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Approaches to inhibit biofilm formation applying natural and artificial silk-based materials

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ABSTRACT

The discovery of penicillin started a new era of health care since it allowed the effective treatment of formerly deadly infections. As a drawback, its overuse led to a growing number of multi-drug resistant pathogens. Challenging this arising threat, material research focuses on the development of microbe-killing or microbe repellent agents implementing such functions directly into materials. Due to their biocompatibility, non-immunogenicity and mechanical strength, silk-based materials are attractive candidates for applications in the biomedical field. Furthermore, it has been observed that silks display high persistency in their natural environment giving reason to suspect that they might be attractive candidates to prevent microbial infestation. The current review describes the process of biofilm formation on medical devices and the most common strategies to prevent it, divided into effects of surface topography, material modification and integrated additives. In this context, recent state of the art developments in the field of natural and artificial silk-based materials with microbe-repellant or antimicrobial properties are addressed. These silk properties are controversially discussed and conclusions are drawn as to which parameters will be decisive for the successful design of new bio-functional materials based on the blueprint of silk proteins.

1. Introduction

Due to the overuse of broad-spectrum antibiotics, there is an urgent need for new materials that provide antimicrobial properties without the danger of facilitating the rapid emergence of multi-drug-resistant pathogens [1]. These materials need to be suitable for a broad spectrum of applications to prevent microbial infestation and biofouling from implants and wound dressings to air pollution and wastewater purification. Especially the formation of biofilms displays a difficult task in these applications. Once a biofilm is formed and matured, it becomes increasingly difficult to disperse it and free the surface from the infestation [2]. Therefore, many approaches in developing microbe-resistant materials focus on systems that can inhibit surface adhesion of microbes in the first place and minimize the risk of biofilm initiation [3,4].

A glance into nature reveals a big variety of strategies and materials to avoid biofouling and bacterial infestation of exposed surfaces in an environment full of microorganisms [5]. One specific class of materials, that aroused increasing attention among material scientists in the last decades, is based on silk proteins. Initially silk textiles have been used due to their luster and appearance, but also due to their suppression of bad odor. Based on the latter, one potential microbe repellent features came into focus [6]. It is reported that in ancient Greece, wounds were treated with honey and vinegar for deep cleaning and subsequently covered with cobwebs or spider webs, which dried out on the surface over time and effectively inhibited bacterial infestation in the wound [7]. Nowadays, researchers worldwide investigate the vast family of silks to reveal and understand the basic mechanisms that contribute to such properties. The ability to produce silks evolved presumably 250 million years ago and is nowadays present in a variety of arthropods, most prominently in silkworms and spiders, but also in the families of e.g. bees, wasps and ants [8]. The silk proteins produced by these animals differ in amino acid sequence and protein structure as they evolved independently and adjusted to the needs of their producers resulting in a versatile class of proteins [9]. The discovered applications of natural silk materials range from sperm support and egg covering to structural elements in nest and burrow building to more exotic ones like building

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material for underwater tunnels, floating rafts for eggs and prey storage [8]. Silkworms of the *Bombyx mori* species are prominently known for their use of silk fibers as building material for their cocoons to provide protection for the larvae during their metamorphosis [10]. Silkworm silk fibers consist mainly of three different proteins, namely a light chain fibroin (25 kDa), a heavy chain fibroin (350–500 kDa) and sericin (10–400 kDa). The latter connects and covers the fibroin double filament in the final spun state [11]. Due to its long history in human trade and cultivation leading to nowadays large-scale production and commercial availability in big quantities, *Bombyx mori* fibroin has been studied extensively. A closer look on silk produced by spiders shows that spider silks display a much higher degree of diversity. For example, orb web weaving spiders like *Nephila clavipes* are known to be able to produce up to 7 different silk types, needed for different parts of their webs or for reproduction [12,13]. This range allows them to produce dragline fibers with high mechanical stability and at the same time add sticky features for prey capturing when building webs. Since most spiders are distinctly territorial and cannibalistic, it was not possible to create profitable farms to harvest sufficient amounts for large scale applications, and availability for research is also limited [14]. Regarding other silk materials like bee or wasp silk, which are mainly used as protective covering for their offspring during maturation stages, only very few publications provide depth insights into material features [8,15,16].

All these silks have developed over millions of years of evolution resulting in a vast cornucopia of physio-chemical properties based on their amino acid sequences which are adapted for specific applications in the lifestyle of the respective species. The unexploited potential of such natural materials inspired researchers for decades and was further fueled by the development of recombinant production strategies that allow easier manipulation as well as production of sufficient amounts of silk proteins with high purity [17–20].

2. How to inhibit biofilm formation and growth

To develop materials that can effectively kill microbes or at least inhibit microbial growth and biofilm formation, it is essential to understand how biofilms are formed. The process of microbial attachment and initial biofilm formation has been extensively studied within the last decades. A biofilm is described as an adherent community of microorganisms embedding themselves in a self-generated extracellular polymer matrix (EPS) [2]. This matrix is a severe threat in fighting biofilm related diseases as it builds a protective layer and enhances the resistance of encapsuled pathogens against antibiotics [21]. Depending on the bacterial strains, it consists of binding proteins, pili, flagella, extracellular deoxyribonucleic acids (DNA) and adhesive fibers [22].

2.1. Biofilm formation

The process of biofilm formation can be divided into (1) reversible bacterial adhesion; (2) quasi-irreversible attachment; (3) biofilm maturation; and detachment (4) [23,24]. A schematic overview on biofilm formation with respect to the single stages is shown in Fig. 1.

Reversible attachment: Microbial cells are able to attach reversibly to almost every surface by using a variety of extracellular organelles (e.g., flagella, pili, fimbriae, curli fibers) and outer membrane proteins [25,26]. Before attachment, pre-conditioning of the surface naturally occurs with organic or inorganic macromolecules depending on the environmental conditions. These soluble components adsorb on surfaces and partly cover the intrinsic physical and chemical properties of the underlying materials. There are similarities between adhesion of bacteria to these “preconditioned” surfaces and the attachment and spreading of mammalian cells on substrates that are remodeled by the adsorption of matrix proteins and DNA [27]. This initial reversible adhesion is mainly driven by locomotive appendages and initiated as a response to environmental factors such as pH, temperature and surface topology. Additional factors like the fluid flow rate over the contact

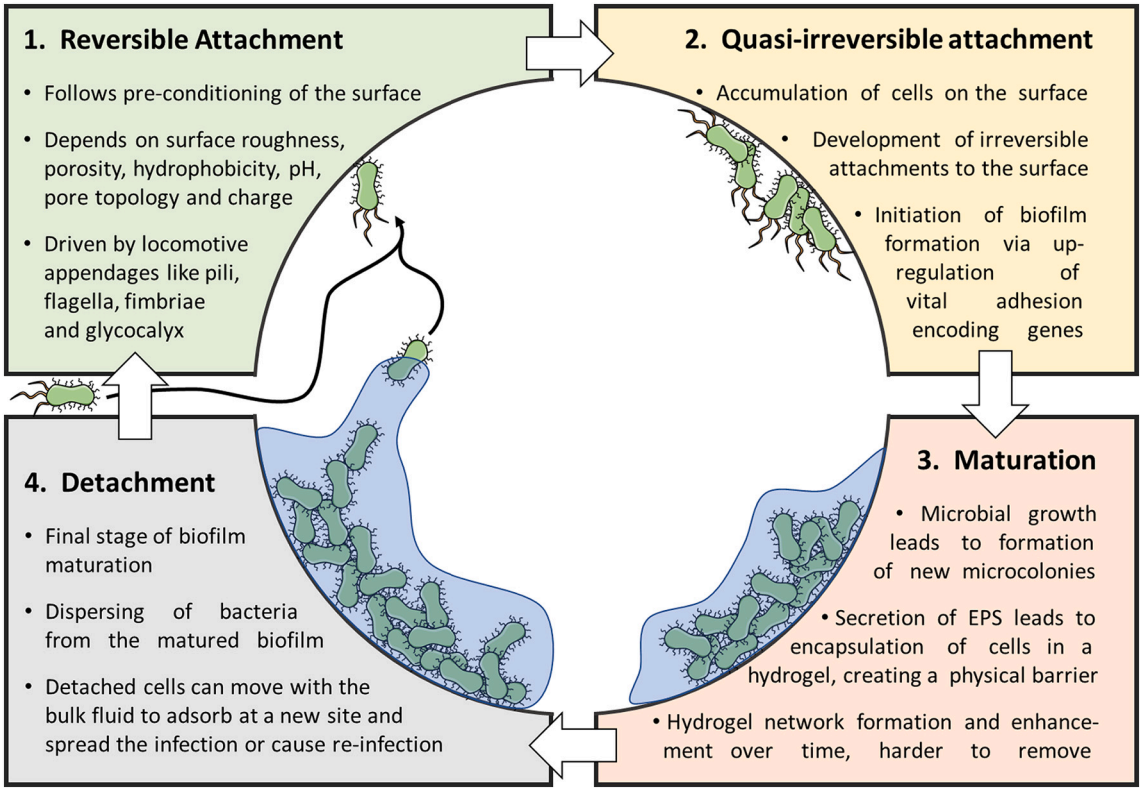

Fig. 1. Biofilm cycle with 4 stages of formation after bacterial infestation.

surface, duration of bacteria-surface contact, nutrient availability, surface hydrophobicity and van der Waals or electrostatic interactions trigger the complex interplay between bacteria and exposed surfaces [21,28–30]. Simultaneously, the presence of flagella, pili, fimbriae and glycocalyx significantly affect the degree of attachment as they help bacteria to remain attached even in presence of repulsive forces [31]. The crucial role of such appendages was exemplarily shown by Abbot et al. demonstrating that *Streptococcus pyogenes* lacking functional pili were unable to bind to tonsil epithelium or human keratinocytes [32].

Quasi-irreversible attachment: After accumulation on the targeted surfaces, the bacterial cells begin to develop quasi-irreversible attachments leading to the initiation of biofilm formation [21,23]. DNA, proteins, lipids, and lipopolysaccharides build up the extracellular polymeric substance (EPS) which is secreted for facilitating adhesion between cells and surfaces [33]. This process is driven by a variety of complex mechanisms involving protein fibrils such as pili and bacterial surface anchor proteins. During this critical step, the production of surface binding proteins is enhanced by the upregulation of the respective genes, leading to an increased surface attachment and promoted biofilm formation [32,34,35].

Maturation: Following surface attachment, subsequent maturation strengthens the adhesion accompanied by microbial multiplication and the formation of a complex three-dimensional architecture [21,24]. Biofilm formation is controlled largely by quorum sensing (QS), which is the best-characterized example of chemical communication in bacteria. QS is one of the mechanisms that cells use to query their extracellular environment and modulate cellular functions like pathogenesis, nutrient acquisition, conjugation, motility, and secondary metabolite production [36]. The EPS occupies the intercellular space between bacteria, encapsules the microbial colonies in a layer of hydrogel and forms a physical barrier between the bacterial community and the extracellular environment [37]. Two characteristics are strongly correlated with biofilm forming bacteria: the increased production of EPS and the development of antibiotic resistance [21]. The formation and secretion is stimulated by growth conditions and chemical communication between cells, which is influenced by the varying composition of EPS depending on the cell type [38]. The resulting network is unique for every cell type and environmental condition and is based on by its dipole associations, hydrophobic and electrostatic interactions and covalent or hydrogen bonds. Therefore, the composition and quantity of EPS is time dependent and varies between 50 and 90% of the total organic matter in a biofilm, which is also affected by different environmental factors [37]. Although at the initiation of microbial interaction the EPS–bacteria bond is weak and can be easily destroyed by flowing water, it gets strengthened with time and the attachment becomes quasi-irreversible. A biofilm that has reached this stage may require stronger treatment for cell detachment and subsequent removal such as scrubbing or scraping.

Detachment: In the final stage, bacteria can disperse from the matured biofilm to start a new cycle. Reduced flow rate, shearing effects and environmental stimuli (e.g., changes in microenvironment, temperature, pH, nutrient concentration, microbial variability, cell density) can lead to the decrease of cell mass and cell detachment from the biofilm. After leaving the EPS matrix, cells disperse into the bulk fluid, where they may adsorb on new surfaces and form biofilms in new environmental niches [39,40]. This can cause the spreading of infections within hosts and results in a threat of re-infection [30,41,42].

2.2. Antimicrobial, microbe repellant & contact killing materials

The mechanisms of biofilm formation offer 3 inhibition strategies, namely microbe repellence, microbiocide and contact killing. Microbial repellence is the most attractive one as it performs a direct inhibition of bacterial adherence on a surface that prevents biofilm formation in the first place. Therefore, in contrast to the other techniques, no actual microbial killing is required. Microbiocide inhibition strategies though use additional agents like drugs or nanoparticles hindering cell reproduction and hence prevent biofilm formation. Lastly, contact killing materials make use of physical interactions between the surface and microbes to disrupt and kill microbes as soon as they attach to the surface. These different approaches can be distinguished according to their basic defense mechanism by A) surface topography, which morphologically disturbs and inhibits the initial adhesion, B) material modification, where intrinsic chemical and physical properties result in microbe-repellence and C) additives to the bulk material, which induce repellence or killing. Fig. 2 gives an overview over the different biofilm inhibition strategies.

Surface topography: A generic approach to achieve antimicrobial material properties is tailoring the surface topography irrespective of intrinsic material properties or additives. Consequently, all possible positive or negative effects on bacterial adhesion result from surface structure, area, pore size and distribution. Intensified surface roughness increases the accessible surface area for bacterial attachment and additionally protects the bacterial cells from fluid shear forces which may disperse the biofilm during formation [43,44]. A study on the commonly used titanium could show that roughness on the nanometer scale – and not micrometer scale – significantly increases the attachment of bacteria [45]. It was concluded that surface topography is the most influential factor for bacterial adhesion in this system, while other parameters like e.g., surface charge, surface energy and surface zeta potential, had little or no influence. Important parameters that influence the bacterial activity of a nanostructured surface are shape, size, spacing and arrangement of the nanoscale features and overall surface structure [43,46,47]. Such nano-structural surface features were successfully demonstrated to potentially serve as contact-killing based on a physical mechanism. When challenged with the surfaces, bacteria will try to settle on the nanostructured surfaces by increasing the contact area with multiple anchoring points. As soon as the cell wall reaches a threshold limit of strain during the stretching acting on it, cell wall rupturing can take place (comparable to a bed of nails) [48]. These assumptions were also mechanically and thermodynamically validated applying theoretical models [49,50]. As suggested by Nowlin et al. and Kelleher et al. [48,51] this strategy can be more sufficient than repelling the bacteria from the surface in the first place which would be the alternative mechanism. As an example, the chemical etching of poly(vinyl chloride) (PVC) to introduce nanoscale roughness with pikes up to 50 nm reduced the initial attachment of bacterial strains [52]. It has been suggested that there may be an optimal feature size on the microscale that decreases the attachment of bacteria to surfaces. They identified 13 nm pikes to have increased adhesion properties and pikes of 95 nm to have reduced adhesion. Further, they stated that cell responses were modified by the shape of nanofeatures and that these changes are related to the interactions of filopodia and focal adhesion complexes with the nanofeatures. Thus, the influence of the distance between nanofeatures must be considered as a function of the distance between focal adhesions. This distance appears to be around 50 nm, the minimal spatial variation that cells can sense being less than 1 nm. Moreover, the spacing between nanofeatures is also important in order to permit the cell membrane to bend between the nanofeatures [53]. However, it is unlikely that there is a “one-size-fits-all” relationship between roughness and attachment, as bacterial strains can vary significantly in size and shape [47,54]. Furthermore, while most studies indicate a positive correlation between surface roughness and microbial adhesion rate, the discussion in the scientific community is controversial whether surface roughness or hydrophobicity is the most influential factor for attachment and biofilm formation [55–58]. Air entrapment can result from hydrophobic nanostructured surfaces as the repulsion of surrounding liquid media is strong enough to generate air pockets within the nanostructures. Such gas-liquid interfaces were shown to effectively inhibit bacterial adhesion directly at regions with entrapped air and reduces the available solid-liquid interface [59]. This mechanism can be further expanded to create self-cleaning surfaces [60]. Based on the same principle, fluid entrapments could be used as an additional future strategy to prevent

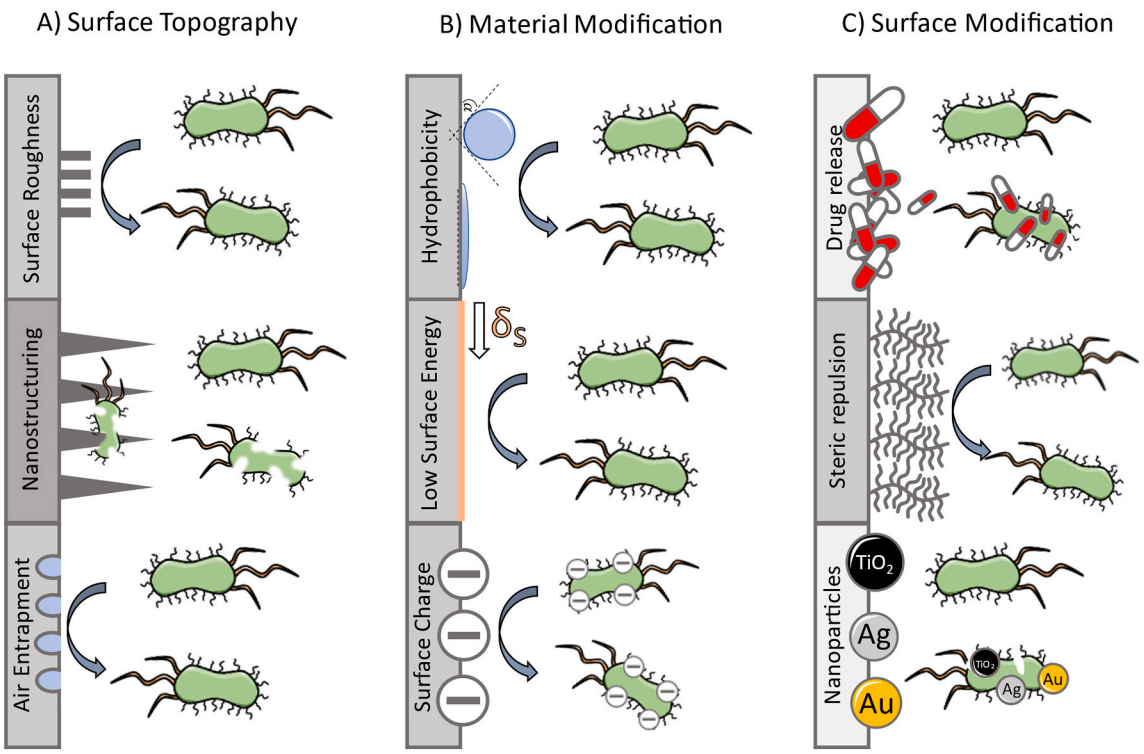

Fig. 2. Common antimicrobial mechanisms in regard of surface topography, material modification and additives. Microbe repellant surfaces in grey, microbiocide mechanisms in brighter grey and contact killing materials in darker grey.

bacterial adhesion. The so-called Slippery Liquid-Infused Porous Surfaces (SLIPS) manage to inhibit stable anchoring of microbial cells and lead to reduced surface adhesion [61,62]. Moreover, several examples of antifouling nanopatterns can be found in nature, such as on the riblet structure of shark skin, common sandfish scales or lotus flower leaves. Based on such blue prints, soft photolithography, micro-molding or nanopatterning can be applied to design bioinspired antifouling surfaces [5]. In conflict with these findings is the point, that increased roughness is considered beneficial in the field of biomedicine as it correlates with faster and firmer integration of implants in the surrounding tissue, e.g., for bone replacements [63].

Material modification: The hydrophobicity of a surface is crucial for the interaction with microbes as their most preferred environment is aqueous. The physical interactions between hydrophobic surfaces and flagella, fimbriae, and pili facilitate the attachment of bacteria to non-polar, low-energy substrates [26,64]. The displacement of water molecules near surfaces enhances hydrophobic interactions and promotes close contact between cells and surfaces [65]. Previous studies have shown that bacterial growth on surfaces can be related to the surface hydrophobicity as they need an optimized environment to enable growth and reproduction [66]. During pre-conditioning of the surface, a protein layer is formed on the surface and promotes bacterial adhesion facilitating the formation of a biofilm. It is generally considered that proteins tend to adsorb more favorably onto surfaces with contact angles of 60–90° and on hydrophobic surfaces [67]. However, superhydrophobic surfaces have been found to have low protein adsorption and facilitate protein detachment [68,69], thus resulting in lower bacterial adhesion [70,71]. With the creation of superhydrophobic surfaces (>150° contact angle), self-cleaning effects could be translated to prevent bacterial adhesion and subsequent biofilm formation. Superhydrophobicity can be achieved by a combination of chemical composition, surface structuring on the micro-/nanoscale and the introduction of low-surface-energy compounds [72]. Privett et al. [73] demonstrated that the adhesion of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was reduced significantly on a superhydrophobic coating (water contact angle of 167°) obtained from fluorinated silica colloids. Crick et al. [74] reported reduced *Staphylococcus aureus* and *Escherichia coli* adhesion on their AACVD (aerosol assisted chemical vapour deposition) coated superhydrophobic surface (water contact angle of 165°) compared with an uncoated plain glass (water contact angle of 60°) and a dip-coated elastomer glass (water contact angle of 95°). Hizal et al. [75] claimed that the combination of superhydrophobic surfaces and fluid shear stress reduced the adherence of *Staphylococcus aureus* and *Escherichia coli*, due to the slipperiness of the superhydrophobic surface. PEO is contrarily an example of an antimicrobial material which is hydrophilic. The most widely investigated mechanism employs hydration forces and/or steric repulsion. While steric repulsion may play a role, it is now understood that hydrophilicity alone can impart surfaces that possess a tightly bound water layer, which creates a physical and energetic barrier and causes interactions with approaching proteins or bacteria to become thermodynamically unfavorable [76].

Another mechanism to reduce bacterial adhesion can be via electrostatic forces. Most bacterial genera have a net negative charge as determined by zeta-potential measurements [77,78]. The differences in cell wall composition as well as membrane proteins in Gram-positive and Gram-negative bacteria result in a broader diversity in attachment mechanisms [79,80].

Bacteria attach rapidly and tightly to positively charged surfaces, and electrostatic repulsion destabilizes cell contacts with negatively charged surfaces [81]. Destabilizing interactions between cells and anionic surfaces during the initial stages of attachment can be overcome by extracellular organelles that promote adhesion, including fimbriae, flagella, curli, and pili [25]. Moreover, it must be considered, that the layer of the bacteria cell wall that is in contact with the extracellular environment is complex and exposes many different functional groups that may interact with substrates. These functional groups include carboxylate, hydroxyl, phosphate, and amine moieties [78].

Additives and Coatings: The third category of inhibition strategies is modifying the surface with other types of materials to inhibit initial attachment or kill the microbial cells. Firstly, the use of antibacterial

agents is a successive and simple option to prevent biofilms. Though, microbes are more protected in a biofilm due to the adaptability of the EPS layer protecting the bacteria from antibiotics as this is ascribed to long-standing bacterial infections [82]. The advantage of rendering material surfaces antibacterial is to locally increase the concentration of the antibacterial agent without transcending the toxicity limits, when compared to traditional delivery methods for antibiotics. In this case, antibacterial substances get used only at the point of care to prevent biofilm formation, thus eliminating resistance activity and obviating the potentially deleterious systemic incidences. Strategies that rely on the release of antibacterial agents from coatings can be inter alia separated into active (triggered) [83–86] and passive approaches [87,88]. Another approach of antimicrobial biofilm inhibition is the use of antimicrobial peptides (AMP). AMPs are short-length peptide antibiotics (between 15 and 30 amino acids), the majority of which are cationic, amphipathic, gene-encoded and directed to the cell membrane [89–91]. They are part of the innate immune system of animals and plants but can also be found in bacteria and fungi [89,91]. Mammalian AMPs are generally expressed and easily induced in epithelial surfaces to repel assault by bacteria, viruses, fungi and parasites [92]. General advantages rely on reducing the chance of bacterial resistance [93,94], effectivity against already resistant bacterial strains [91,95,96] and host-non-host microbe specificity [94]. While conventional antibiotics usually act by inhibition of cell wall synthesis or DNA, RNA and protein synthesis [95], most AMPs permeabilize microbial membranes, inducing either a large-scale failure or small defects that dissipate the transmembrane potential, which results in cell death [95,96]. An overall problem with this inhibition approach is the restricted longevity of their functionality. Hence, the surface doesn't have an unlimited supply of antimicrobial agents, this technique is mainly useful for overcoming shorter periods of time until antimicrobial properties become more and more ineffective.

Steric repulsion is the second additive technique and prevents the microbial attachment in the first place instead of killing adhering cells. General strategies for the design of substrate surface chemistry include covalent modification and degradation of polymeric surfaces [97]. These strategies have been successfully used to control bacterial attachment as they repel foulants by forming a barrier based on a hydration layer through hydrogen bonding and/or ionic solvation [98]. When proteins approach the surface, water molecules are released from the surface and the polymer chains are compressed. This leads to an increase in enthalpy due to polymer dehydration and decrease in entropy due to chain compression. According to thermodynamics, both of these events are unfavorable and hence these surfaces tend to repel proteins or other foulants by the mechanism of steric repulsion [76,99]. To address this mechanism, there are various materials that can be applied like e.g. poly(N-isopropylacrylamide) (PNIPAAm) [100,101], dextrans [101], poly(ethylene oxide) (PEO) [3,102–104], poly(ethyleneimine) (PEI) [105,106], poly(sulfobetaine methacrylate) [107], chitosan [108], hydroxyapatite, albumin [67], heparin [109] and poly (N-isopropylacrylamide). A more recent study successfully demonstrated formation of an oriented and dense layer of flagellin proteins on a hydrophobic surface, resulting in an effective bacterial cell repellent feature [110].

Furthermore, nanoparticles embedded in the surface of biofilm-exposed surfaces can show antimicrobial properties. Silver ions in solution have been demonstrated to be antibacterial and have been vastly used in medicine [111]. Moreover, silver can be manufactured into silver nanoparticles (AgNPs) to increase ion release and lead to improved physical, chemical, and biological properties [112–114]. Due to their huge relative surface area, nanoparticles significantly pronounced physiochemical and biological impact as compared to their bulk materials. It has been reported that AgNPs can anchor to the bacterial cell wall and infiltrate it. This action will cause physical damage in the bacterial membrane, which results in cellular contents leakage and bacterial death [114,115].

3. Intrinsic antimicrobial properties of silk-based materials

The emergence of multi-drug resistant germs, caused by the overuse of traditional antibiotics, poses a severe risk in the health sector. To counteract this phenomenon, sustainable solutions must be established. One strategy is to identify and examine natural systems displaying high microbe-repellency and to transfer such principles into bio-inspired technical material systems. In this context, silk is considered a multipurpose material providing mechanical and chemical stability, biocompatibility and bio-persistence against microbial degradation. Naturally produced silk materials and classical modifications like coating with metallic nanoparticles are nowadays facilitated by tailored recombinant silk variants for specific applications. Therewith, the whole range of known antibacterial mechanisms can be exploited [111,116]. Strikingly, there is an ongoing discussion in the scientific community concerning the intrinsic properties of silk proteins in context of microbe defense [117–120]. One core misunderstanding in the discussion is the often-neglected distinction between the mechanisms of microbe repellency, microbiocide and contact killing that is elaborated in chapter 2.2. The advantageous properties of silk are believed to lie within their ability to prevent bacterial attachment to the surface and biofilm formation [121]. Materials with microbe repellant properties will effectively inhibit the adhesion process of bacteria but will not lead to a reduction in total number of bacteria in an incubation broth approach or show distinct inhibition zones in a radial disc assay. On the other side, also the importance of the structure-function relationship, as demonstrated by Kumari et al. [117], is often not considered sufficiently. They could show that it is possible to switch microbe repellant properties on and off by changing the amino acid sequence of recombinant spider silk proteins resulting in nano-structural changes of crystalline patches on the surface of their tested materials. It is known that the secondary structure of silk materials significantly influences their properties, from mechanical performance to cell adhesion, surface charge and hydrophobicity [122]. These factors must be considered when investigating effects on microbes and the underlying mechanisms of real intrinsic silk properties. The following section will give an overview on recent findings investigating properties solely based on silk materials without applying any bioactive additives.

3.1. Silk fibroin

Its traditional use in textile industry makes silk fibroin the most common industrially processed type of silk and, therefore, it has been investigated thoroughly. Despite the mentioned discrepancies in experimental setups, there were some systematic approaches with emphasis to rule out influencing factors like washing detergents or organic solvents when testing silk fabrics in different states that could not identify significant antibacterial features [120,123]. Kaur et al. investigated the cocoons of four different silk worm strains (*Bombyx mori*, Philosamia Cynthia ricini, Antheraea assamensis and Antheraea mylitta) and used a set of extraction protocols to compare the components suspected to be antibacterial (raw/degummed/demineralized cocoons, degummed powder, sericin and silk solution), but could not find significant evidence [120].

3.2. Sericin

In the natural silk fiber, a double filament of silk fibroin is covered by different molecular weight sericin proteins as a coating. Therefore, sericin and not silk fibroin is exposed to microbes when the first adhesion attempts take place. This causes a systematic error in many approaches where sericin is removed during the washing step in silk regeneration. As Pedregal-Cortés et al. [124] proposed, sericin is the bioactive compound in the silk fiber, being able to create a 100 μm sized water exclusion zone along with a proton gradient of more than two pH units that acts as a physical and chemical barrier to effectively prevent

biofouling. Based on that, sericin was investigated as a coating for different synthetic fibers like polypropylene in wastewater treatment [125] or Nylon and Polyester for polluted air treatment [126], showing in both studies improved antifouling properties of the treated surfaces. When prepared with tannic acid, sericin proved to be also an effective coating for metallic surfaces as it significantly reduced bacterial adhesion on treated titanium foils [127].

Apart from coatings, sericin proteins can be processed into different morphologies like fibers or particles [128]. Electrospun fibers of sericin mixed with chitosan showed very strong antibacterial properties that are proposed to be based on ionic interactions. The protonated amino groups of sericin and chitosan can interact with negatively charged bacterial membranes and lead to loss of membrane integrity enabling the penetration of low molecular weight sericin proteins leading to a collapse of cell function [129]. It was confirmed that sericin can also show high antibacterial efficiency when processed into nanoparticles. Dependent on the size and the applied surface potential upon the addition of poly-L-lysin, the disruption of the bacterial cell membrane was observed to be strongest for nanoparticles of 37–49 nm mean diameter with positive surface potential. The authors propose that these nanoparticles show the best results, as the specific size and surface potential allows adherence and subsequent diffusion through the bacterial membrane, followed by disruption of the cytoplasmic membrane by increased generation of reactive oxygen species, ultimately leading to cell death [130].

Like for silk fibroin, the secondary structure of sericin is crucial for its bulk properties. Aramwit et al. [131] could show that extraction of sericin with different extraction methods including heat, acidic, alkaline and urea extraction drastically changed the percentage of preserved beta sheets and helical structures in the regenerated protein. These changes significantly influenced the property of regenerated sericin to inhibit biofilm formation, with the alkaline extraction completely removing functionality while the urea extraction method provided the best performing material that was able to even kill bacteria residing within the biofilm. These findings can explain the differing results of testing sericin in experimental setups where the extraction method was different and not taken into account. They further emphasize the hypothesis, that secondary-structural features might be a decisive factor in the microbe-repellant properties of silk materials.

To avoid the problem of protein degradation during regeneration, Matsumoto et al. [132] presented a transgenic silkworm that produced cocoons purely made of sericin. The generated fibers were called intact sericin and compared to commercially available degraded sericin. It could be shown that the intact sericin did not kill bacteria but significantly reduced the colony size of *E. coli* and *S. enterica* on culture media compared to degraded sericin by approximately 41% and 56%, underlining the importance of intact high molecular proteins in the application against bacterial infestation.

3.3. Silkworm AMPs

A number of studies, that focused more on the properties of the whole cocoon system, used stifled and washed cocoons in different maturation stages of the larvae and found the 18-wheeler (18w) protein being a promising candidate acting as AMP within the silkworm proteome [10]. The 18-wheeler protein is known to be expressed in the larval fat body, which is the primary organ for antibacterial peptide synthesis. It was shown previously that bacterial infection triggers increased transcript levels of 18-wheeler, and the absence of the 18w receptor leaves larvae more susceptible to bacterial infections, as it is incorporated in the cocoon [133]. Tests with the bulk cocoon material after washing and processing revealed that only weak antibacterial properties are present in the fibroin itself [10,133], leading to the conclusion that other factors are introducing antibacterial properties to silk fibers and that materials from regenerated silk fibroin must be further modified to gain effective anti-fouling properties.

One already established example is the creation and cultivation of transgenic silkworms that make use of other known AMPs present in the *B. mori* genome [134]. Overexpression of Cecropin B, an AMP that is naturally present in the genome of *B. mori*, by a transgenic silkworm resulted in cocoons which showed increased antibacterial properties against infestation with *E. coli* [119,135]. The effectiveness of using natural antibacterial peptides in combination with *B. mori* silk was furthermore demonstrated via surface coupling of e.g. Cys-KR12 motifs on nanofiber membranes and cecropin B coupling to regenerated silk films [136,137].

3.4. Spider silk

The successful establishment of recombinant production routes for spider silk proteins at the beginning of this millennium paved the way to large scale production of highly pure materials. Furthermore, the possibility of precisely controlling silk protein design and modifications opened the door to fundamental research on the structure-property-relationship of tailored silk materials. Nevertheless, although silk materials are extensively studied nowadays, regarding microbe-persistency no generic hypothesis could be formulated so far. While one study reported enhanced bacterial growth when seeded directly on spider silk webs and proposed that spider silk proteins can be used as a nutrient source by several microbes [138], other studies provided clear indications that spider silk can be microbe repellent [121,139–142]. It must be noted that this is true for selected spider silks and does not apply for spider silk in general [138,139,143].

The difficulty in comparing these results is determined by the non-uniform application of test setups, silk preparation procedures and moreover by using silk from different spider species. A closer look at the different natural applications of spider silk reveals that some cases, like prey storage, permanent nest constructions, cocoons or underwater tunnels require a much higher degree of resistance against microbial degradation than other types such as dragline silk that is usually recycled by the distinct spiders on a daily basis [8,143,144]. To rule out these external factors and investigate the intrinsic material features that arise from the amino acid sequence of the protein, simplified repeating building blocks from the natural spider silk protein were engineered and proteins made thereof investigated accordingly [14,17,19]. Through engineered variation of the protein sequence, a deeper understanding of the general structure-function relationship could be generated and applied to design tailormade proteins [14]. Harris et al. [145] created a recombinant spider silk protein (rSSp) based on the dragline sequence of *Nephila clavipes* MaSp1 and MaSp2 and found an intrinsic ability to inhibit biofouling due to thrombosis when processed via aqueous-based solvation and coating of a wide array of substrates. Another study by Kumari et al. [117,146] applied different variants of engineered *Araneus diadematus* fibroin to demonstrate that intrinsic microbe-repellency of a negatively charged eADF4(C16) can be switched off by changing the amino acid sequence towards neutral charge resulting in nano-structural changes of films and hydrogels. It was concluded that size and distribution of crystalline patches are crucial to create anti-fouling surfaces. Furthermore, bio-selectivity was achieved by introducing the cell adhesion motif RGD to eADF4(C16) resulting in selective mammalian cell adhesion and simultaneous microbe-repellency. Moreover, coatings made of recombinant spider silk proteins on silicone surfaces have previously shown enhanced microbe-repellent properties against four opportunistic infection-related strains [147]. A different effect, due to triboelectric charging of a film made from rSSP based on the MaSp1 sequence, was reported by Zhang et al. [148]. Their self-powered triboelectric nanogenerator builds up electrical charges of up to 135 V, leading to a potential difference between the positively charged surface and the bacteria. An extracellular transmission of electrons impairs the bacterial morphology and leads to death of the bacteria by inducing a burst of reactive oxygen species inside the bacterial cytoplasma.

3.5. Honeybee silk

The range of silk producing species is huge, but only few other silks than silkworm and spider silk have been investigated regarding their structural and bio-functional properties [9]. Nevertheless, considering that they belong to the same material class, there is no reason to rule out the same antimicrobial potential in silk produced e.g., by honeybees, paper wasps or ants [8,15,149]. Since honeybee silk proteins can be produced recombinantly [150], first investigations started to explore their potential application as AMPs by screening the various building blocks of the honeybee silk proteome [16]. Among the 19 screened peptides derived from the AmelF3 gene, 9 were able to kill more than 98% of the tested *E. coli* bacteria and all except two killed at least 50%, displaying significant activity in a laboratory assay with peptide concentrations of 100 μg/ml in solution.

4. Modification of silk-based materials to enhance or generate antimicrobial properties

4.1. Silk fibroin

Modification of the surface of materials to introduce antibacterial properties is a strategy that is often applied if intrinsic material properties are not satisfying. A common application is the introduction of metallic nanoparticles to textiles or medical surfaces to render the surface bactericidal. Since *Bombyx mori* silk is produced at relatively low costs (less than 5$/kg) and available at high amounts (roughly 200,000 metric tons per year worldwide), the introduction of surface modifications of fabric is more economic than recombinant production (more than 300$/kg) [151–153].

A straightforward approach to create a strong antibacterial effect on silk fabric, which can easily be upscaled, would be the plateless silver coating technique as proposed by Yu et al. [154]. Other often-used modifications include the introduction of silver or gold nanoparticles via different coupling mechanisms [155–157]. The mechanism of these metallic nanoparticles was reported extensively in the past and allows fast and effective bactericidal modification of large amounts of fabric [158]. If more delicate fibrous materials are needed, AgNPs can be mixed in the spinning dope of regenerated silk solutions for electrospinning to create antibacterial electrospun nonwovens [159]. For a stronger coupling of the AgNPs on the silk protein, they can be synthesized via a green chemistry route using silk as reducing agent that allows dispersion and stabilizes the generated nanoparticles [160]. Silver is also used in the morphology of nanowires, which then allow the creation of a superomniphobic surface when embedded in PDMS on a silk substrate, strongly inhibiting any kind of surface adhesion process [161]. Recently published approaches reported strong antibacterial behavior by the incorporation of nano diamonds in electrospun silk nanofibers but also the creation of more sophisticated multi-layer fibers for future suture applications by wet spinning and addition of carbon nanotubes (CNT), growth factors or graphene quantum dots [162,163].

A different approach was reported by Tullii et al. [164] as they applied soft photolithography and were able to create patterned silk films with topographical features in the micron and submicron range. Based on bioinspired antifouling mechanisms, they created stripes with 800 nm grooves and 800 nm distance between each groove as well as microwells with 1–2 μm and 3.5–5 μm diameter. They reported a decrease in bacterial adhesion of more than 65% on the structured surfaces compared to flat films and proposed steric effects by features one size lower than the bacterial dimensions and hydrophobic effects induced by the micro-structured surfaces as driving forces. In both cases, the available surface area for microbes to adhere was significantly reduced, with the microwells of diameter 1–2 μm being the most effective.

4.2. Sericin

Due to the more pronounced intrinsic antibacterial properties of sericin, modification strategies are less investigated than for silk fibroin but nevertheless some studies report optimization of sericin based materials.

The addition of TiO_2 nanoparticles to regenerated sericin, cross-linked with and without polycarboxylic acid to cotton fabric showed stronger effect against *S. aureus* than against *E. coli* and a more pronounced antibacterial behavior than sericin alone when used as a coating [165]. Surface-linked TiO_2 particles result in photo holes inducing reactive oxygen species upon UVA radiation. This photocatalytic reaction leads to oxidative damage in the outer cell membrane of bacteria and allows permeation of the damaged membrane by low molecular weight sericin and TiO_2, causing loss of cell integrity and subsequent cell death. Blends of sericin with PVA and AgNPs were investigated in the form of films and hydrogels showing remarkable inhibition zones in radial diffusion assays and significantly reducing bacterial growth during the log phase of a growth curve assay [166,167]. SEM micrographs revealed bacterial lysis by destroyed cell membranes due to the presence of AgNPs. Similar to silk fibroin, sericin can be used to produce AgNPs in situ by using sericin as reducing and capping agent [168]. The generated particles effectively inhibited the growth of *E. coli*, *S. aureus* and *K. pneumoniae* in radial diffusion tests.

The next step in the development of sericin based antimicrobial materials is the application of genetic engineering methods to produce specially designed fusion proteins with AMPs as shown by Thomas et al. [169]. They reported the successful production of a recombinant sericin based on a simplified repetitive sequence and a fusion protein of sericin and cecropin B in *E. coli*, *P. pastoris* and a cell-free approach [169,170]. The resulting purified proteins displayed a strong antibacterial effect against Gram-positive and Gram-negative bacteria. The authors state that the antibacterial activity of sericin can be attributed to their 38-amino acid serine-rich repetitive motif and was further enhanced by the fusion with cecropin B, leading to membrane blebbing of bacterial cells and subsequent cell death.

4.3. Spider silk

The aforementioned issues in harvesting sufficient amounts of naturally generated spider silk emphasizes the more pronounced need of recombinant production routes as compared to well-available *B. mori* silk. This development promoted and facilitated the introduction of modifications on the primary sequence level of spider silk proteins and enabled the creation of fusion proteins.

Modifications of recombinant variants of spider silk proteins included the introduction of heparin binding sites and the coupling of various natural AMPs like HNP-2, HNP-4, Hepcidin, 6mer-HNP-1, SAL-1 and dispersin B to create a whole range of fusion proteins with superior antibacterial and in some cases also biofilm dispersing effects [116,171–173]. Furthermore, the introduction of silver binding sites in recombinant spider silk proteins from *Nephila clavipes* MaSp1 dragline silk allowed for effective coupling of Ag Ions from a solution and showed strong inhibitory effects on Gram-positive and Gram-negative bacteria by the chimeric silver-bound silk protein [174]. On the other side, Huang et al. reported that the antibacterial properties of Selenium nanoparticles could be further enhanced by coating them with positively charged recombinant eADF(κ16) dragline silk proteins, while cytotoxicity testing showed that the coated particles are safe to use at certain concentrations in combination with Balb/3 T3 mouse fibroblasts and HaCaT human skin keratinocytes [175]. As for other silks, spider cobwebs were proposed as a useful reducing agent to synthesize AgNPs in a green chemical process [158]. The resulting AgNPs displayed considerable inhibition against the growth of bacteria strains like *E. coli*, *S. aureus, K. granulomatis* and *P. aeruginosa*, as well as the fungi *A. flavus*, *A. fumigatus* and *A. niger*.

4.4. Honeybee silk

Based on the recombinant production of honeybee silk proteins and the highly potent AMP pexiganan, Trueman et al. investigated the different efficiencies of silk-pexiganan fusion proteins in comparison to silk containing entrapped AMP or AgNPs [176]. The chimeric proteins showed a reduction in *E. coli* cell number after degradation of the protein leading to the release of a truncated version of the AMP, while entrapped pexiganan was leached from films at rates of <1% per day. The films containing AgNPs showed good antibacterial activity, which correlated with the amount and morphology of the AgNPs.

5. Conclusion and outlook

Bioinspired strategies to create new materials that inhibit bacterial attachment and biofilm formation are a promising approach to tackle the growing problem of multi drug-resistant pathogens emerging through the overuse of antibiotics in medicine. Among those strategies, silk-based materials are of high interest due to their intrinsic combination of attractive material properties. While basic properties can also be found in other materials, silk-based materials are unrivaled in combining excellent biocompatibility, superior mechanical performance, processability into various morphologies as well as distinctive bio selectivity. Apart from textile industry, these unique properties lead to numerous potential applications in medical products [177–180], for tissue engineering [181–183] or drug delivery systems [184,185].

Two silk classes, namely silkworm silk and spider silk, are standing out from the vast range of silk materials, dominating scientific interest. Strikingly, even when mainly focusing on these silk classes, this review has demonstrated an exploding diversity resulting from the matrix of material origin (species, silk type), production (natural, recombinant/transgenic), processing (films, coatings, fibers, nonwoven meshes, particles…) and additives (antimicrobials, AMPs, nano-particles…), which is further spreading by the use of different testing methods and organisms (Fig. 3). Consequently, creating an integral hypothesis on the function of silk materials against microbes is not possible, and unraveling the respective controversial uncertainties can only be achieved by differentiation and clear definition of the aforementioned aspects.

Reviewing the findings considered in this article on a generalized comparative level as shown in Table 1, it becomes obvious that introducing established mechanisms of antimicrobial additives such as nanoparticles and antibiotics is frequently successful. Nevertheless, these strategies are not necessarily specific for silk materials and would apply for many other polymers. Thus, the most elegant way is still to make use of intrinsic microbe-repellant silk properties. In this regard, Table 1 demonstrates a broad diversity, even for similar materials such as stifled cocoons of *B. mori* [10,120]. The differing findings indicate the sensitivity of antimicrobial silk properties to external factors such as sample conditioning, experimental design, and analytical tools. On the other hand, there's clear evidence, that silk materials have the potential of microbe-repellency emphasizing the need of systematic and reproducible testing, which can e.g. be achieved by working with recombinant silk proteins providing perfectly defined monodisperse materials with high purity. These approaches are also the most promising ones to unravel the mechanisms behind inherent anti-fouling properties of silk proteins. Recent work showed that structural changes in the crystal distribution and conformation, as induced by slightly changing the silk amino acid sequence, resulted in inhibition of the microbe-repellant properties [117]. It was hypothesized, that homogeneously distributed hydrophobic patches formed by assembly of beta-sheet crystals can inhibit microbe adhesion as they don't provide sufficient anchoring sites for microbes. Importantly, the patches must be small enough (<10 nm), which is the case for various spider silk types [186,187]. In contrast, it is suspected, that the crystalline size of *B. mori* fibroin is big enough (~ 14

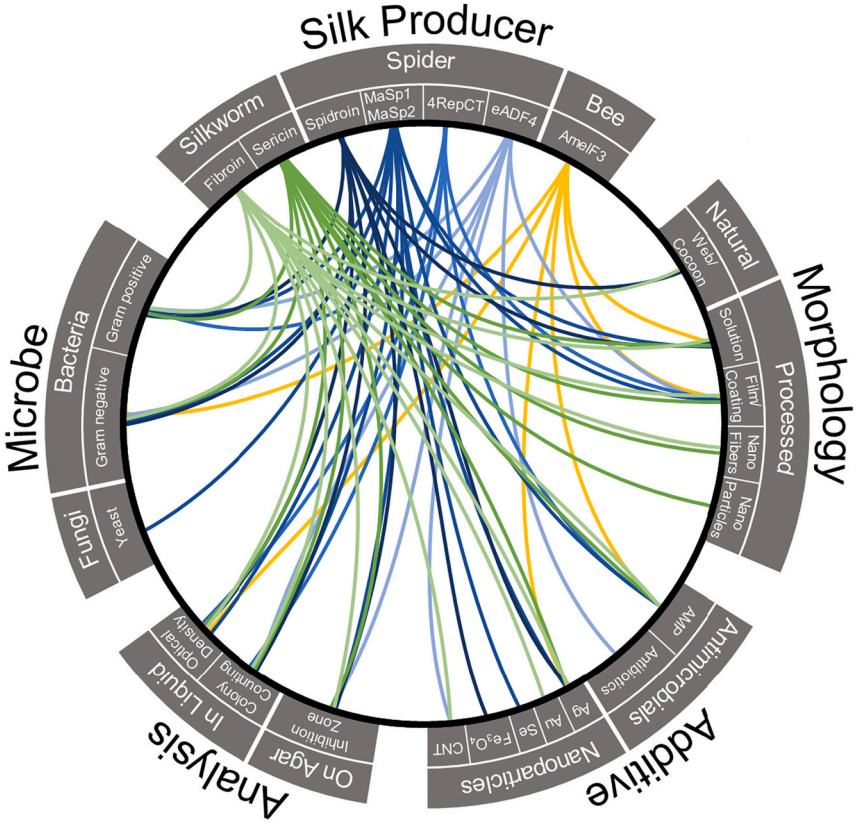

Fig. 3. Test matrix of silk-based materials, connecting lines represent possible connections of the different parameters present in publications. Different colors are assigned to the origin of the silk proteins of interest.

Table 1
Overview with focus on antimicrobial properties for silk-based materials.

Animal	Protein	[a]	Species	Morphology	Additives	Incub.	Time	Analysis methods	Tested microbes (Gram negative/positive)		Effect	Ref.
Silkworm	Fibroin	**N**	*Bombyx mori*	Multi-layer suture	CNT, GM-CSF, GQD	Liquid	30 min	Colony counting (CC)	⊖ *Escherichia coli*	⊕ *Staphylococcus aureus*	+	[163]
		N	*Bombyx mori*	Electrospun nanofibers	PEO, nanodiamonds	Liquid	18 h	Bacterial staining/ SEM	⊖ *Escherichia coli* ⊖ *Pseudomonas aeruginosa*	⊕ *Staphylococcus aureus*	+	[162]
		N	*Bombyx mori*	Membrane	Ag nanowires in PDMS	Dry	–	Visual evaluation			○	[161]
		N	*Bombyx mori*	Raw fabric	Heat treatment in Argon	Liquid	18 h	CC	⊖ *Escherichia coli*		○	[123]
		N	*Bombyx mori*	Film	Surface Nanostructure	Liquid	24 h	Bacterial staining (BS)	⊖ *Escherichia coli*		++	[164]
		N	*Bombyx mori*	Stifled cocoons	AgNP	Agar	24 h	Inhibition zone (IZ)	⊖ *Escherichia coli*	⊕ *Staphylococcus aureus* ⊕ *Mycobacterium tuberculosis*	+	[159]
		N	*Bombyx mori*	Fabric	AgNP	Liquid	24 h	CC	⊖ *Klebsiella pneumonia*	⊕ *Staphylococcus aureus*	+	[155]
		N	*Bombyx mori*	Nanofiber	Cys-KR12	Liquid	4 h	CC/FE-SEM	⊖ *Escherichia coli* ⊖ *Pseudomonas aeruginosa*	⊕ *Staphylococcus aureus* ⊕ *Staphylococcus epidermidis*	++	[137]
		N	*Bombyx mori*	Degummed fabric	Ag plating	Liquid	–	CC	⊖ *Escherichia coli*	⊕ *Staphylococcus aureus*	+	[154]
		N	*Bombyx mori* *Philosamia cynthia ricini* *Antheraea assamensis* *Antheraea mylitta*	Stifled cocoons		Liquid	18 h	CC	⊖ *Escherichia coli*		–	[120]
		N	*Bombyx mori*	Processed fabric	AuNP	Liquid	18 h	CC	⊖ *Escherichia coli*		+	[157]
		N	*Bombyx mori*	Solution	AgNP	Liquid	24 h	CC		⊕ *Methicillin-resistant Staphylococcus aureus (MRSA)*	+	[160]
		N	*Bombyx mori*	Stifled cocoons		Agar	24 h	IZ	⊖ *Escherichia coli* ⊖ *Klebsiella pneumonia* ⊖ *Pseudomonas aeruginosa*	⊕*Staphylococcus aureus* ⊕*Bacillus cereus*	+	[10]
		N	*Bombyx mori*	Film	Cecropin B	Liquid	2 h	CC	⊖ *Escherichia coli*	⊕ *Staphylococcus aureus*	+	[72]
		T	*Bombyx mori*	Degummed yarns	Cecropin B & Moricin	Liquid	24 h	CC	⊖ *Escherichia coli*		++	[119]
		T	*Bombyx mori*	Grounded cocoons	GFP & Cecropin B	Liquid	8 h	CC			○	[135]
		R	*Bombyx mori*	Film	mKate2	Liquid	1 h	CC	⊖ *Escherichia coli*		○	[191]
		N	*Bombyx mori*	Freeze dried		Liquid	24 h	CC/BS/SEM		⊕ *Streptococcus mutans*	++	[131]
	Sericin	**N**	*Bombyx mori*	Surface coating	Tannic acid	Liquid	4 h	CC/OD/BS/SEM	⊖ *Escherichia coli*	⊕ *Staphylococcus aureus*	+	[127]
		N	*Antheraea mylitta*	Nanoparticles	Poly-L-lysine	Liquid	24 h	IZ	⊖ *Escherichia coli*	⊕ *Staphylococcus aureus*	++	[130]
		N	*Bombyx mori*	Fiber coating	AgNP	Agar	24 h	IZ	⊖ *Escherichia coli*	⊕ *Staphylococcus aureus*	+	[192]
		N	*Bombyx mori*	Nanoparticles	Ag	Agar	24 h	IZ/OD	⊖ *Escherichia coli* ⊖ *Klebsiella pneumonia*	⊕ *Staphylococcus aureus*	+	[168]
		N	*Bombyx mori*	Hydrogel	AgNP	Liquid	36 h	OD/BS/SEM	⊖ *Escherichia coli* ⊖ *Pseudomonas aeruginosa*	⊕ *Staphylococcus aureus*	+	[167]
		N	*Bombyx mori*	Fiber coating		Liquid	3 h	IZ/OD/Pressure drop			○	[125]
		N	*Bombyx mori*	Film	AgNP	Liquid	30 min	CC	⊖ *Escherichia coli*	⊕ *Staphylococcus aureus*	○	[166]
		N	*Bombyx mori*	Nanofibers	Chitosan	Liquid	12 h	CC	⊖ *Escherichia coli*	⊕ *Bacillus subtilis*	+	[129]
		N	*Bombyx mori*	Coating of cotton fabric	TiO_2	Liquid	3 h	IZ	⊖ *Escherichia coli*	⊕ *Staphylococcus aureus*	○	[165]
		N	*Bombyx mori*	Suture coating		Agar	48 h	CC			○	[126]
		T	*Bombyx mori*	Film		Agar	48 h	IZ/OD			+	[132]

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Table 1 (*continued*)

Animal	Protein	[a]	Species	Morphology	Additives	Incub.	Time	Analysis methods	Tested microbes (Gram negative/positive)		Effect	Ref.
									⊖ *Escherichia coli* ⊖ *Salmonella enterica*	⊕ *Staphylococcus aureus* ⊕ *Bacillus subtilis*		
		R	*Bombyx mori*	Solution	Cecropin B	Agar	12 h	CC	⊖ *Escherichia coli*	⊕ *Staphylococcus aureus*	+	[169]
Spider	Unspecified	**N**	*Linothele fallax* *Linothele megatheloides*	Raw web		Liquid	18 h	OD	⊖ *Escherichia coli* ⊖ *Pseudomonas aeruginosa*	⊕ *Staphylococcus aureus* ⊕ *Enterococcus faecalis*	−	[138]
		N	*Pardosa brevivulva*	Dissolved web		Agar	24 h	IZ	⊖ *Klebsiella pneumonia* ⊖ *P. aeruginosa* ⊖ *Proteus vulgaris* ⊖ *Salmonella Typhi*	⊕ *Staphylococcus aureus* ⊕ *Bacillus megaterium* *Div. Fungi*	○	[193]
		N	*Eriovixia excelsa*	Dissolved web		Agar	24 h	IZ	⊖ *Acinetobacter* spp. ⊖ *Pasteurella* spp.	⊕ *Staphylococcus* spp. ⊕ *Streptococcus* spp.	○	[142]
		N	*Nephila pilipes* *Cyrtophora moluccensis*	Raw web		Agar	24 h	IZ	⊖ *E. coli* ⊖ *Enterobacter bugandensis*	⊕ *Bacillus altitudinis* ⊕ *Bacillus subtilis*	+	[121]
		N	*Parasteatoda tepidariorum*	Egg cocoons		Liquid	72 h	CC			○	[194]
		N	*Pholcus phalangioides*	Dissolved web		Agar	24 h	IZ	⊖ *Acinetobacter baumannii* ⊖ *Pasteurella multocida* ⊖ *Klebsiella pneumonia*	⊕ *Staphylococcus aureus*	○	[141]
		N	*Tegenarica domestica*	Solution	AgNP	Agar	48 h	IZ	⊖ *E. coli* ⊖ *Klebsiella granulomatis* ⊖ *P. aeruginosa*	⊕ *Staphylococcus aureus*	+	[158]
		N	*Stegodyphus dumicola*	Raw web		Agar	24 h	IZ		⊕ *Bacillus thuringiensis*	○	[144]
		N	*Crossopriza lyoni*	Degummed fiber	Fe3O4 NP	Liquid	24 h	OD	⊖ *E. coli* ⊖ *Pseudomonas stutzeri*	⊕ *Bacillus licheniformis*	+	[195]
		N	*N. pilipes*	Raw web		Agar	24 h	IZ	⊖ *E. coli* ⊖ *P. aeruginosa* ⊖ *Klebsiella pneumonia*	⊕ *Staphylococcus aureus*	○	[139]
		N	*Pholcus Phalangioides*	Dissolved web		Agar	24 h	IZ	⊖ *E. coli*	⊕ *Listeria monocytogenes*	○	[196]
		N	*Tegenaria domestica*	Raw silk		Liquid	24 h	OD	⊖ *E. coli*	⊕ *B. subtilis*	+	[140]
	MaSp1	**R**	*Araneus diadematus*	Particle coating/ film	SeNP	Liquid	4 h	CC	⊖ *E. coli*		++	[175]
	MaSp1	**R**	*A. diadematus*	Film/Hydrogel		Liquid	60 h	BS/SEM/AFM	⊖ *E. coli* Yeast: *Pichia pastoris*	⊕ *Staphylococcus aureus* ⊕ *Streptococcus mutans* Fungi: *Candida albicans*	+++	[117]
	4RepCT FN-4RepCT	**R**	*Euprosthenops australis*	Film	Dispersin B	Liquid	24 h	OD		⊕ *Staphylococcus aureus*	++	[173]
	4RepCT	**R**	*Nephila clavipes*	Coating silicone sheet	Heparin binding motif	Liquid	24 h	CC/BS		⊕ *Staphylococcus aureus*	+	[172]
	MaSp2	**R**	*N. clavipes*	Coating for suture	HNP1	Liquid	24 h	CC/BS/SEM	⊖ *E. coli*	⊕ *Methicillin-resistant Staphylococcus aureus (MRSA)*	++	[116]
	MaSp1 MaSp2	**R**	*E. australis*	Coating	SAL-1, PlySs2, DspB	Liquid	1 h	CC/BS		⊕ *Staphylococcus aureus*	+	[118]
	eADF4 (C16) variants	**R**	*N. clavipes*	Cast membrane	Graphene & CNT	Liquid	30 min	CC	⊖ *E. coli*	⊕ *Staphylococcus aureus*	+++	[148]

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Table 1 (*continued*)

Animal	Protein	[a]	Species	Morphology	Additives	Incub.	Time	Analysis methods	Tested microbes (Gram negative/positive)	Effect	Ref.
	eADF4 (k16)	**R**	*N. clavipes*	Coating	Antibiotics, Heparin	Agar	24 h	IZ	⊖ *E. coli* ⊖ *P. aeruginosa* ⊖ *Serratia marcescens*	⊕ *Staphylococcus aureus Fungi-C. albicans*	++	[145]
	MaSp1	**R**	*N. clavipes*	Solution	HNP-2, HNP-4, Hepcidin	Agar	24 h	IZ	⊖ *E. coli*	⊕ *Staphylococcus aureus*	++	[171]
	MaSp1	**R**	*N. clavipes*	Film	AgNP	Liquid	24 h	IZ/OD	⊖ *E. coli*	⊕ *Staphylococcus aureus*	+	[174]
Bee	AmelF3	**N**	*Apis mellifera*	Solution		Liquid	12 h	OD	⊖ *E. coli*		++	[16]
	AmelF3	**R**	*A. mellifera*	Film	Pexiganan & AgNP	Liquid	2 h	OD	⊖ *E. coli*		+++	[176]

Most often applied analysis methods are colony counting (CC), inhibition zone measurement (IZ), staining of bacterial membrane (BS), scanning electron microscopy (SEM) and optical density measurements (OD). The described effect in the publication is evaluated from "accelerating microbial growth" (−) over "no reliable effect" (○), to detectable (+), moderate (++) and strong (+++) inhibition of microbial growth.

[a] The origin of the used protein is classified as either N = natural, T = transgenic or R = recombinant.

nm) [188] for various microbes to be applied as adhesion sites, which is in accordance with the presented studies investigating different material states of silk cocoons, finding no evidence for intrinsic microbe-repellant properties [120,123]. The basic idea of such a nano-structural mechanism could be further confirmed for naturally derived *B. mori* sericin [131]. Microbe-repellant properties were significantly affected by the way, sericin was extracted (heat, acidic, alkaline or urea). This was attributed to the respective changes in the percentage of preserved beta sheets and helical structures of the regenerated protein. Consequently, attributing properties to well-defined nanocrystalline structures is currently the most valid theory, which must be further validated via correlation of nano-structural features with standardized microbial examinations of various silk materials. Systematically unraveling such mechanisms would also open the door to design new polymeric materials and apply synthetic production routines for block-copolymers.

As a simplified outcome for future research in this field, three key learnings could be generated from reviewing the broad and controversial scientific discussion concerning the antimicrobial nature of silk proteins.

Not all silks are the same: Biological studies on the huge family of silk producing species indicate that not all silks are antimicrobial and that their properties heavily depend on their application in the animal's environment. A careful choice of the material's origin is crucial to develop functional products.

You get what you measure: The expected mechanism for unmodified silk materials is microbe repellency and not contact killing. The test setups must be designed accordingly, otherwise no effect can be measured.

Function follows structure: The properties of materials from silk proteins are highly dependent on their secondary structure. Careful choice of solvents and processing conditions is crucial in preserving anti-fouling properties.

Following these learnings, future studies should focus on understanding the mechanism of hydrophilic/hydrophobic distributed patches rendering silk surfaces cell selective or microbe-repellant. As transitional solution, additives can be used to introduce new properties but on the long run, the application of systematic optimization through genetic engineering will lead to advanced genome design. This will enable the precise tuning of final bulk material properties on the level of amino acid sequences. Another important aspect is the development of more sophisticated processing strategies. Currently, silk materials are often processed in a similar way as synthetic polymers, neglecting that the increased complexity of a protein requires a higher level of know-how when the full potential of the material should be unveiled. Better control of the processing conditions to form films, fibers or hydrogels is a crucial step to introduce the desired secondary structures already during processing and make better use of the intrinsic properties. Promising examples of microfluidic approaches, mimicking the conditions in the spinning glands, showed superior mechanical properties in the resulting fibers compared to conventional wet spinning [189,190]. This further confirms the impact of processing on the structure-function relationship. It is a strong hint that other intrinsic properties can also be optimized during processing stages, unraveling the full potential of silk-based materials.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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