease, Parkinson's disease, and systemic amyloidosis, as well as preventing infection by gastrointestinal bacteria and HIV following

sexual exposure. Several examples of approaches to prevent amyloid formation are already under development, and more will likely be developed in the coming years [65, 97, 101].

Acknowledgments

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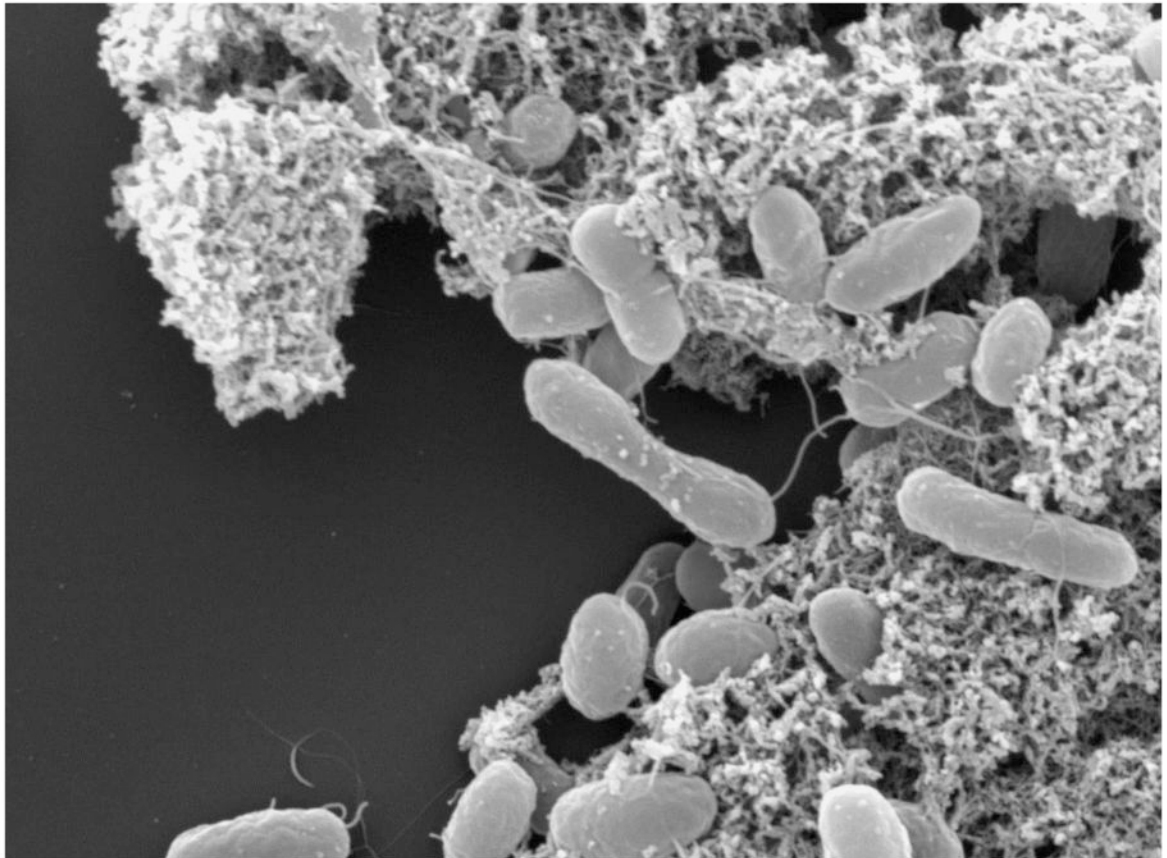
Fig. 1.
Human defensin 6 (HD6) nanonets entangle enteric pathogens in human ileal fluid.
Scanning electron microscopy image of *Salmonella typhimurium* treated *ex vivo* with HD6
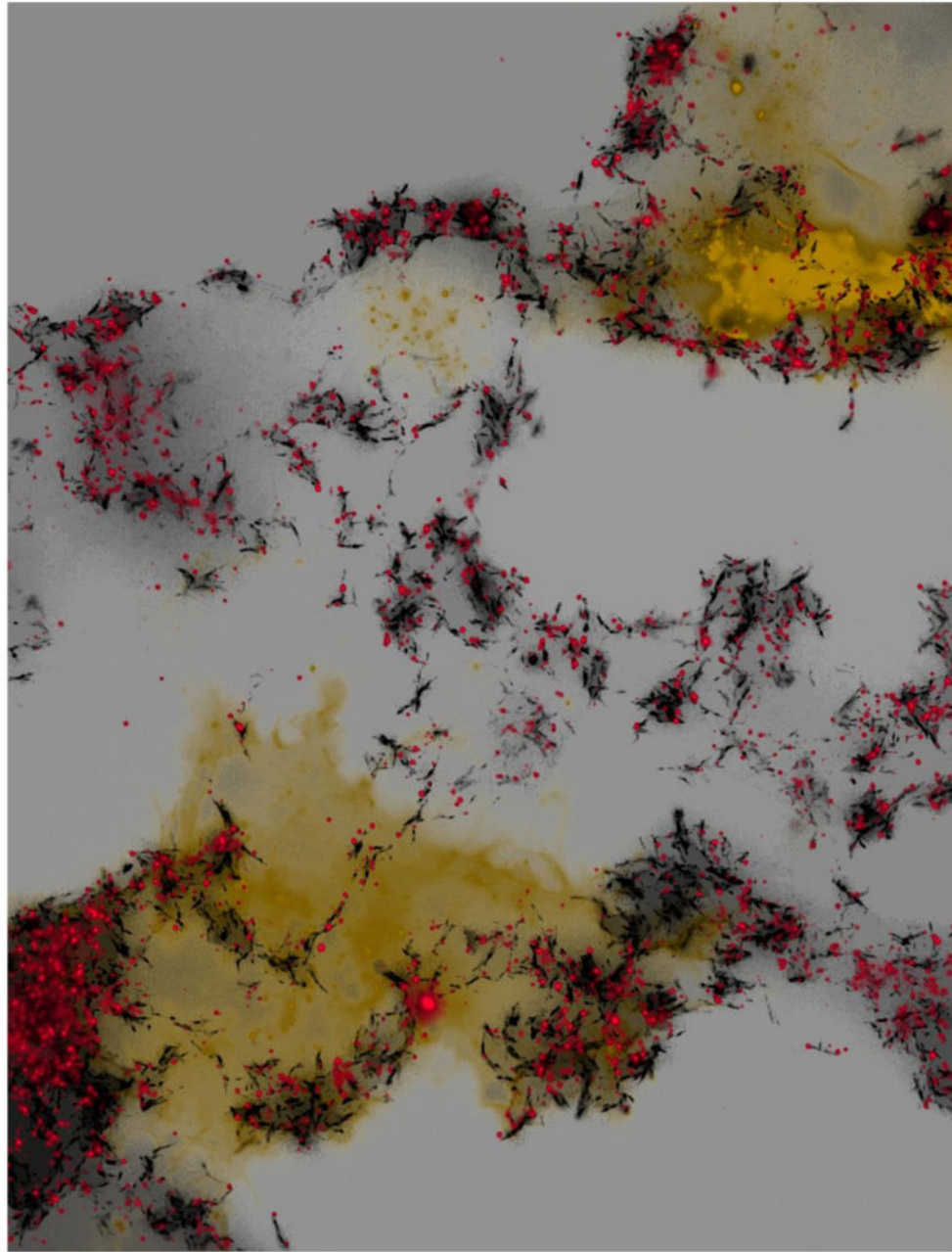(10 μg/mL) in clarified human small intestinal luminal fluid aspirate (reproduced from [9]).

Fig. 2.
Mechanism of semen-derived enhancer of viral infection (SEVI)-mediated enhancement of HIV-1 infection. SEVI amyloid fibrils (black) sequester HIV-1 particles (red) and bind to the surface of target cells (yellow), thereby increasing viral attachment and fusion rates. The image was generated by Walther Mothes, Joseph Luna, and Pradeep Uchil, using a Nikon TE2000 microscope. Z-stacks of images were deconvoluted by using Volocity software from Improvision, and the final image was pseudocolored in Adobe Photoshop.
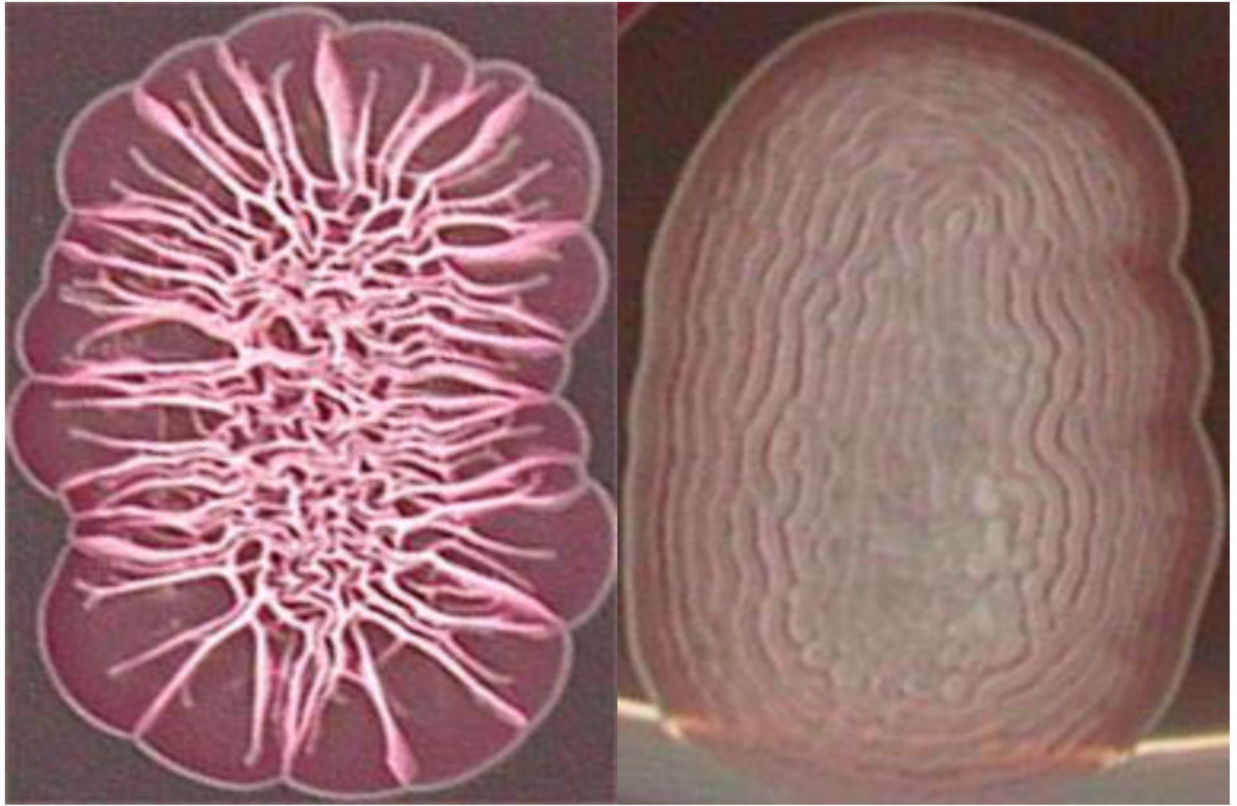
Fig. 3.
Salmonella typhimurium strain 4037/65 producing curli and cellulose (left) and a curli-only producing mutant (right). Reproduced from Werner Bokranz, PhD thesis.

Table 1

Key studies of amyloid formation in health and disease

Amyloid formation in human disease	Study
Parkinson's disease	[1, 102]
Alzheimer's disease	[1, 102]
Amyloidosis	[102]
Functional amyloid formation in humans	
Melanin synthesis	[5]
HD6	[9]
Semen-derived fibrils	[10, 31, 68]
Functional amyloid formation in bacteria	
Curli	[2, 7, 74–76]
Biofilm	[86, 87]

HD6, human defensin 6.